Ramsey and Muspratt

DAVID KELLIN, 1887–1963

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Obituary Notice

DAVID KEILIN, 1887–1963

The sudden death of David Keilin on 27 February 1963 at the age of 75 years brought to an end a lifetime of research covering a most exceptional range of interests. It is doubtful if in the future any one man will achieve fame, as did Keilin, in such diverse fields as descriptive morphology of protists, fungi and insects, and the biochemistry of respiratory enzymes and metalloprotein compounds. Though the fields of research were so diverse, throughout there was an underlying unity of thought, an interest in the living cell, which guided his research from his early morphological work to its culmination in the discovery of cytochrome and the studies on respiratory enzymes. The breadth of his interests makes it difficult, if not impossible, for one person to write an appreciation which would cover adequately all his activities. It is for this reason that we decided to delay writing this appreciation of David Keilin and his work so that we could attempt to cover both the biological and biochemical fields of his work and to pay special attention to aspects of this which may be of particular interest to microbiologists. We were both closely associated with Keilin for about 40 years, one of us (P.T.) on the biological side and the other (M.D.) on the more biochemical aspects of his work. More details of his purely biological work will be found in an article published in *Parasitology* (1965, **55**, 1–28, by P.T.) and articles paying more attention to his biochemical work have been published by E. F. Hartree (*Biochem. J.* **89**, 1–5), T. R. R. Mann (*Biogr. Mem. Fellows R. Soc.* **10**, 185–207) and E. C. Slater (*Enzymologia*, **26**, 313–20). David Keilin always strongly expressed the opinion that a scientist was best remembered not by personal characteristics, but by the work he had accomplished, and that a complete list of publications was the best indication of what a man had achieved in his research. We, therefore, concentrate mainly on Keilin's scientific achievement and give as complete a list as we can of his publications, arranged in chronological order. On the more personal side our own recollections have been enriched by those of his numerous friends and collaborators in various scientific departments in Cambridge and elsewhere. To his widow, Dr Anna Keilin and his daughter, Dr Joan Keilin, we are indebted for the particulars of his early life before he came to Cambridge in 1915.

David Keilin was born in Moscow on 21 March 1887. His parents, who were of Polish nationality, were then temporarily living in Moscow but soon returned to Warsaw. David was the fourth of their seven children. His father was a business man and small landowner and his mother was a wise and energetic woman. The parents were devoted to their children and sought to give them a good education. David was not a robust child and suffered from asthma which persisted throughout his life and was always a severe handicap to him. Until the age of ten he was taught at home by a governess. For the next seven years he went to the Górski School, a well-known private school. There he proved to be an average pupil in most subjects but showed an aptitude for literature, mathematics and languages, studying Russian,

French and German in addition to his native Polish. The mastery of languages was of much help in his scientific career as it made much scientific literature easily accessible to him and fostered the close personal contacts he maintained with other scientists from all over the world. He also derived much pleasure throughout life in reading the works of great authors in the original language.

David Keilin began his university career in 1904 in Belgium at the University of Liège. There he read Natural Sciences, including the elements of logic and philosophy, and one of his teachers was Edward van Beneden. He obtained the Diplôme de la Candidature des Sciences Naturelles, having completed a two-year course in one year and, in July 1905, he moved to Paris There he at first occupied himself by reading at the Bibliothèque St Geneviève, attending lectures on philosophy by Professor Henri Bergson at the Collège de France and visiting art galleries. A chance encounter with Professor Maurice Caullery at the Laboratoire d'Évolution des Êtres Organisés led to his entering upon a career in biology. One day a heavy rainstorm made him seek shelter in a building and he found his way into the room in which Professor Caullery was lecturing. He was so deeply impressed with the lecture that he continued to attend the course three times weekly and thereby became acquainted with Caullery, and the two men started a life-long friendship. Keilin always acknowledged that Caullery had greatly influenced his scientific career and it was at his invitation that he began biological research in the Laboratoire d'Évolution and, acting on his advice, that he entered the Sorbonne to study zoology under Professor Alfred Giard, geology under Professor E. Haug, and also botany and embryology. Keilin had now found his real life-interest and he quickly became not only a laboratory biologist but an ardent field naturalist; he collected enthusiastically in the neighbourhood of Paris and also, during the vacations, at the Marine Stations at Wimereux, Roscoff and Banyuls. Though in later years he became more and more restricted to bench work he never lost his interest in field work and to him the living animals were always the real object of study.

Keilin continued to work in the Laboratoire d'Évolution until 1915 when he moved to Cambridge. His work in Paris was mainly on insects but here again his field of study was determined by chance. Caullery had suggested that he should start his research by investigating the protozoa parasitic in earthworms. While he was collecting material for this purpose, Keilin came across many other organisms, both free-living and parasitic, and he became especially interested in the life-cycles of dipterous flies and the adaptations of their immature stages to the parasitic mode of life. These studies were incorporated in a thesis for which he was awarded the D.Sc. Degree in the Sorbonne in 1915.

During the First World War Keilin came to Cambridge, at the instigation of Stanley Gardiner, then Professor of Zoology in the University, to join G. H. F. Nuttall, who was then Quick Professor of Biology. Keilin arrived in Cambridge in February 1915 and he was given a Research Studentship in Medical Entomology to work with Nuttall in the Quick Laboratory, and, in the following year, 1916, he was appointed Assistant to the Quick Professor. He joined Magdalene College, thus beginning an association which lasted to the end of his life and which always gave him great pleasure and satisfaction through the social and intellectual contacts he made, both with distinguished workers in all fields of knowledge and with the younger members of the College. He took the B.A. Degree in the University of Cambridge by research and in due course became M.A.

In those days the Quick Laboratory in which Keilin joined Nuttall and his co-workers, was housed in one room in the old Department of Pathology situated at the corner of Downing Street and Corn Exchange Street. Conditions there were very difficult owing to over-crowding, inefficient ventilation and lack of equipment. However, Keilin found there a congenial group of very active workers and conditions for work changed drastically when, in 1921, the Quick Laboratory moved to the newly built Molteno Institute for Research in Parasitology. The building and equipping of this Institute had been made possible by a munificent gift of Mr and Mrs P. A. Molteno. It was located on the Downing Site, and in those days it was pleasantly situated in restful, almost rural, surroundings.

In spite of the improved laboratory conditions, Keilin remained very insecure financially for some years and had to depend on various research grants for support. He held first Junior and then Senior Beit Memorial Fellowships from 1920 to 1927, and, although he was appointed University Lecturer in Parasitology in 1925, his meagre University stipend had to be supplemented by grants from other sources. Nevertheless, these years were very productive scientifically and he published many important papers in rapid succession, including not only many on insects and protists but also his main paper on the discovery of cytochrome, discussed later.

In 1931 Professor Nuttall retired and Keilin was appointed to succeed him as Quick Professor of Biology, but in conformity with a change in Keilin's work towards more biochemical lines, the subject of the Professorship was changed to 'Study of the Biology of the Cell'. At the same time Keilin was appointed Head of the Department of Parasitology and Director of the Molteno Institute, the title of which was changed in 1934 to 'Molteno Institute of Biology and Parasitology'. He continued to hold these posts until he reached the retiring age in 1952. Keilin now had a securely established position although the funds for research purposes were still limited. However, generous grants from the Rockefeller Foundation provided him with money for assistance and equipment, and additional support was given at times by the Agricultural Research Council, the Medical Research Council and the Royal Society.

Although he retired from his University offices in 1952, Keilin did not decrease his scientific activity, and he continued to work in the Molteno Institute, free from administrative commitments, for the rest of his life. During this period he received financial support from the Nuffield Foundation and, later, from the Wellcome Trust. These eleven years were devoted to his research and to writing a book on *The History of Cell Respiration and Cytochrome* which was nearly completed at his death and which has been published posthumously. David Keilin died suddenly on the afternoon of 27 February 1963 after a morning spent in the laboratory engaged on his usual activities with his customary energy and cheerfulness. Thus the end came when he was still in full activity mentally and physically and with much still unaccomplished, but with a record of scientific achievement which it would be difficult to exceed in one life-time.

It is curious that it was in 1925, the year of Keilin's appointment to a University Lectureship in Parasitology, that the orientation of his work changed and thereafter became progressively more biochemical, whereas previously it had been mainly more in the nature of descriptive biology. The publication of his first paper on cytochrome in 1925 marks the point of divergence of the two lines of research. In

considering his scientific work his career falls into three periods: (1) Paris 1909–15; (2) Cambridge 1915–25; (3) Cambridge 1925–63.

Paris, 1909–15: Research on insects and protozoa

As will appear later Keilin's discovery of cytochrome was an outcome of his interest in the respiration of insects. It is, therefore, noteworthy that his interest in insects was stimulated by an event which, at the time, must have been a bitter disappointment to him. As has already been mentioned, Caullery suggested that Keilin should start research on the protozoa parasitic in earthworms and he spent several years working assiduously on this subject; but before he had published any of the work it was forestalled by the appearance in 1909 of a monograph on the subject by Hesse ('Contribution à l'étude des monocystidées des oligochètes', *Archs. Zool. exp. gén.* **43**, 27–301). In the course of this work Keilin had collected material extensively but he found a very fruitful and convenient collecting ground in the small garden of the Laboratoire d'Évolution, which was situated near the Panthéon and was surrounded by the large built-up area of the Quartier Latin. He found that this small area formed a virtual oasis where many organisms lived and reproduced in great numbers in close proximity, so that transmission of parasites from host to host was facilitated and it was easy to collect different stages of organisms and their parasites at will. Keilin later maintained that such limited habitats were almost analogous to culture conditions and he attributed much of his success in finding hitherto undescribed organisms and elucidating their life-cycles to the intensive examination of material from restricted habitats.

Among the earthworms he collected he found that those of one species, *Allolobophora chlorotica* were often parasitized by dipterous larvae and when he bred out adult flies from these larvae they proved to be a very common cosmopolitan fly, *Pollenia rudis*, the cluster fly. This fly frequently invades houses in great masses in autumn and remains clustered in dormant masses throughout the winter. In spite of its prevalence and wide distribution, the life-cycle of this fly was completely unknown and it had long been regarded as an entomological mystery. To counteract his disappointment over his work on the protozoa of earthworms Keilin turned to the study of the insect material he had gathered but which he had hitherto regarded as of less importance.

Keilin's first scientific publication was a short note on the parasitism of the larval stages of *Pollenia rudis* in the earthworm *Allolobophora chlorotica* published in 1909. This was the basis of his work on the larval stages of Diptera and their parasites. As his work on *Pollenia rudis* was such an important phase in his scientific career and aroused great interest among entomologists all over the world a brief account of his results will be given.

Keilin found that the mature female *Pollenia rudis* deposits eggs in the soil in late August or early September. There they hatch in 4–5 days and the young first-stage larvae burrow in the soil until they come in contact with a suitable earthworm. The larva, on meeting a suitable worm, enters the vesicula seminalis through the male genital pore and there it ceases activity and begins a period of dormancy, or diapause, which lasts throughout the winter. The following spring, about the end of April or beginning of May, the larva becomes active and migrates to the anterior end of the worm. When it reaches the prostomium of the worm, the posterior

end of the larva is directed forwards and ruptures the wall of the prostomium so that the post-abdominal spiracles of the larva are in contact with the air. Now that it has access to an ample supply of oxygen, the larva begins to grow rapidly, feeding on the earthworm which has become inactive and no longer feeds. The larva feeds voraciously, grows rapidly, undergoes two moults and becomes a third-stage larva in about 10 days. Meantime the larva has gradually become free from the body of the worm, only the posterior segments of which remain unconsumed. Then the fully grown larva becomes detached, burrows deeper into the soil and pupates. The pupal stage lasts 35–45 days and then the adult fly emerges, makes its way to the surface and so completes the life-cycle. The demonstration by Keilin that this abundant widely distributed and familiar fly had such a strange and complex life-cycle was received by entomologists all over the world with amazement and, at first, in some cases with frank incredulity; but soon the correctness of the account was generally accepted. Keilin had found that the winter diapause of the larva was caused by low temperature and that the quiescent larvae could be reactivated at any time by placing the worms containing the inactive larvae at a temperature of 19–21°; under such conditions flies emerged in the laboratory as early as January.

Consideration of the life-cycle of *Pollenia rudis* stimulated Keilin's interest in two themes which were to dominate his future work. These were: (1) the adaptations of dipterous larvae to the parasitic mode of life; (2) the overriding importance of respiratory requirements in controlling the life-cycle of parasitic and free-living insects. These factors are well exemplified in the life-cycle of *P. rudis* in which the larva does not grow during the period of quiescence in the genital organs of the earthworm or during the migration to the prostomium while the supply of oxygen is limited but, once the spiracles communicate with the air, ample oxygen is available and rapid growth begins.

In the meantime Keilin had also studied the comparative morphology and biology of many other groups of Diptera and had published a number of important papers detailing the results of his studies. The most important results of his work in Paris were collected in a thesis which was submitted for the D.Sc. Degree of the Sorbonne in 1915 and was published under the title 'Recherches sur les larves de Diptères cyclorhaphes: I. Cycle évolutif de *Pollenia rudis* Fabr. parasite d'*Allolobophora chlorotica* Sav. II. Biologie comparée des larves de Diptères' (*Bull. scient. Fr. Belg.* **49**, 15–198).

Among the many interesting generalizations which emerged from this period of his work a few of the more important may be mentioned here. He emphasized the almost universal occurrence of three larval stages in cyclorrhaphous Diptera and the close correlation of the morphology of the larvae with the mode of life, especially as regards the cephalo-pharyngeal armature, so that it is usually possible to determine the mode of life of the larvae from an examination of the mouthparts. A very interesting discovery was the presence on the ventral surface of the thoracic segments of the dipterous larvae of three pairs of groups of sensory hairs which were associated with the openings of the stalks of the imaginal buds of the legs of the adult flies. Keilin suggested that all external organs, locomotory or otherwise, have in addition to their obvious function, a primary sensory one and that when, through parasitic adaptation or otherwise, the main organs are lost, vestiges of them remain in the form of sensory structures, such as setae or papillae. He also believed that the

lack of external appendages and the very simple sensory organs and some other structural features indicate that all Cyclorrhapha have, in the course of their phylogeny, passed through phases when the larvae were adapted to a parasitic mode of life, during which external appendages and mouthparts were lost and sensory organs were greatly reduced; and that the existing free-living dipterous larvae have become so by secondary adaptations. The secondary adaptation to the free-living mode of life has led to the development of new methods of locomotion and new, cuticular mouthparts and so on; thus, in Keilin's view, following Dollo's law of the irreversibility of evolution.

Cambridge, 1915–1925: Research on insects and protists

During his early years in Cambridge Keilin's work continued along the same general lines upon which he had been engaged in Paris. It dealt largely with insects, with special attention to life-cycles and adaptations in parasitic dipterous larvae. Again his capacity for exploiting specially favourable sources of material was shown by intensive study of the larvae and their parasites which are found in slime-fluxes and rot-holes of trees. The ten years after his arrival in Cambridge were scientifically very productive and papers on very diverse themes appeared in rapid succession. Among them were important comparative studies of the immature stages of many different families and genera of Diptera. During the First World War the Quick Laboratory undertook work of importance to the armed forces, especially the study of human lice, and Keilin joined in this work. An outcome of this work was the publication by Nuttall and Keilin of a paper on 'Hermaphroditism and other abnormalities in *Pediculus humanus*'. They found that hermaphroditic forms might amount to 20% in families of lice derived from crosses between *P. corporis* and *P. capitis*, whereas in populations of 'wild' lice hermaphrodites amounted to only 0·2–8% of the lots in which they were found. Consequently Nuttall and Keilin concluded that the hermaphrodites which sometimes occur in wild populations arise from fortuitous crosses between *P. corporis* and *P. capitis*. Nuttall and Keilin intended to produce a large monographical study of *Pediculus humanus* and they had done much work on the subject when other interests diverted both of them so that the work was not completed. In its incomplete form it was published in 1930 as 'Iconographic studies of *Pediculus humanus*', a notable feature of which is the beautiful illustrations, many of which were drawn by Keilin.

During this period Keilin had been accumulating material for a general survey of the biology and morphology of the early stages of Diptera. This work was written in French in preparation for publication but it was then put aside and not published until 1944 when it was re-written in English and the literature citations brought up-to-date. This important work appeared under the title 'Respiratory systems and respiratory adaptations in larvae and pupae of Diptera'. It was divided into two parts 'Part 1. Respiratory systems in dipterous larvae', and 'Part 2. Respiratory adaptations of larvae and pupae of Diptera'. In this paper Keilin embodied much original work and put forward many new ideas and generalizations on the structure and adaptations of the respiratory systems in Diptera. In addition, all the important work on the subject was summarized and critically discussed. Although this paper forms a veritable landmark in the literature on the immature stages of Diptera, it was originally designed by Keilin to be but a preliminary to an extended study of

the physiology of insect respiration. The reasons why this plan never reached fruition were given by Keilin himself in the introduction to the paper. He wrote (page 2):

> I should like to add that this paper represents the results of work of more than 20 years and forms a part of a more ambitious plan of investigation covering both the morphological and physiological aspects of insect respiration. The biological and morphological study of the respiratory organs of larvae required, however, continual collection of fresh living material, long periods of observation, breeding of immature larvae, numerous dissections, reconstructions of complicated structure and numerous drawings. All this work absorbed much of my time and was continuous only during the first few years of this investigation. It began soon to suffer unavoidable interruptions increasing in frequency and duration, and had to be abandoned finally when certain purely biochemical aspects of insect respiration compelled me to enter a much wider, although intimately connected, field of research on intracellular respiration. The physiological and biochemical aspects of insect respiration have been, however, purposely excluded from the present study in the hope that they will be dealt with separately in papers published elsewhere.'

In fact, the further work he did on this subject was incidental to his studies on intracellular respiration and he did not publish any papers dealing with the physiology of insect respiration. Nevertheless, his previous studies had led him to the general conclusion (page 57) that 'The dominating factor in the respiratory adaptation of parasitic dipterous larvae is that usually they cannot feed and grow actively without bringing their highly developed tracheo-spiracular system into communication with air. This is reflected in certain structural features of these larvae, their behaviour within the host and their life histories.' Having studied the mechanisms for ensuring an adequate supply of oxygen, it was logical that Keilin should wish to carry the work a stage further and investigate the utilization of oxygen when it entered the organism, or in other words to undertake the study of intracellular respiration. This was the starting-point of his studies on the biology of the cell which occupied him for the rest of his life.

Before passing to an appreciation of his work on cellular biology some account must be given of his contributions on the morphology and biology of protists. Although, as already mentioned, Keilin's first research was intended to be on the protozoa parasitic in earthworms, the work that he published on the subject arose incidentally to his studies on dipterous larvae. It was accomplished between 1914 and 1923, and the results were published in 11 papers. No less than 8 new genera and 12 new species of protists were described, all of them parasitic in larvae of insects. He had found them when he was collecting material for his entomological studies and his success in finding so many new organisms he attributed largely to his concentrated study of limited habitats. This is exemplified by the fact that five of the new genera and seven of the new species were found in larvae living in slime fluxes from wounds in two trees growing near the laboratory. A list of the organisms described gives a clear indication of how wide was his range of interests and how ready he was to tackle problems in fields remote from his main line of research.

Fungi *Monosporella unicuspidata* gen.nov., sp.nov.
Coelomomyces stegomyiae gen.nov., sp.nov.
Helicosporidium parasiticum gen.nov., sp.nov.

Protozoa

Amoebaea *Entamoeba mesnili* sp.nov. [now *Dobellina mesnili*]

Gregarinomorpha	*Leidyana tinei* sp.nov.
	Dendrorhynchus systeni gen.nov., sp.nov.
	Caulleryella aphiochaetae gen.nov., sp.nov.
	Allantocystis dasyhelei gen.nov., sp.nov.
	Schizocystis legeri sp.nov.
	Lipotropha macrospora gen.nov., sp.nov.
	Lipotropha microspora sp.nov.
Ciliata	*Lambornella stegomyiae* gen.nov., sp.nov. [now *Tetrahymena stegomyiae*]

Two of these protists are of more than usual interest. *Helicosporidium parasiticum* differed markedly from all known protists and Keilin thought that it formed a new type of organism which could not be placed within existing classification, but he temporarily placed it in the Sporozoa. Recently, however, Weiser (1964) has suggested that it is really a fungus allied to *Monosporella* and he has removed it from the Protozoa. The other species of special interest is *Coelomomyces stegomyiae*. Keilin described it from a single mosquito larva which had been collected by W. A. Lamborn in Malaya. This is another organism of uncertain systematic position which Keilin considered to be a fungus, probably belonging to the Phycomycetes, but showing some resemblance to the Chrytridinae. Since his original description about twenty-two more species have been described and named and the genus *Coelomomyces* is now classed among the aquatic fungi in the order Blastocladiales. With three exceptions all the species described are parasitic in mosquito larvae. The fungus has been found in forty-six genera of mosquitoes, but twenty-five of the species in which it occurs belong to the genus Anopheles. This fact and the wide distribution of the parasite which has now been recorded from every continent, have suggested that it might be used as a means of biological control of mosquitoes. The possible use of the parasite for biological control has aroused much interest, and experiments to test it have been made in various parts of the world. So far the results have been inconclusive and the experiments are made difficult owing to the failure to cultivate the organism and the difficulty of obtaining infection of mosquitoes under laboratory conditions.

Hitherto Keilin's work on both insects and protists had been almost entirely descriptive morphology and biology, but early in the decade 1920–30 he became more experimental in his approach to problems and this is shown by papers on the nephrocytes in larvae and pupae of the fly *Lonchaea chorea*, on the absence of paedogenetic multiplication in the blowfly (*Calliphora erythrocephala*) and on the appearance of gas in the tracheae of insects. The real change to experimental work came when he was studying the respiratory adaptations of the horse bot-fly (*Gasterophilus intestinalis*). This fly is parasitic in its larval stages in the horse. Eggs are laid by the female fly attached to hairs on the legs of horses and when the horse licks the area, stimulated by the warmth and moisture of the tongue, the eggs hatch and release a first-stage larva. The larva burrows into the mucosa of the tongue and migrates to the pharynx and from there to the stomach which it reaches about September. It has undergone one moult and is now in the second-stage and fixes itself to the mucosa of the stomach wall by its mouth parts. It grows, undergoes a second moult and develops into a third-stage larva increasing in weight from about 10 to

450 mg. or more. In early summer the mature larvae are passed out in the faeces, they burrow into the soil, pupate and the adult flies emerge after 4–5 weeks. Thus the larvae spend about seven months in the horse's stomach and during this time they get their supply of oxygen from the bubbles of air which the horse swallows with its food. The posterior spiracles are adapted for this purpose and have valves which open when they are in contact with air but close tightly when they are submerged in fluid. The larva has therefore an intermittent supply of oxygen and shows an adaptation to such conditions by having tracheal cells which contain haemoglobin. This haemoglobin enables the larvae to take up and store oxygen when contact is made with bubbles of air, so that oxygen is available for use when there is no contact with air bubbles.

It was the study of the respiratory requirements of *Gasterophilus* larvae in the stomach of the horse that led Keilin to the discovery of cytochrome and directed his future work towards cellular biology with particular reference to problems of intracellular respiration and the role therein of metalloprotein compounds. Keilin was interested as to whether the haemoglobin in the tracheal cells of *Gastrophilus* larvae derived from the haemoglobin of the horse or was synthesized by the larvae. He was studying the material spectroscopically when he noticed that, in addition to haemoglobin, another substance was present which showed a spectrum with strong absorption bands which were visible spectroscopically under reducing conditions, that is, under conditions in which the spectrum of haemoglobin would be almost imperceptible.

Cambridge, 1925–1963: Research on cytochrome and oxidizing enzymes

It was in 1923 that Keilin made the observation mentioned above, which was to direct the whole trend of his research thereafter towards biochemistry and enzymology. He was investigating the fate of the haemoglobin in *Gasterophilus* larvae which disappears during metamorphosis, and he observed with the direct-vision spectroscope that the two-banded spectrum of the haemoglobin in the larva was replaced in the adult by a completely different four-banded spectrum. At first he thought it possible that this was the spectrum of a product formed by modification from the haemoglobin which disappeared, but on examining a number of other insects, the larvae of which were devoid of haemoglobin, he found the same four-banded spectrum in the adults, showing that this could not be true. The spectrum was therefore due to a quite distinct cell pigment, appropriately named by him 'cytochrome'.

Keilin soon extended these observations to micro-organisms. It happened that he had been using pure cultures of both *Bacillus subtilis* and of baker's yeast (*Saccharomyces cerevisiae*) in experiments on the feeding of *Gasterophilus* larvae, and he found that both showed the four-banded spectrum, the yeast very strongly. Of Keilin's subsequent work on cytochrome, so much was done with yeast and with heart-muscle preparations that it is not generally realized that a great deal of the early work was done with bacteria and other micro-organisms. This is partly due to the fact that his first paper on cytochrome was deemed by the editors of the journal to which he submitted it to be too long, and most of the work on bacteria had to be removed in order to comply with editorial requirements. Later he was deterred from publishing the omitted work in full by the publication of research on the subject from

other laboratories, but some of it has now appeared in chapter 12 of his recently published book (Keilin, 1966).

In 1923–24 he devoted much time to an extensive study of the distribution of the pigment in a great variety of organisms. At that time he was not aware that cytochrome underwent oxidation and reduction. One day in the early summer of 1924, however, he was examining a suspension of yeast shaken in water, and failed to find the spectrum. While he watched, the spectrum suddenly appeared, but disappeared at once on shaking the suspension with air. This was the key, not only to the nature and function of the pigment, but also to the mechanism of the main intracellular respiratory system of aerobic organisms.

Keilin at once realized that the cytochrome was undergoing continuous reduction by reducing enzymes of the cells and re-oxidation by atmospheric oxygen. It was therefore acting as an intracellular respiratory pigment, although in a very different way from the recognized respiratory pigments such as haemoglobin. The cytochrome was in fact acting as an intermediate carrier of reducing power from the reducing enzymes to oxygen, by becoming itself alternately reduced and oxidized, thus behaving as a chemical catalyst. The haemoglobin, on the other hand, remained reduced throughout its action, merely taking up oxygen molecules and giving them up again unchanged as required, thus behaving as a physical O_2-transporter, and not as a catalyst.

At that time Keilin did not know that the four-banded spectrum had been observed previously, but a search of the older literature which he carried out shortly afterwards revealed that as far back as 1886 it had been seen in a wide variety of vertebrate and invertebrate tissues by C. A. MacMunn, who had shown that the pigment could be oxidized and reduced by chemical reagents and that the spectrum was that of the reduced form. MacMunn believed that it was a respiratory pigment of haemoprotein nature, related to haemoglobin. Keilin always freely acknowledged MacMunn's priority in the discovery. It must be remembered, however, that it was Keilin and not MacMunn who showed the real nature and significance of the pigment. MacMunn believed it to be a single compound which acted very much like haemoglobin, combining with oxygen conveyed to it in the blood, holding it for the purposes of metabolism and parting with carbon dioxide in exchange for the oxygen it took up. He believed that its respiratory capacity was far inferior to that of haemoglobin because it did not take up the oxygen in a loose combination but as a far more stable compound, and he thought that the pigment could be reinforced or replaced by haemoglobin at times when extra activity of internal respiration was required. His conception of its action was thus entirely different from Keilin's.

The four-banded spectrum was a difficulty at first, since there were no substances giving such spectra among haemoglobin derivatives. Keilin soon showed, however, partly by comparative studies, partly by extraction, partly by the use of inhibitors and partly by the preparation of derivatives, that the spectrum was not that of a single substance, but was due to a mixture of several rather similar pigments, each giving a spectrum of the typical haemochrome type with two bands in the visible region. He thought at first that the normal cytochrome complex consisted of three such haemochromes, which he called 'components *a*, *b* and *c* of cytochrome', in descending order of wavelength of their main visible bands. At that time he used the

name 'cytochrome' for the complex as a whole, but later the components were regarded as separate cytochromes and called 'cytochrome *a*' 'cytochrome *b*' and 'cytochrome *c*'. It was only about 15 years later that he was able to show that the normal cytochrome complex consisted not of three but of five distinct cytochromes, called *a*, a_3, *b*, *c* and c_1, the 'a' and the 'c' bands of the spectrum each being due not to one but to two cytochromes with superimposed bands.

Keilin's extensive survey of the cytochrome spectra of a wide variety of cells showed that what may be called the normal spectrum, due to the cytochromes just mentioned, occurs in aerobic cells of a great many different kinds, in animals, plants and micro-organisms. There were, however, a number of departures from this normal grouping, especially among bacteria. Although some bacteria showed the normal spectrum, in others one or more of the above-mentioned cytochromes was either missing or was replaced by one with a different spectrum. In fact, including more recent observations by many other workers, the number of known cytochromes has now increased to over 30 (see the list given by Dixon & Webb, *Enzymes*. 2nd ed. (1964), London: Longmans). Most of these, however, occur in particular situations and have specialized functions not forming part of the respiratory system studied by Keilin.

The story of how, starting from the simple observation of the normal cytochrome spectrum of aerobic cells, Keilin developed the whole picture of the nature and mechanism of the main cell-respiration system, is a fascinating one. It is rare in science that a whole field is charted in all its essentials by one man, as this was by Keilin. At the same time it should not be overlooked that the scientific climate of the neighbouring biological departments at Cambridge was at that time particularly favourable for the development of work on haemoproteins and the mechanisms of cell-respiration. In the Department of Physiology, J. Barcroft was completing his great work on haemoglobin, and was in close touch with Keilin during the early studies on cytochrome. Also H. Hartridge had just completed the development of the reversion spectroscope, and had used it for the study of the combination of haemoglobin with carbon monoxide, an inhibitor of respiration which was to become of great importance to Keilin's work. In the Department of Biochemistry, Frederick Gowland Hopkins had recently put forward the idea of intermediate carriers of reducing power in relation to glutathione (which, however, did not turn out to be the main carrier in cell-respiration, as he had hoped), R. Hill had acquired an expert knowledge of the chemistry and spectroscopy of pigments, especially of derivatives of haemoglobin and haemochromes, while M. Dixon was working on oxidizing enzymes and on the development of manometers for the study of respiratory systems. Both of these biochemists developed close relationships with Keilin not long after the discovery of cytochrome, collaborating in several papers, and they remained in close contact with him along their lines of mutual interest for the rest of his life. Thus there were many lines of work proceeding at that time which presented aspects particularly helpful and relevant for the study of haemoprotein catalysts of cell-respiration and oxidation. Keilin availed himself of contributions from all these lines, bringing them together in a masterly fashion in the synthesis of the general picture of the action of the cytochrome system.

If the scientific atmosphere was favourable for this work, however, the conditions

of work were not. For the next 10 years he had to pursue the research on cytochrome with very little financial support or assistance. He had to carry out the work almost single-handed, apart from some help from P. T., R. H. and M. D. In 1934, however, he was joined by E. F. Hartree, who was to be his collaborator for the next thirty years, and with whom he was to publish no less than 45 joint papers. From then on conditions improved and he was joined by a succession of collaborators, among whom may be mentioned especially T. Mann and E. C. Slater.

For the study of the mechanism of the respiratory system, Keilin naturally selected biological material which the spectroscope had shown to be particularly rich in cytochrome. Consequently a large part of the work was carried out either with baker's yeast or with heart-muscle preparations. He used two main techniques, the manometric measurement of oxygen utilization, by which he could follow the overall action of the system, and the spectroscopic observation of the oxidation and reduction of the various cytochromes, by which he was able to observe the working of the intermediate steps of the mechanism. His success was largely due to the fact that he continually coordinated the results of the two methods.

Since the materials were optically dense and opaque, the usual spectroscopic methods could not be used, and it was necessary to adopt the technique (also used by MacMunn) of concentrating a strong beam of light onto the under side of the object, collecting as much as possible of the scattered light emerging from the upper side and observing the spectrum with a low-dispersion direct-vision spectroscope. This was achieved with a microscope with a substage condenser and a spectroscopic eyepiece, and it gave Keilin some satisfaction to be able to claim that he had observed all the essential features of the system with this simple and inexpensive outfit, and to point out that a more elaborate high-dispersion instrument would have been useless for the purpose, since a low dispersion is essential to show up the absorption bands clearly by optical contrast.

Heart muscle contains large amounts of myoglobin, which tends to obscure the cytochrome spectrum. It was therefore necessary to remove this by extracting the minced muscle with large volumes of water; the myoglobin was extracted, but the cytochrome system remained in the insoluble part of the tissue. The water extraction had another beneficial effect, shown by Thunberg a few years before in his studies on the oxidizing enzymes (dehydrogenases) of muscle, namely that it removed the endogenous oxidizable substances (substrates) and therefore the 'blank' respiration, and made it possible to study the oxidation of particular substrates by restoring them one by one. The heart-muscle preparation was later much improved and became known as the Keilin–Hartree preparation; it has been extensively used by many workers. We now know that it consists of a suspension of minute particles derived by fragmentation of the mitochondria, but at the time of Keilin's early work on the system mitochondria had not been isolated and it was not known that they had a special connection with respiration, still less that the cytochrome system was located within them. In fact Keilin did comparatively little work with intact mitochondria.

The situation at the time of Keilin's discovery of cytochrome was that on the one hand it was known that the tissues contained dehydrogenases which had been shown to catalyse the oxidation of their substrates by artificial hydrogen acceptors, though not by oxygen; and on the other hand Warburg had shown that in respira-

tion the oxygen reacted in the first place with an iron-containing catalyst, the 'respiratory ferment', which showed certain characteristic reactions with several inhibitors, notably cyanide. It was fairly clear that these two systems formed the two ends of the respiratory mechanism, but between them there was a gap in knowledge. Keilin bridged that gap by showing that the cytochrome became reduced on the addition of substrates, especially succinate, and the reduced cytochrome became re-oxidized on the admission of oxygen. It was shown that the cytochrome was not reduced by the substrates alone without the dehydrogenases, and it was not re-oxidized by oxygen alone, but required a special enzyme to catalyse this oxidation. This enzyme was identified with an oxidase already known under the name indophenol oxidase, and this name was used by Keilin in his early papers; it is now called cytochrome oxidase, a name suggested in 1929 by M. D.

Within two years, Keilin had identified this oxidase with the respiratory ferment of Warburg, on the grounds of their identical behaviour towards inhibitors. The evidence from carbon monoxide was particularly convincing. This inhibits by competition with oxygen, and it was possible by kinetic measurements to determine a constant related to the affinity of the catalyst for the inhibitor. Moreover, this constant is greatly affected by light of certain specific wavelengths, but relatively little by other wavelengths. In all these respects the oxidase behaved precisely like Warburg's catalyst. Since the latter had already been shown to be responsible for the respiration, at any rate of the cells studied, it followed that the respiration of these cells was due to the oxidation of cytochrome by the oxidase. It was later possible to measure the rate of oxidation and reduction of the cytochrome in the respiring cells and to show that it corresponded with the rate of oxygen consumption. There could therefore be no doubt that in cytochrome Keilin had found the main intracellular respiratory carrier.

This, however, raised a problem, since cytochrome was not a single substance but a mixture of several quite distinct substances, all of which normally appeared to be oxidized and reduced simultaneously. Did they act independently in parallel, each bridging the gap between the dehydrogenases and the oxidase? Or did they act in series, forming a carrier chain in which each component reacted only with the one next to it? And if so, in what order were they arranged in the chain? Owing to two fortunate properties of the system, Keilin was able before long to show that the cytochromes do in fact act in series as a chain of carriers, but it has taken many years of patient work to establish that the order is:

$$\text{dehydrogenases} \rightarrow \ldots \rightarrow b \rightarrow c_1 \rightarrow c \rightarrow a \rightarrow a_3 \rightarrow O_2$$

and even now not every worker will agree on all points. It is not possible here to give the evidence for this order in detail.

The first favourable circumstance was that urethane, one of the inhibitors used by Warburg, was found by Keilin to act not at the right-hand end of the above chain (as does cyanide, stopping the oxidation of all the components), but between cytochromes *b* and c_1. In the presence of succinate and oxygen, therefore, the addition of urethane caused *b* to become completely reduced and all the others to become completely oxidized. At the same time the oxidation of the succinate by oxygen became inhibited. The cutting off of *b* from oxygen and from the other cytochromes (but not from the succinate) and the cutting off of the other cytochromes from the

succinate (but not from oxygen) could hardly be explained if they were acting in parallel, but was readily explained if they formed a chain. The second circumstance was that one of the cytochromes, namely *c*, is soluble in water and can be extracted while the others remain. The preparation is then no longer capable of oxidizing several substances, but the oxidation power is regained when cytochrome *c* is restored, showing that the oxidations went through *c* but could not be catalysed by the others apart from *c*. It could be shown that the oxidation of reduced *c* went through the *a* cytochromes, but was not inhibited by urethane. Again, this could hardly be explained in any other way than by a chain of carriers. Much evidence has accumulated since.

Two things were quite new about these results. One was the idea of haemoproteins as carriers of reducing power, acting by becoming alternately oxidized and reduced, an entirely new function for haemoproteins. The other was the idea of a chain of successive carriers rather than a single carrier. The purpose of this arrangement only became clear much later from studies on oxidative phosphorylation by other workers. Keilin did not study this aspect of the system, largely because the Keilin–Hartree preparation had lost the power of phosphorylation possessed by intact mitochondria.

The haemoprotein nature of the cytochromes had already been deduced by Keilin from spectroscopic studies not only of the unmodified compounds but also of a number of derivatives formed from them by chemical reagents. The isolation of cytochrome *c* in the pure state greatly contributed to the study of their nature through direct analysis. Recently the complete chemical structure of its molecule has been determined.

At the time that we have been considering, neither of the cytochromes a_3 or c_1 was known. Their existence was established by Keilin by spectroscopic observations much later, for a_3 in 1938 and for c_1 in 1949. The discovery of a_3 in particular led to important results. Keilin had believed that none of the cytochromes of the respiratory system combined with carbon monoxide. He noticed, however, that when the reduced preparation was exposed to CO a shading appeared on one side of the main visible band of cytochrome *a*. A more marked effect was produced on the band in the ultraviolet region which had been attributed to cytochrome *a*. Keilin interpreted this effect as indicating that the *a* spectrum was due to two *a* cytochromes with bands in the same positions, and that only one of these could combine with CO, the combination with CO displacing the spectrum towards shorter wavelengths, where it was no longer masked by the bands of the unchanged component. He retained the name cytochrome *a* for the latter component, and called the one which combined with CO cytochrome a_3, a_1 and a_2 having been already used for cytochromes which occur in micro-organisms.

Not all workers found the spectral evidence for the existence of cytochrome a_3 immediately conclusive, and for many years, in fact almost up to the present time, it has been doubted by some. Recently, however, convincing evidence has been obtained in a number of different ways.

The fact that a_3 appeared to be the only component which could combine with CO, the characteristic inhibitor which had been shown to combine with cytochrome oxidase, led Keilin immediately to consider whether a_3 could itself be the oxidase. At first he rejected the idea, but he later became convinced of its truth. The fact

that CO had been shown to compete with oxygen was evidence that a_3 reacted directly with O_2; moreover, the oxidase was known to combine with cyanide, and Keilin showed that cyanide produced changes in the *a* part of the spectrum while leaving the other parts unaffected. Strong evidence was the fact that those particular wavelengths of light which had been found by Warburg to dissociate the CO-compound of the respiratory ferment corresponded exactly with the absorption bands of the CO-compound of cytochrome a_3. Recently conclusive evidence has been obtained by other workers from the study of pure preparations of cytochrome oxidase.

This identification means that the earlier statement, that the cytochrome system links the dehydrogenases with the respiratory ferment or cytochrome oxidase, is not quite accurate. Since the latter is itself one of the cytochromes, it would be more correct to say that the cytochrome system links dehydrogenases with oxygen. It has now become clear also that still other carriers come between the dehydrogenases and the first of the cytochromes.

Keilin had noticed that the position of the absorption band attributed to cytochrome *c* in the heart-muscle preparation did not quite correspond with the band of extracted and purified *c*. He tested a number of possible explanations of this effect, and came to the conclusion in 1949 that the only possible one was that the band was actually due to the fusion of two bands differing slightly in position, one that of cytochrome *c* in its normal position and the other that of a new cytochrome. An optical mixture of reduced cytochrome *c* with the preparation in the oxidized state (and therefore not showing the band of the new cytochrome) gave the *c* band in its normal position. Cytochrome *c* is more thermostable than the others and it was found that after heating the heart muscle preparation its band now appeared in the normal *c* position (presumably owing to the destruction of the new component but not of *c*).

Keilin then applied the technique which he was then developing for intensifying and sharpening absorption bands by cooling to liquid air temperature, and found that the two *c* bands were now separated and the band of the new cytochrome could be clearly seen between the bands of *b* and *c*. A band had been seen in this position a few years previously by Japanese workers and had been ascribed by them to a new cytochrome c_1, but this had been attributed by others to denatured haemoprotein. As Keilin had now confirmed their observations, he called the new cytochrome c_1, and showed that it was an essential component of the chain of cytochromes.

The work on the cytochrome system, which did so much to elucidate the mechanism of cell-respiration, was Keilin's greatest contribution to biological chemistry. But he by no means confined his work to this main line; at every stage he branched out from it into related investigations, doing most distinguished researches in many fields of biochemistry and enzymology.

For example, he carried out extensive studies on other haemoproteins, including methaemoglobin and its compounds with H_2S, H_2O_2, NO, etc., and the haemoprotein enzymes catalase and peroxidase. He investigated the nature and behaviour of the prosthetic groups of these two enzymes, and the mechanism of their catalytic action; he also discovered that catalase had the ability to oxidize ethanol by H_2O_2, and showed that the system aldehyde oxidase plus catalase brought about

a very interesting cyclic oxidation of ethanol by oxygen. He also discovered haemoglobin in certain plants, yeast, moulds and protozoa.

He had obtained some indications that cytochrome oxidase contained copper atoms as well as haem groups, and this led him to study the properties of other copper-proteins. In 1938–39 he purified the two plant phenol oxidases, catechol oxidase of mushrooms and laccase, showed that they were both copper-protein enzymes, studied their specificity and mode of action, and showed that the former enzyme was strongly inhibited by CO but the latter was not, although they are both inhibited by cyanide. He found, moreover, that the inhibition of the former by CO was unaffected by light.

He also isolated two crystalline copper-proteins, haematocuprein and hepatocuprein, from blood and liver respectively. Twelve years earlier he had studied turacin, a copper-porphyrin pigment of the feathers of certain African birds, and had compared it with haematin. In addition, he studied the properties of urate oxidase, now known to be a copper-protein, and showed it to be inhibited by cyanide but not by CO.

Furthermore, he studied a non-oxidative metalloprotein enzyme, carbonic anhydrase of blood, which he purified and identified as a zinc-protein. He further made the interesting observation that this enzyme is strongly inhibited by sulphanilamides.

A number of oxidases apart from those already mentioned were also studied, particularly glucose oxidase of moulds. This enzyme was already known to be a flavoprotein, but he identified its prosthetic group as flavin-adenine dinucleotide. With this enzyme he carried out one of the best studies of specificity ever done with any enzyme, showing the extraordinarily high degree of adaptation of its active centre to fit every part of the glucose molecule. During the course of this work he discovered and studied another enzyme, mutarotase, which catalyses the interconversion of the two forms of glucose. In addition he carried out experiments on coupled oxidations brought about by peroxide formation by several flavoprotein oxidases.

Apart from all this work Keilin made a number of simple but valuable contributions to the technique of spectroscopy and manometry. The intensification and sharpening of absorption bands by cooling in liquid air has been particularly important. During work on this he rediscovered the strong absorption bands in the visible region due to the O_4 molecule, which had been largely forgotten. In 1958 he devised a method for correcting for light scattering errors the absorption spectra obtained with suspensions of pigmented particles. He also devised a spectrophotometer cell from which oxygen could be removed by evacuation, as well as a widely used modification of the Thunberg tube in which substances could be added under anaerobic conditions. For manometry, the small 'Keilin cup' for adding substances quantitatively without disturbing the temperature of the vessel has been invaluable, and an ingenious tap for introducing alkali into the vessel during an experiment made possible a method for measuring respiratory quotients.

Cambridge, 1915–1963: General activities and personal qualities

Although Keilin ceased work on parasitology soon after he entered the field of cellular biology, he maintained a deep interest in the subject and published a number of papers, alone or in collaboration, embodying results of work undertaken years before but put aside when he became increasingly preoccupied with the bio-

chemical aspects of respiration. Thus he became editor of *Parasitology* in 1934 and in spite of increasing demands upon his time and energy by other activities he continued to edit the journal until his death. For many years he lectured on parasitology to candidates for the Cambridge Diploma in Tropical Medicine and Hygiene until the Diploma was discontinued in 1931. He had also given short courses for candidates reading pathology and zoology in the Natural Sciences Tripos Part II and he continued to give these courses until 1936. Thereafter the teaching in parasitology was taken over by a newly appointed lecturer in parasitology (P. T.). In 1937 Keilin began a series of lectures on 'Respiration and respiratory carriers' which soon increased to a course of 16 lectures illustrated with striking and meticulously prepared demonstrations. This course was very popular and attracted large audiences not only of undergraduates but also of research workers from many other departments in the University. The demonstrations were prepared in conjunction with E. F. Hartree who, in later years, gave some of the lectures together with E. C. Slater, R. Hill and P. Nicholls. Keilin's lectures were models of lucidity and he spared no pains in preparing them. He particularly valued his teaching because it brought him into close contact with young workers. A notable feature of Keilin's character was his interest in, and sympathy for, young workers and, although his formal lectures were always stimulating, he was at his best among a group of young workers discussing informally problems arising out of their research. Keilin had the gift of rapidly getting to the root of a problem and of suggesting fresh lines of approach towards the solution of the most intractable problem.

The wide range of his scientific interests was shown not only in his own research but in the way he encouraged and helped the development of new teams of workers both in his own department and elsewhere. Thus, he was instrumental in the formation of the Medical Research Council's group for 'Research in Chemotherapy' under Dr A. Bishop, and for Dr H. Laser's work on 'Radiobiology'. Another group of workers who owed much to Keilin in their early struggles to establish themselves are the group who, under Dr M. F. Perutz and Dr J. C. Kendrew, formed the basis of the present Medical Research Council's Laboratory for Molecular Biology. Dr Perutz in a letter to *The Times* a few days after Keilin's death wrote of the debt they owed to Keilin for being one of the first to see the potentialities of their physical approach to biochemistry and for giving them bench space in the Molteno Institute and helping them to grow protein crystals; and, later, for the part he played in ensuring the continuation of their work under the Medical Research Council. The Agricultural Research Council's 'Virus Research Unit', under Dr Kenneth Smith and Dr R. Markham, was much helped and encouraged by Keilin in its early work and for many years had laboratory accommodation in the Molteno Institute. Keilin also aided the development of Dr T. Mann's work which led to the formation of the Agricultural Research Council's 'Unit of Reproductive Physiology and Biochemistry'.

Although Keilin took a full share in serving on Boards and Committees of the University and of Magdalene College and, especially during the war years 1939–45, on Government and Royal Society Committees, he was essentially a research worker and he was never happier than when he was busy manipulating apparatus or working at his microspectroscope at the bench in his laboratory. Owing to the high standard he set himself he found writing tiresome and he would frequently express

his satisfaction, after a period of writing, at returning to work at his bench 'for a rest'! He had the capacity to work long hours with great concentration and even towards the end of his life he customarily spent 10 hours or more in the laboratory each day and, in addition, he did much reading and writing at home. He always maintained that his ideas came to him best when he was actually handling material and performing experiments in his laboratory. He died quite suddenly while he was attending a Board Meeting after he had spent an active and happy morning working in his room in the Molteno Institute.

In spite of his devotion to his own research, Keilin always had time to see members of the Institute or those from other University Departments or visitors from elsewhere and to listen sympathetically to their troubles and difficulties, whether scientific or personal, and to give them encouragement and advice. He was always very mindful of the human being within the scientist and with him the youngest research worker was as sure of a patient hearing and kindly advice as were the most distinguished scientific personalities. He had a delightful sense of fun and liked to tease, in a good-natured way, his friends, especially if they felt themselves aggrieved, so that they soon realized that their troubles were less important than they had believed and they regained a sense of balance and good humour returned. He was by temperament optimistic and he had a great gift for inspiring confidence in others and stimulating them to attack their problems along new lines with renewed vigour.

He was small in stature but trimly built, with a fine head and very delicate and supple hands. Although asthma was a constant handicap to him he was quick and active in his movements and of a cheerful disposition. All who knew him will remember his cheery greeting 'How you are?' for he never lost some delightful inversions of phrases arising from his polyglot upbringing. In fact, he spoke Russian, Polish, French and English fluently and read German with ease. For relaxation or when unwell, he read much of the works of the great authors of these countries in the original languages. Owing to his liability to asthmatic attacks he travelled little but compensated for this by his readiness to meet and welcome in the Institute and in his home scientists from all over the world and to keep in touch with them by correspondence. To Keilin, an interest in science was a sufficient introduction to ensure a warm welcome to any visitor from any country.

He inspired lasting bonds of admiration and affection in all those who were privileged to work in collaboration or in association with him, and the 'Old Boys of the Molteno Institute' soon became an informal fraternity at international gatherings, united by ties of common respect and regard for Keilin and his Department. It would take too much space to list all those who visited the Molteno Institute, for long or short periods, to enjoy the stimulating atmosphere and the excellent facilities which were available in the Institute. Their number and the wide variety of their subjects of research may be judged from the list of references of the publications of Keilin, either alone or in collaboration, which is printed after this appreciation. Of course, this list does not include the numerous papers which were published independently by workers who did not collaborate directly with Keilin.

We have tried to make the list of his publications as complete as possible and, as he himself would have wished, we let his reputation as a scientist rest upon the firm foundation of the work recorded therein. His friends and colleagues have

endowed a *Keilin Memorial Lecture* to be given every two years under the auspices of the Biochemical Society.

Although he never sought honours, many were bestowed upon him and he derived much pleasure from them. He was elected a Fellow of the Royal Society in 1928, was Croonian Lecturer in 1934, received the Royal Medal in 1939, the Copley Medal in 1952 and was Leeuwenhoek Lecturer in 1958. He was elected an Honorary Member of the Society for General Microbiology in 1958. In 1947 he was made Membre Correspondant and, in 1955, Membre Associé Etranger of the Académie des Sciences, Paris. In 1959 he was elected a Foreign Honorary Member of the American Academy of Arts and Sciences and also a Member of the Polish Academy of Sciences, Warsaw. Honorary degrees conferred upon him included, D.Sc. Brussels, 1946; D.Sc. Bordeaux, 1947; M.D. Liège, 1951 and M.D. Utrecht, 1957.

Throughout his scientific career, Keilin was supported by a singularly happy home life. His wife Dr Anna Keilin was a fellow-student studying medicine at the Sorbonne, and for many years she was a much respected and loved medical practitioner in Cambridge. She survives him together with their only child, a daughter Joan Keilin who is now well known as a biochemist and who has edited and prepared for publication the manuscript of a book upon which he had been working for a number of years. This book has just appeared with the title *The History of Cell Respiration and Cytochrome* (Cambridge University Press, 1966). Dr Anna Keilin helped him not only directly by making beautiful and scientifically accurate illustrations for some of his lectures and published papers, but she always took a great interest in his work and all those associated with the Molteno Institute. She made their delightful home a meeting place where all his friends and colleagues, and visitors from all over the world were sure to find a warm welcome, interesting company, generous hospitality and stimulating conversation in a truly cosmopolitan atmosphere.

Keilin's scientific achievements will assure him a high place among the leading scientists of his generation but his friends will also remember him affectionately for the warmth, humanity and loyalty of his personality.

M. DIXON

P. TATE

LIST OF SCIENTIFIC PUBLICATIONS BY DAVID KEILIN

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The Adaptation of *Aerobacter Aerogenes* to the Stress of Sublethal Doses of Formaldehyde

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SUMMARY

This paper deals with some biochemical events that occur when a population of *Aerobacter aerogenes* is treated with a sublethal dose of formaldehyde. It was discovered that during the bacteriostatic period there was a change in the base ratio of the non-ribosomal RNA. Along with this change there was the appearance of induced enzymes capable of metabolizing the formaldehyde at an increased rate. As soon as the formaldehyde concentration had been decreased by metabolism, growth resumed and the base ratio of the RNA returned to a normal value.

INTRODUCTION

Biological systems are constantly being exposed to some form of stress from the environment in which they are located. The challenge, which may be in the form of chemical or physical agents, elicits various reactions from the organism, ranging from lethal to beneficial. These reactions are very important since biological evolution is influenced to a great extent by the organism's response to the challenge. Indeed, it may be stated that without stress there would be no evolution and without evolution there would be no life as we know it. One of the problems in studying the biochemical events associated with stress is the length of time involved in determining its effects on succeeding generations. Bacterial systems because of their short generation times overcome this difficulty. Stress may be applied at controlled periods of growth and the influence on future generations may be observed during a 24-hr period.

My work with the action of formaldehyde on *Aerobacter aerogenes* (Neely, 1963*a*, *b*, *c*) led me to look on this situation as a means of elucidating some of the events by which bacteria adapt to stress. The investigations cited in the above references were concerned with gaining a better understanding of how formaldehyde exerts its antimicrobial activity. Substerilizing concentrations of ^{14}C[HCHO] were added to a growing culture of *Aerobacter aerogenes* and the rate of $^{14}C[CO_2]$ evolution was measured. This work demonstrated that such concentrations of formaldehyde had an initial bactericidal action followed by a bacteriostatic period. As soon as the formaldehyde had been depleted to a certain critical concentration by metabolism, the culture resumed its normal growth pattern. By using this same system, it was the intention of the present work to gain a better understanding of how a population of microorganisms, specifically, *A. aerogenes*, reacts to the stress of the presence of formaldehyde. The purpose will be to correlate the biochemical events with the physiological observations.

METHODS

Growth of organism. Aerobacter aerogenes ATCC 8308, was grown and maintained at 30° as described previously (Neely, 1963*a*) in the defined medium of Warren, Ells & Campbell (1960).

Analytical techniques. The procedures for measuring growth, viability and $^{14}C[CO_2]$ evolution were similar to those used in the earlier investigation (Neely, 1963*a*). The $^{14}C[CO_2]$ evolution curves are the result of a continuous recording of the ^{14}C activity as measured by a Cary Model 31 vibrating reed electrometer (Applied Physics Corp.). The ionization chamber used in this investigation had been previously calibrated (Neely, 1963*a*). By means of this calibration (25·6 mV were equivalent to 0·0458 μc) the activity of the CO_2 evolved could be determined from the area under the curve. In all cases reported in this investigation, the amount of formaldehyde metabolized was in the range from 87 to 90%. In the metabolism experiment, a modification was made to allow periodic sampling (0·2 ml. samples) of the culture for making colony counts. A hypodermic needle was inserted into the air inlet hose next to the Warburg flask. A length of tubing was attached to the needle to reach the bottom of the flask which contained the culture. In this manner the viability and rate of $^{14}C[CO_2]$ evolution were measured on the same culture.

Chemicals. The ^{14}C-labelled formaldehyde (specific activity of 10 mc/m-mole) was supplied by the New England Nuclear Corporation (Boston, Massachusetts, U.S.A.) Parke-Davis Co., (Detroit, Michigan, U.S.A.) brand of chloramphenicol was used in the investigation. All other chemicals were of reagent grade.

RNA base composition. The cultures were grown aerobically with and without formaldehyde (50 μg./ml.) in 5-l. volumes with constant agitation to ensure maximum growth. The formaldehyde was added to the culture at the beginning of the exponential growth phase. Samples (1000 ml.) were removed as indicated in Table 1 and the bacteria were centrifuged down, and washed with 0·9% NaCl solution. The sample for the resting culture (Table 1) was obtained by centrifuging 1000 ml. of culture which had just reached exponential growth, these bacteria were washed in 0·9% NaCl solution, re-suspended in 0·2% glucose solution, maintained for 7 hr at room temperature and then were recentrifuged.

Total RNA was purified by a modification of the method of Volkin & Astrachan (1956). The centrifuged bacteria were placed in the Omni Mixer (Ivan Sorvall, Inc., Norwalk, Conn.) with an equal weight of glass beads and water. The mixture was chilled and disrupted for 15 min. centrifuged at 14,000 *g* for 15 min. and the supernatant fluid extracted with an equal volume of a chloroform+isoamylalcohol mixture (20+1 by vol.). The liquid layers were separated and the top (aqueous) layer re-extracted. To this aqueous layer was then added 1·5 volumes of ethanol and the mixture allowed to stand in the refrigerator for 1 hr. The precipitated RNA was centrifuged and dissolved in N-KOH and allowed to hydrolyse at 37° for 16 hr. Sufficient 71% (v/v) perchloric acid was then added to bring the concentration to 0·55 N after neutralization of the KOH. After 45 min. in the cold, the solution was centrifuged, the supernatant fluid adjusted to pH 8·0 with ammonium hydroxide and the nucleotides resolved by ion-exchange chromatography, as described later.

Ribosomal RNA was prepared according to the method outlined by Roberts, Britten & McCarthy (1963). The bacteria cells were chilled and disrupted for 15 min.

by using an ultrasonic generator (the Narda Ultrasonics Corp., Westbury, L. I., New York). The ribosomal pellet resulting from two centrifugations in the Beckman Spinco Ultracentrifuge at 92000 *g* was dissolved in N-KOH and the hydrolysis procedure as described above was done, before chromatographic analysis.

The nucleotide mixtures were resolved on Dowex 1 ion exchange resin in the chloride from (2% cross-linked; 200–400 mesh) using a 20 cm. × 1 cm. column. The procedure was essentially that described by Gallant & Suskin (1962). The fractions were collected by using the Vanguard Fraction Collector equipped with an automatic ultraviolet analyser for scanning the eluate at 260 mμ (see Fig. 1 for a typical resolution). The tubes representing each nucleotide were pooled and examined spectrophotometrically. The final concentration was determined from the published extinction coefficients (Beaven, Holiday & Johnson, 1955).

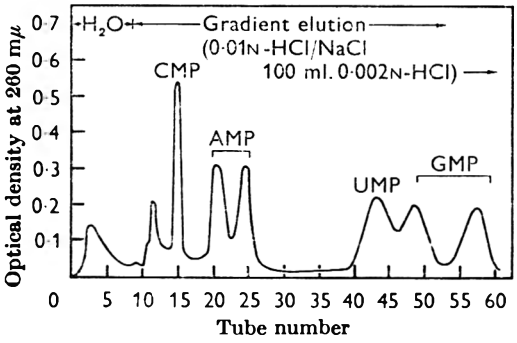

Fig. 1

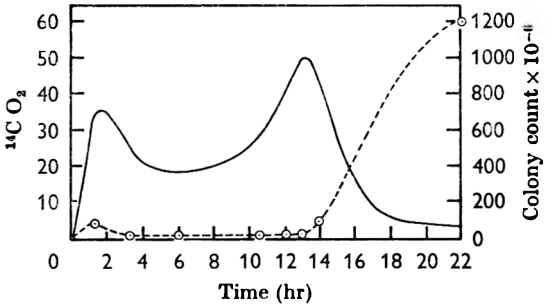

Fig. 2

Fig. 1. Chromatogram of RNA nucleotides isolated from *Aerobacter aerogenes*. Fractions (5 ml./tube) were eluted as shown from a 20 cm. × 1 cm. column at a flow rate of 1 ml./min. The peak appearing before the cytidylic acid has not been identified.

Fig. 2. $^{14}C\ O_2$ evolution by *Aerobacter aerogenes* treated with labelled formaldehyde (0·5 μc/4 ml.) at a concentration of 50 μg./ml. The corresponding colony count (equiv. no. organisms/ml. culture) of the culture is shown by the broken curve. In Fig. 2, 3 and 4, 10 units on the $^{14}C\ O_2$ ordinate are equivalent to 3 mV (25·6 mV = 0·0458 μc).

RESULTS

Demonstration of increased metabolic capacity for formaldehyde

To a culture of *Aerobacter aerogenes* which had reached the exponential growth phase, sufficient ^{14}C-HCHO was added to give a final concentration of 50 μg./ml. and a total radioactivity of 0·5 μc. Periodically, 0·2 ml. samples were removed and the appropriate dilutions were made for colony counts. The results of plotting the colony count (equiv. no. organisms/ml. culture) and the evolution of ^{14}C-CO_2 against time are shown in Fig. 2. The initial burst of CO_2 evolution followed by the decline to a constant rate is parallel with the viable count measurement. The second burst of CO_2, however, preceded the increase in viable count by 5 hr. To a duplicate of the culture illustrated in Fig. 2 a second portion of formaldehyde (equal to the first) was added 8 hr after the first addition. This time period was chosen because it coincided with an increase in the rate of ^{14}C-CO_2 evolution and it was before any change in the viable count. No detectable lag in the ^{14}C-CO_2 evolution was noted as a result of this

second addition of formaldehyde (Fig. 3). By measuring the area under the ^{14}C-CO_2 curves in Figs. 2 and 3 it was calculated that the area in Fig. 3 was twice that of Fig. 2. Finally, to help exclude strain selection as opposed to induction of formaldehyde-metabolizing enzymes the following experiment was performed. A culture which had been exposed to formaldehyde 50 μg./ml. was allowed to recover (Fig. 2; 22 hr); this culture was then used as a source of inoculum for a repeat of the experiment shown in Fig. 2. No difference in the pattern of ^{14}C-CO_2 evolution or viable count was noted.

The action of chloramphenicol

To culture which had reached the exponential phase of growth (similar to the previous experiments) ^{14}C-HCO (0·5 μc; 50 μg./ml. culture) was added. At subsequent time intervals, chloramphenicol was added to a final concentration of

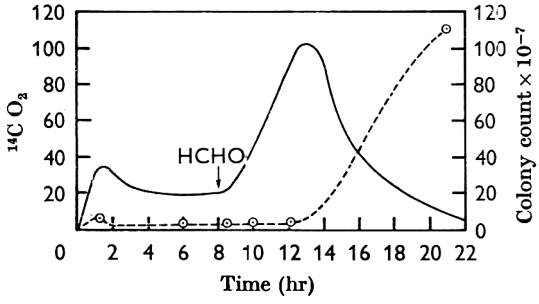

Fig. 3

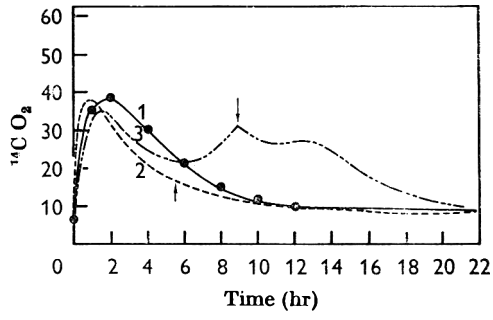

Fig. 4

Fig. 3. ^{14}C-CO_2 evolution by *Aerobacter aerogenes* treated with labelled formaldehyde at 0 hr. and at 8 hr. Each portion was equivalent to 0·5 μc/4 ml. at a concentration of 50 μg./ml. The corresponding colony count (equiv. no organisms/ml. culture) is shown by the broken curve.

Fig. 4. $^{14}C\ O_2$ evolution by *Aerobacter aerogenes* treated with labelled formaldehyde 0·5 μc/ml.) at a concentration of 50 μg./ml. Curve 1, chloramphenicol 25 μg./ml. added at 0 hr; Curve 2, chloramphenicol 25 μg./ml. added at 5·5 hr; curve 3, chloramphenicol 25 μg./ml. added at 9 hr.

25 μg./ml. The results of these experiments are shown in Fig. 4. The curves are quite dramatic and indicate that the second large burst of CO_2 was prevented by the presence of the antibiotic. Curve 3 is noteworthy since in this case the chloramphenicol was added after the ^{14}C-CO_2 evolution had indicated an increase in rate.

Base ratio analysis

The base ratio analyses for the total RNA and ribosomal RNA for the normal culture were independent of the time of isolation. The data in Table 1 are in close agreement with those reported for *Aerobacter aerogenes* by Midgley (1962). The striking result in Table 1 is the base ratio for the total RNA from a culture that was under the stress of formaldehyde treatment. An analysis of variance of the data in Table 1 showed a significant decrease in the amount of cytidylic acid and a significant increase in the amount of uridylic acid, as compared to the normal ratio. This analysis is reported in Table 2. All other samples of RNA including the RNA isolated

Table 1. *Base composition of total and ribosomal RNA of Aerobacter aerogenes synthesized in the presence and absence of formaldehyde*

Sample	Moles % of: Cytidylate	Uridylate	Adenylate	Guanylate*
Culture grown in absence of formaldehyde				
(*a*) Beginning of log growth				
Total RNA†	21	22	26	30
	19·5	24	25·5	31
	21	23	26·5	30·5
Ribosomal RNA	20·5	23·5	25·5	30·5
	19·5	24·5	26	30
(*b*) Resting culture				
Total RNA	20	24	25	30·5
	20·5	23·5	25·5	31
(*c*) 24-hr culture				
Total RNA†	21	24	24	30
	19·4	23·4	26	31
	20	23·7	25	30·5
Ribosomal RNA	19·5	23	25	31
	19·5	23·5	24·5	31·5
Culture grown in presence of formaldehyde				
(*a*) 7 hr after addition of formaldehyde (see Fig. 2)				
Total RNA†	17·5	25·5	26	31·5
	18	25·5	26·5	30
	18	26	26	30
Ribosomal RNA	21	23	26·5	29·5
	20	23·5	26	30·5
(*b*) 24 hr after addition of formaldehyde				
Total RNA†	21	22·5	26	30·5
	19·5	23	25·5	31
	21	23·5	26	29·5

* Each horizontal row represents an individual experiment on a separate culture of *A. aerogenes*.
† These results were used for the analysis of variance shown in Table 2.

Table 2. *Analysis of variance of the data shown in Table 1. The analysis was made on the results for cytidylic and uridylic acid*

Source of variation	Degrees of freedom	Average variance	Variance ratio*
1. Age level of culture	1	0·04	
2. With and without formaldehyde	1	0·04	
3. Nucleotide	1	100·86	219·3
Interactions			
1 × 2	1	0·05	
1 × 3	1	6·83	14·8
2 × 3	1	6·83	14·8
1 × 2 × 3	1	15·35	33·4
Replicates	16	0·46	

* Significant at 0·01 probability level.

from the 7-hr static culture and the ribosomal RNA from the formaldehyde-treated culture were similar to the base ratio found for the untreated population.

DISCUSSION

The experiment shown in Fig. 2 indicates that the increase in the rate of metabolism of formaldehyde (8 hr after addition of HCHO) was due to the increased ability of *Aerobacter aerogenes* to convert HCHO to CO_2. This conclusion is established by the fact that during the increase in rate of CO_2 evolution the concentration of viable organisms in the culture was constant. Normal growth as judged by increase in colony count occurred only when most of the HCHO had been removed. The initial burst of ^{14}C-CO_2 followed by a decline to a steady state may be correlated with the decrease in colony count. Fig. 2 indicates that the rate of ^{14}C-CO_2 evolution decreased from a high value of 36 mV to 18 mV; during this interval there was a corresponding decrease in the colony count. These results suggest that induced enzymes might be responsible for the second large burst of CO_2. If induced enzymes were not responsible for the observed increase in rate of ^{14}C-CO_2 evolution then a second addition of HCHO at 8 hr in Fig. 3 should prolong the lag. This did not occur; the second portion of HCHO was metabolized at an increasing rate even though the colony count was constant. A possible explanation for this observation is that the initial HCHO had caused the induction of the necessary enzymes; consequently the culture was able to metabolize the second quantity of HCHO without the usual lag period. An alternative explanation might be that a small fraction of the bacteria population was capable of metabolizing HCHO and then to grow in its presence. If this were the case, then two events should have occurred. (1) the increase in the rate of ^{14}C-CO_2 evolution at 8 hr should have coincided with an increase in the population; (2) an inoculum resulting from a population that had been exposed to HCHO and allowed to recover, i.e. a 24-hr culture should have shown no lag. Neither of these results occurred, thus lending further support to the thesis that the increase in the capacity of the organisms to handle HCHO was due to an increase in the concentration of HCHO-metabolizing enzymes.

Since chloramphenicol has been shown to be an inhibitor of protein synthesis (Hunter, Brookes, Crathorn & Bolter, 1959), and of inducible enzyme formation (Mandelstam, 1957; Pollock & Kramer, 1958; Eisenstadt & Klein, 1961), the experiments with this compound demonstrated that *de novo* synthesis of new protein was required for the metabolism of HCHO. Curve 3 in Fig. 4 shows that the lag in appearance of increased metabolic activity must be due to the time involved for non-dividing cells to mobilize the means necessary to cause the synthesis of the necessary proteins. It has been previously shown (Neely, 1963*c*) that HCHO during this bacteriostatic period is an inhibitor of cytoplasmic and nuclear syntheses which are necessary for new growth. However, during this lag there is a continual low degree of metabolism of HCHO, consequently, a point is reached where the inhibition is removed to such an extent as to allow the synthesis of new protein. One of the first proteins to be made must be the system for metabolizing HCHO at an increased rate, thus allowing the culture to return to its normal physiological state.

The base ratio analysis would imply that the HCHO caused a change in the non-ribosomal RNA. This result comes from the observation that no change in the base

ratio of the ribosomal RNA was detected; however, there was a significant change in the cytidylic and uridylic acid in the total RNA from the HCHO treatment. Once the culture had depleted the HCHO and normal growth had resumed, the base ratio of the RNA also returned to normal. This evidence suggests that the change in ratio might be related to the appearance of enzymes responsible for the increased metabolism of HCHO. At the concentrations used HCHO was bacteriostatic. The possibility existed, therefore, that the altered base ratio in the RNA was merely due to the physiological state of bacteriostasis and was not related directly to the metabolism of HCHO. Since there was no change in the RNA as a result of the prolonged period of bacteriostasis (see results for resting bacteria in Table 1) this possibility seems excluded.

Another observation from the analysis of variance in Table 2 is that the decrease in cytidylic acid was exactly compensated by an increase in uridylic acid. It is interesting to speculate on the possibility that HCHO prevents the conversion of uridine to cytidine and in this manner causes the observed alteration in the base ratio.

The following items are presented as the sequence of events which enable a population of *Aerobacter aerogenes* to adapt and recover from a sublethal dose of formaldehyde. 1. A certain number of bacteria are killed as indicated by the initial decrease in colony count (Neely, 1963*a*). 2. The loss in viability is neutralized by the reaction between formaldehyde and homocysteine to form 1,3-thiazane-4-carboxylic acid (Neely, 1963*b*). This reaction blocks methionine biosynthesis and successfully counteracts the potential death from unbalanced growth (Neely, 1963*c*). 3. As the present work indicates, during this static period of growth the physiology of the population is altered in the direction of an increased potential for metabolizing formaldehyde. This potential is expressed as soon as the static culture has decreased the formaldehyde concentration to the point where new protein synthesis can occur.

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Continuous Culture of *Torulopsis utilis*: A Kinetic Study of Oxygen Limited Growth

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SUMMARY

A small single-phase continuous fermentor system for convenient long-term operation at steady state with small population densities is described. A method by which the effect of extracellular limiting nutrient on growth rate can be evaluated from the resulting data is discussed. This method was used for *Torulopsis utilis* to determine the Michaelis constants for growth with oxygen and glycerol; these were found to be $1{\cdot}4 \times 10^{-5}$ M and $4{\cdot}9 \times 10^{-5}$ M. Size of organisms and yields were found to decrease with growth rate. Average volume of organisms decreased from 60 to 41 μ^3 and the yield decreased from 1·8 to 1·2 g. dry wt./organism/g. oxygen consumed. An endogenous metabolism rate constant determined from yields at growth rates above 30% maximum was 0·056 g. oxygen used/g. dry wt. organism/hr.

INTRODUCTION

Among the advantages of continuous cultures of micro-organisms is the production of a culture grown at a fixed rate by the controlled extracellular concentration of a single nutrient. The fact that growth rate is determined by a limiting nutrient concentration and that population density is fixed by the amount of limiting nutrient absorbed can be utilized to relate growth to limiting substrate concentration. This relationship was shown to fit the well-known Michaelis–Menten equation by Monod (1942) where the Michaelis constant K_s was defined as the concentration of limiting substrate at which microbial growth velocity was half maximum. Monod's method of determining K_s was to measure growth rates in batch culture at known initial sugar concentrations. Herbert, Elsworth, & Telling (1956), in an extension of the original continuous culture theory presented by Monod (1950), attempted to measure K_s for glycerol by analysis of the effluent of a glycerol-limited continuous culture. Davies, Karush & Rudd (1965) used the same technique to analyse fermentor effluents for extracellular and limiting quantities of radioactive amino acids. It was recognized by Rao (1962) that decreasing steady-state populations at decreasing substrate concentrations in the feed of a continuous culture predict, when extrapolated to zero population size, the substrate concentration which surrounds the organisms at a particular growth rate. Rates of oxygen disappearance in static culture have been measured polarographically by Longmuir (1954) at various oxygen concentrations. The purpose of the present work is to

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relate microbial growth rates to dissolved oxygen concentration in the medium under conditions of oxygen limited growth.

Theory. A continuous culture is a continuous flow stirred tank reactor of the type described by Danckwerts (1954) adapted to grow micro-organisms. The autocatalytic growth reaction therein utilizes part of the limiting nutrient and the remainder is removed with the reaction product organisms. In a completely mixed reactor, the most efficient for autocatalytic processes (Herbert *et al.* 1956), this effluent substrate concentration is the same as the nutrient concentration which surrounds the organisms. The growth velocity of a culture limited by the concentration of a single limiting nutrient proceeds as a monotonic increasing function of substrate concentration (Monod, 1942) and the population of organisms proceeds directly to an equilibrium steady-state concentration (Button, 1964) fixed by the concentration and the rate at which substrate is supplied to the reactor. The above is widely recognized and can be formulated mathematically as follows:
Rate of change of concentration of organism = growth rate − output rate,

i.e.
$$V(dX/d\theta) = \mu XV - FX, \tag{1}$$

where V is the volume of the reactor, X the cell population, μ the specific growth rate of the organisms $(1/X)(dX/d\theta)$, F the feed rate of fresh medium and θ time. At steady state, the concentration of organisms is constant with respect to time, $dX/d\theta = 0$ and
$$\mu XV = FX. \tag{2}$$

Designating the specific through-put rate as F/V and dividing through by V gives:
$$\mu = r. \tag{3}$$

Thus at steady state the growth rate is equal to the dilution rate and can be set at any desired value between zero and the maximum growth rate of the organism, μ_{max}, by suitable adjustment of the feed rate F.

The limiting nutrient balance in reactor. Rate of substrate concentration change = input rate − output rate − rate of substrate utilization for growth; i.e.
$$V(dS/d\theta) = FS_0 - FS - \mu(X/\gamma)V, \tag{4}$$

where S_0 and S are the feed and reactor limiting nutrient concentrations, respectively, and γ is a yield constant relating dry weight of organisms produced to mass of limiting substrate utilized, or $X/(S_0 - S)$. Then at steady state where X and S are at equilibrium values and $dS/d\theta = 0$,
$$F(S_0 - S) = \mu(X/\gamma) \tag{5}$$

since $r = F/V$ and $\mu = r$, one can solve for S to give concentration of limiting nutrient in the reactor surrounding the organisms:
$$S = S_0 - (X/\gamma). \tag{6}$$

The value of S can be determined at any given growth rate by measuring the population density X at a series of feed substrate concentrations S and extrapolating to zero population size according to the equation
$$X = \gamma(S_0 - S). \tag{7}$$

Since the growth rate μ can be varied by changing the dilution rate of the system according to equation (3), the growth rate can be related to the limiting nutrient concentration by determining S as above at a series of dilution rates. The yield constant γ has no effect on the extrapolation to S, hence different yields at different growth rates do not affect this method of substrate analysis. The only assumptions made in deriving the above equations are: (1) The growth rate is a function only of the concentration of a single limiting substrate, $\mu = f(S)$. (2) A range of steady-state stable conditions can be maintained in the fermentor. This implies that the net behaviour of the cells does not change with respect to time under steady-state conditions. (3) Perfect mixing exists in the fermentor. (4) All the organisms produced are viable.

METHODS

Extracellular nutrient concentration determination by the above method improves with operation of smaller population densities, where the extrapolation to zero population density is short. At these low population densities, a non-homogeneous distribution of organisms (part of the organisms growing on the fermentor

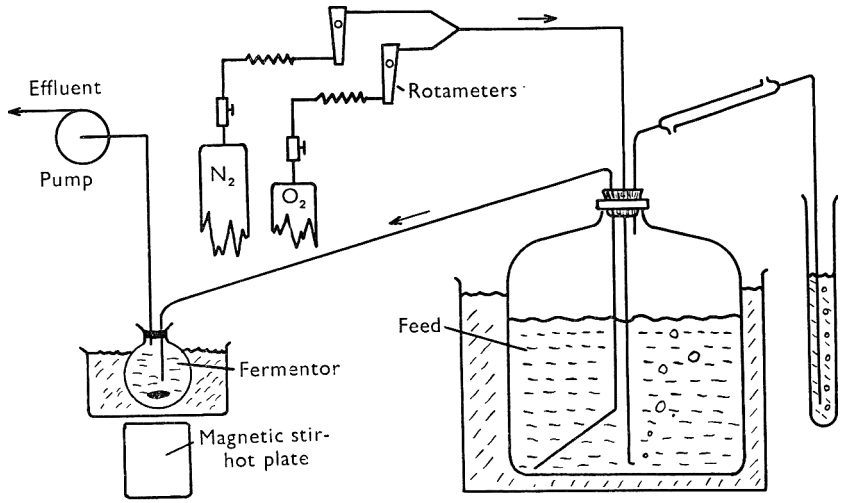

Fig. 1. Small single phase continuous fermentor.

walls, for example) render this method of nutrient concentration determination useless. A continuous culture system was thus designed specifically for operation at small population densities with special attention directed toward maintaining a uniform population as well as accurate regulation of the culture volume, feed rate, and limiting substrate concentration.

Apparatus. The continuous culture apparatus used consisted of a 40 l. Pyrex feed carboy, a 500 ml. round bottom flask reactor and a pump connected as shown in Fig. 1. Oxygen was supplied to the fermentor as one of the components dissolved in the feed. The fermentor was completely filled, thus eliminating a gas phase and associated problems in the growth portion of the system.

Feed-dissolved oxygen content was regulated by sparging the desired oxygen + nitrogen + dry air ratio into the feed carboy. Higher oxygen concentrations were

obtained by mixing streams of the pure gases at fixed flow rates. These flow rates were regulated by controlling the pressure above a resistance bed of 300 μ diam. glass beads packed into a section of capillary tubing. The streams were fed through calibrated rotameters for measurement before joining. Gas pressure was decreased and controlled with a series of three diaphragm pressure regulators. Compressed gas cylinders of oxygen in nitrogen were purchased (with analysis; The Matheson Company, Inc., Joliet, Illinois, U.S.A.) for obtaining the lower oxygen concentrations. Effluent gas was discharged into a column of ethylene-glycol to provide positive hydrostatic pressure at the level of the pump.

The 40 l. carboy was mounted in a constant temperature bath, all of which could be detached from the rest of the system and autoclaved as a unit. Ports were provided, in a rubber-stopper which closed the carboy, for a thermometer well, feed siphon, substrate additions and gas sparging. Additions to the carboy were injected through a syringe needle piercing a rubber diaphragm which covered one of the ports.

Feed was pumped into the fermentor, a 500 ml. round-bottom flask, positioned in a constant temperature bath over a magnetic stirrer. A 2 in. stir bar which mixed the system was driven at about 600 rev./min.; it dissipated a small volume of injected dye in less than a second. Feed and fermentor temperatures were accurately controlled at 35°.

Flow rate through the system was metered with a positive displacement Milton Roy controlled volume pump. The pump discharged effluent culture through a section of thermometer capillary, thus providing back pressure to prevent siphoning through the system. This arrangement allowed feed rates to be set within a few parts per thousand. Large-bore capillary tubing joined with sections of rubber hose connected the various components. These joints were wrapped with Mylar tape to decrease oxygen diffusion into the system; however, this precaution turned out to be unnecessary.

Organism. Several months of effort failed to find a suitable medium or fermentor surface which would allow the use of *Escherichia coli* as a test organism because of its tendency to adhere to solid surfaces. Although not apparent to the eye for the first few days, inconsistencies in the data indicated that a film of organisms was forming on the fermentor wall; this could later be seen. Therefore a different organism, *Torulopsis* (*Candida*) *utilis* 3, was chosen on the basis of its larger size and rapid growth in simple media. This organism did not adhere to the reactor walls and handled well. Later Mr D. Weinshank of this laboratory found that *Bacillus megaterium* was suitable for work at small population densities. The marine yeast, *Cryptococcus albidus*, is free from sticking tendencies in continuous culture (D. K. Button, unpublished observations).

Medium. The medium used was designed to study the effect of extracellular dissolved oxygen on the growth rate of *Torulopsis utilis* at small population densities. Thus additional required nutrients were adjusted between limits of concentration large enough to insure lack of growth limitation and small enough to insure absence of inhibition over the range of population densities required. Available inorganic analyses of yeast data were used to suggest the proper ratio of these constituents.

The medium was designed to accommodate a population density equivalent to about 0·05 g. dry wt. organism/l. Trace metal concentration adjustments were made to give a minimum lag phase before exponential growth in batch fermentations at

small population densities. Equilibrium amounts of dissolved oxygen were varied from 0·1 to 23·1 mg. O_2/l. medium as calculated according to Henry's law and gas solubilities in dilute salt solutions. Glycerol was used as a convenient carbon source to avoid anaerobic metabolism confusing the experiments. Figure 2 shows relative steady-state population densities at a fixed oxygen concentration in the feed medium over a range of glycerol concentrations. These data show that the population density when oxygen was limiting was independent of concentration of carbon source. Since the data were collected at half maximum growth rate, the population densities extrapolate to a Michaelis constant for glycerol of 3·5 mg./l. Other constituents of the medium were (per l.): $NaH_2PO_4.H_2O$ 2 g., $(NH_4)_2SO_4$ 325 mg., KCl 40 mg., $MgSO_4$ 25 mg., $CaCl_2.2H_2O$ 5 mg., $ZnSO_4.7H_2O$ 0·7 mg,, $Fe(HN_4)_2(SO_4)_2.6H_2O$ 0·5 mg., $CuSO_4.5H_2O$ 25 μg., $MnSO_4.H_2O$ 1·5 μg., $CoSO_4.7H_2O$ 1·5 μg., MoO_3 0·5 μg., biotin 1 μg. Growth rate was independent of pH value between 4 and 7. The medium was buffered at the lower value to decrease the probability of successful contamination.

Operational procedure. The medium was prepared in 40 l. carboys with distilled water, filtered through 0·45 μ pore 1–1 size filters and autoclaved 45 min at 121°. One carboy was fitted with a siphon to refill the feed carboy through a rubber covered port, connexion being made with a sterile syringe needle. Normally two 40 l. carboys were sterilized per run. Weighed quantities of glycerol were also injected into the feed where required and its concentration calculated with reference to volume calibration marks inscribed on the carboy.

To start a run, shake-flask cultures were inoculated from stock agar slants and a small quantity of exponentially growing organisms introduced into the fermentor. Equipment set-up time was about 2 days; runs lasted 1 to 2 months. The system was generally trouble free; two of seventeen runs were interrupted for culture failure for some unknown reason and one for mechanical repairs.

An appropriate interval was allowed between adjustments to ensure that new steady-state values had been reached. Continuous recording of the population densities after a substrate concentration shift indicated a smooth asymptotic approach to the new steady-state value as predicted by the rate of change of substrate concentration. The composition of the medium in the fermentor will approach that of the feed medium in a mixed system such as this by a factor of $1-e^{-r\Theta}$ in r hours and is 99% complete after 4·6 fermentor volumes have been pumped through. Continuous recording of population densities showed that new steady-state populations were approached in a smooth asymptotic manner. Thus 5–10 vol. were allowed to pass through the fermentor before taking steady-state readings, depending on the magnitude of the change in population density. When the composition of the sparged gas into the feed was changed resulting population densities showed that equilibration was essentially complete in 24 hr. Here a minimum of 2 days was allowed between steady-state readings.

After changing the dilution rate, assuming no lag in the biological response of the system, the approach to new steady-state values should be about 99% complete after dilution by only one fermentor volume at the new rate. For this adjustment at least five fermentor volumes were allowed to pass through for conditions to stabilize.

Population density measurements were made primarily with an electronic counter

calibrated against direct dry-weight determinations; optical extinction measurements and occasional plate counts were also made. The cell suspension was brought to a suitable conductivity with sodium chloride 7·5 g./l. and sodium sulphate 2·5 g./l., the latter being added to remove traces of dissolved oxygen and to stop growth of the oxygen-limited culture during the analysis period. Samples were diluted to 60,000 organisms/ml. or less to decrease coincidence errors where necessary. A 50 μ orifice permitted good electronic sizing. Little orifice fouling was encountered during counts since the media and diluent had been pre-filtered. Oscilloscope pulse height was taken as proportional to individual cell volumes (Kubitscheck, 1956).

Dry-weight measurements were made with a Cahn electronic microbalance. The pans were enclosed in a tube wound with heating coils, which could be evacuated for rapid drying. Samples of organisms were collected on tared 13mm. diam. 0·45 μ pore size filters, washed and then dried in place on the balance pan. About 1 mg. organisms was collected on a 7 mg. filter. Accuracy was limited by the facility with which the filter disc was removed from its holder on humid days. Static electricity made weighing impossible on dry days. Triplicate sample weight variation seldom exceeded 5 %. Samples were removed for analysis through a syringe needle extending into the fermentor and capped with a small cork.

RESULTS AND DISCUSSION

Population density data were collected at a series of growth rates and feed oxygen values to determine fermentor oxygen concentrations according to equation (7). The maximum growth rate was established in batch culture at 0·506 hr^{-1}; this value of $\mu_{max.}$ persisted at the low populations near that of operation. Most data were

Table 1. *Number of organisms/μg. dry weight and volumes of organisms.*

Growth rate (% $\mu_{max.}$)	No. organisms/μg. dry wt. ($\times 10^5$)	Average volume/organism (μ^3)	Most frequent volume/organism (μ^3)
30	1·71	41	34
50	1·04	48	37
80	0·83	60	43

collected at 30, 50 and 80 % of this growth rate. The sizes of the organisms were significantly larger at faster growth rates as shown in Table 1. These data were taken from size distribution curves of the type shown in Fig. 3 and in conjunction with dry-weight measurements. Variation in extinction at 625 mμ/unit mass of organism was more than twofold over the growth rates observed. The yield of organism/g. oxygen utilized decreased from 1·5 g. organism/g. oxygen near $\mu_{max.}$, down to a yield constant of about 1·0 at 5 % of $\mu_{max.}$ as shown in Fig. 4. These data were taken directly from dry-weight measurements at various growth rates with limiting oxygen at 31·5 mg./l. Oxygen utilized was taken as input of dissolved oxygen less a small correction for unused oxygen escaping in the effluent. This correction was made according to the Michaelis–Menten equation, $\mu = \mu_{max.} S/(K_s+S)$ and increased with growth rate. The value of K_s for oxygen was taken as 0·45 mg./l. as determined below. The growth rate was taken as the dilution rate of the continuous culture system.

Fermentor oxygen concentrations at the three growth rates were determined by extrapolation of steady-state population values to zero population as shown in Figs. 5–7. Here population measurements were based on numbers of organisms since the experimental and statistical problems involved in obtaining size distribution data at the very small population densities used prevented accurate cell volume determinations. These small population densities were also below the lower limits for reasonable accuracy of the dry-weight measurements. While extinction measurements were obtained, they were not of a high order of accuracy at the low population densities used.

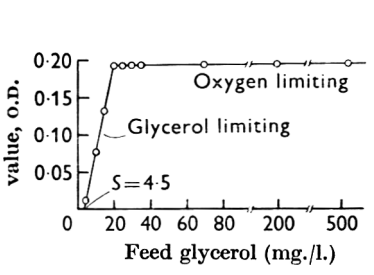

Fig. 2

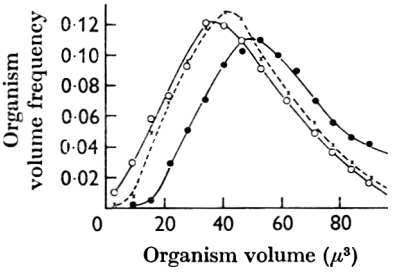

Fig. 3

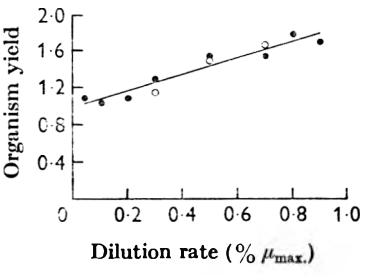

Fig. 4

Fig. 2. Steady-state population density against glycerol concentration. The growth rate is half maximum or 0·253 hr^{-1}. Feed dissolved oxygen is held constant at 6·2 mg./l.

Fig. 3. Volumes of organisms distribution at three growth rates limited by dissolved oxygen concentration. Growth rates are: ○, 30 %; ×, 50 % and ●, 80 % maximum. Curves are reduced to a common total organism volume.

Fig. 4. Steady-state yield of organism from oxygen plotted against dilution rate. ●, mg. organism dry wt. /mg. oxygen used. ○, organism volume (arbitrary units)/mg. oxygen used.

Table 2. *O_2 concentration and yield constants at various growth rates.*

Growth rate (% $\mu_{max.}$)	Oxygen concentration (μg./ml.)	Yield constant*	Yield constant†
30	0·15	1·2	1·2
50	0·5	1·5	1·4
80	1·0	1·8	1·7

* Yield of organism from direct dry-weight measurements (Fig. 4).
† Yield of organism calculated from slope of $X = \gamma (S_0 - S)$ plot (Figs. 5–7).

The fermentor oxygen concentrations obtained from Figs. 5–7 and summarized in Table 2 were computer fitted to the hyperbola described by the Michaelis–Menten equation to obtain the concentration of oxygen at which growth proceeded at half its maximum rate. Values for extracellular oxygen concentrations obtained in a similar manner by using extinction values and plate count data were approximately the same as those shown in Table 2. The relationship between growth velocity and oxygen concentration taken from the electronic counter data is shown in Fig. 8. The curve drawn is the best hyperbola fitting these data. Deviation of the data from the curve drawn is within experimental error. The Michaelis constant for oxygen-limited growth of this yeast was thus determined to be 0·61 mg./l. with a standard error of 0·4 mg./l.

Several assumptions were made to determine that an oxygen concentration of $1{\cdot}4 \times 10^{-5}$ M will allow this yeast to grow at half its maximum rate. Growth rate must be only a function of limiting nutrient concentration. Where this is true a plot of input substrate concentration against population density at a single through-put rate will be linear, as shown in Fig. 9. A curve toward the substrate axis which has

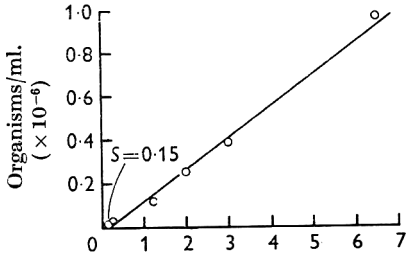

Fig. 5

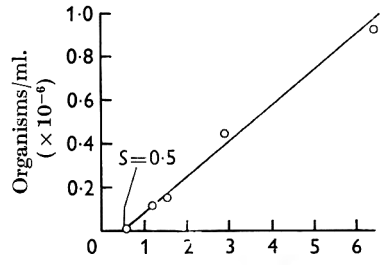

Fig 6

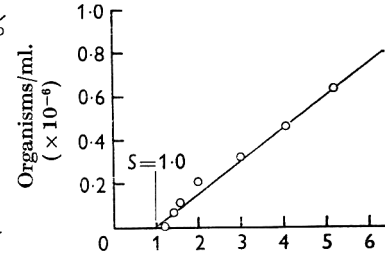

Fig. 7

Fig. 5. Steady-state population densities plotted against oxygen concentration in the feed at 30 % of the maximum rate. The line extrapolates to an extracellular oxygen concentration of 0·15 mg./l. at this growth rate.

Fig. 6. Same as Fig. 5 except growth rate is 50 % maximum.

Fig. 7. Same as Fig. 5 except growth rate is 80 % maximum.

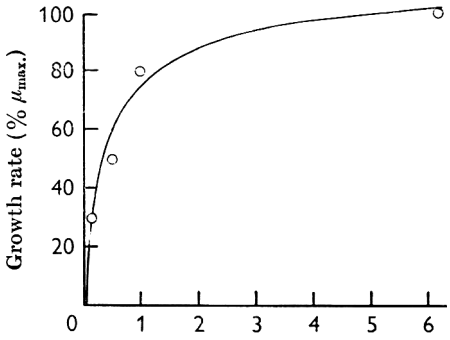

Fig. 8

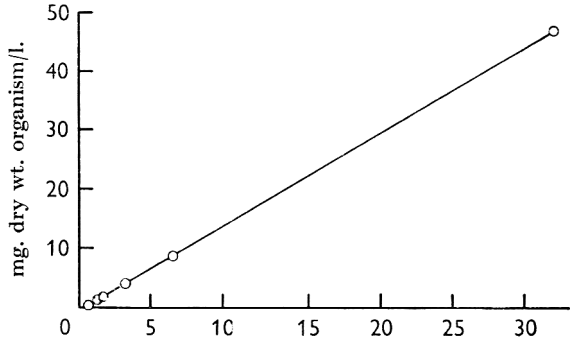

Fig. 9

Fig. 8. Growth rate of *Torulopsis utilis* plotted against extracellular dissolved oxygen concentration.

Fig. 9. Steady state-organism mass plotted against dissolved oxygen concentration in the feed at 50 % of the maximum growth rate.

sometimes been reported probably represents partial limitation by other constituents of the medium or an effect of population density on the yield of organisms from substrate. Such a curve resulted in the present system when the carbon source was glucose and facultatively anaerobic metabolism could occur. Glycerol can only be used aerobically, however, and there was no effect of this carbon source on oxygen-limited population density when the glycerol concentration was varied over a 100-fold range. This is shown by the sharp break in Fig. 2. Oxygen concentration was the only factor which controlled both growth rate and population density. Change in the growth rate did, however, affect the size of the organisms, as

shown in Fig. 3. Extrapolation to a negative substrate concentration in this plot as sometimes found represents a background of limiting substrate in the feed.

The second assumption requiring steady-state operation was not established or closely approximated until operation was shifted to yeast rather than bacteria eliminating the problem of adhesion to the reactor wall. In a larger fermentor, or when working with higher population densities, this phenomenon might well have been overlooked. However, here the effect on resulting data was large; the consistency of the data over extended periods of operation at small population densities substantiates this assumption.

The third requirement is for perfect mixing, which implies a homogeneous dispersion of substrate as well as organisms. Gross mixing in this small fermentor appeared to be better than in larger conventional fermentors as judged from the observation of injected dyes. Improved mixing at the surface of the organisms would probably not affect the data since the diffusivity of oxygen through water is such that a stagnant film of medium a few microns thick around the organisms should have little effect on the rate of growth.

The last assumption, that all the organisms are viable, must hold for the dilution rate to be equal to the growth rate. Steady state could theoretically be achieved with a proportion of the organisms produced being non-viable. In this case, the net growth rate, i.e. the growth rate minus the death rate, is equal to the dilution rate. In these experiments where kinetic data were gathered, i.e. at dilution rates between 30 and 80 % maximum, plate counts and electronic counter data were the same within experimental error. Thus, essentially all of the organisms were viable at these high growth rates. However, as the dilution rate was decreased below 30 % of the maximum the resulting yield data can be interpreted to suggest the production of increasingly large numbers of metabolically inactive organisms. It can be seen from Fig. 4 that increasingly lower yields were associated with lower growth rates. If it be assumed that the culture utilizes a portion of the oxygen for endogenous metabolism at a constant rate dependent only on the mass of organisms, and the rest for growth processes at the associated rate, an endogenous metabolism rate constant can be determined. At growth rates above 30 % maximum where yield data are of greatest precision, a plot of $1/\mu$ versus $1/\gamma$ gives a straight line as predicted from the model with an endogenous metabolism rate constant of 0·057 g. oxygen consumed/g. organism/hr. Marr (1963) determined a similar rate constant, 0·028 hr^{-1}, for *Escherichia coli* with glucose as the limiting nutrient. The yield data presented conform to the above model at lower growth rates only when it is assumed that increaxingly large proportions of the organisms are dead. This assumption predicts that 79 % of the culture would be dead at the lowest growth shown. While the data are not sufficient to present a complete explanation, they clearly suggest matters to be given close attention when working at low growth rates.

The K_s value of $1{\cdot}4 \times 10^{-5}$ M for oxygen is significantly higher than the range of values Longmuir (1953) obtained for several yeast, bacterial, and subcellular preparations; $6{\cdot}45 \times 10^{-7}$ M for Baker's yeast, $2{\cdot}22 \times 10^{-8}$ M for *Escherichia coli*. The data of Longmuir were obtained by measuring polarographically the short-term rate of oxygen removed by an oxygen-starved suspension of organisms. It seems likely that this procedure measures a different phenomenon than the K_s value for growth reported here. The K_s for growth probably measures the rate of some rate-

limiting reaction within the organism which is regulated, perhaps indirectly, by oxygen concentration. Short-term oxygen uptake, however, probably represents the ability of oxygen-starved organisms to absorb nutrient. This is supported by the direct correlation which Longmuir found between K_s for oxygen and size of organism. The limited data available here for *Torulopsis utilis* show no such correlation between K_s values for growth and size of organism.

The values of $1{\cdot}4 \times 10^{-5}$ M for oxygen and $4{\cdot}9 \times 10^{-5}$ M for glycerol are similar to values obtained for growth by other workers. Monod (1942) reported $2{\cdot}2 \times 10^{-5}$ M for glucose with *Escherichia coli* in batch culture. Rao (1962) reported $2{\cdot}1 \times 10^{-5}$ M when using the experimental approach used here with *E. coli*. The direct analytical data of Davies *et al.* (1962) who used a haemolytic streptococcus and growth limited by various amino acids indicated K_s values from 10^{-3} to 10^{-5} M. Extrapolation of their data obtained at various feed amino acid concentrations according to the method presented here yields values that agree well with their own analytical values.

While data given here are not of sufficient precision to make statements about the exact shape of the oxygen-limited growth velocity curve with *Torulopsis utilis*, they do indicate the concentration range of dissolved oxygen at which aerobic growth proceeds at a reasonable rate. The consistency of the data demonstrate the advantage of obtaining growth information from steady-state systems.

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Bacteriocins and Phages Produced by *Serratia marcescens*

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SUMMARY

One hundred and thirty-nine strains of *Serratia marcescens* were investigated for bacteriocin and phage production. Two types of bacteriocins were detected. Group A were all active on serratias and were produced by 71 strains. Some of these bacteriocins also attacked Salmonella, Escherichia and Aerobacter strains. They were produced spontaneously in broth, and higher titres were produced by induction with ultraviolet (u.v.) radiation. These bacteriocins are divided into eight subgroups by their spectrum of activity and resistant mutants of indicator strains. Group B bacteriocins are not active on serratias. Their action is restricted to escherichias, hafnias and aerobacters. Mutant bacteria resistant to any one of these B agents are resistant to all other B bacteriocins. The B agents are serologically similar and are produced in broth by 54 strains. They are produced spontaneously and higher titres are produced after u.v. induction. With one exception the B bacteriocins may be identical. Thirty-five strains produced both types of bacteriocins. The A bacteriocins are resistant to chloroform, trypsin and the proteolytic enzymes of certain organisms, are heat-stable and non-dialysable. Group B bacteriocins are heat-labile, non-dialysable and inactivated by chloroform and the above enzymes. Agents A and B differ from colicins. Thirty-seven of the strains were lysogenic for Serratia species and 19 of the phages productively lysed Salmonella species. Although overlaps did occur it was always possible to distinguish the bacteriocins from the corresponding temperate phage produced by a strain, by means of their spectra of activity.

INTRODUCTION

Strains of *Serratia marcescens* may produce at least three antibacterial substances. Fuller & Horton (1950) described an antibiotic-like substance, marcescin, which has an inhibitory action on *Corynebacterium diphtheriae* and *Staphylococcus aureus*. Hamon & Peron (1961) and Mandel & Mohn (1962) described bacteriocinogenic activity among strains of *S. marcescens*; Prinsloo & Coetzee (1964) found 15 of 31 strains of *S. marcescens* to be lysogenic for other *S. marcescens* and Salmonella strains. Fredericq (1954) showed that colicinogenic factors were transmissible to strains of other *Escherichia coli* and shigellas while Amati & Ozeki (1962) transmitted colicinogenic factors E_1, E_2 from *Salmonella typhimurium* to serratias. In view of the genetic importance of phage and bacteriocinogenic factors, these agents were investigated in a series of 139 *Serratia marcescens* strains.

METHODS

Bacteria. These comprised 15 strains of *Serratia marcescens* obtained from the National Collection of Type Cultures, NCTC 1377, 2446, 2847, 4618, 8015, 3084, 8706, 8900, 9493, 9940, 9741, 9743, 10036, 10211, 4619; one American Type Culture strain ATCC 9986; 123 local strains isolated from human and insect sources. The 123 strains could be differentiated by lysogenicity and phage-susceptibility (Prinsloo & Coetzee, 1964; H. E. Prinsloo, unpublished). The strains were biochemically typical (Martinec & Kocur, 1961) except for SM 8 which was maltose-negative, and SM 52 which was sucrose-negative, and were either non-pigmented or pigmented. Standard colicinogenic strains which produced colicins A to K, V and S1–4 were obtained from Professor F. Fredericq. The indicator used for colicins was *Escherichia coli* 0, while the following bacterial strains were used to determine the spectrum of activity of the antibacterial agents produced by *S. marcescens*: all the Serratia strains, the colicinogenic strains of Fredericq, *Hafnia* spp (H_{1-6}), *Aerobacter aerogenes* (X_{1-7}), *Alcaligenes faecalis* (NCTC 8769), *Proteus hauseri*, *P. morganii*, *P. rettgeri* and the providence cultures previously used (Coetzee, 1963*a*). In addition, 12 *Salmonella typhi* strains of different Vi phage types were used as well as 28 Salmonella serotypes belonging to each of the Kauffman–White groups and 10 unclassified strains, 24 Shigella strains of different serotypes, 10 strains of *Staphylococcus pyogenes*, 10 Bacillus strains, and one toxigenic *Corynebacterium diphtheriae*. The following proteolytic organisms were used: *Bacillus cereus* strain B 2, *S. marcescens* SM 1, 5, 8, 9, 11, 41, 52, NCTC 2847, *P. mirabilis* strain 13–0 (Coetzee, 1963*b*). The bacteria were maintained on nutrient agar slopes at 4°. Cultures were incubated at 32°.

Media. Oxoid No. 2 nutrient broth was used. The solid media contained 1·1% (w/v) agar, 0·8% NaCl (w/v), 1·3% (w/v) tryptone, 0·2% (w/v) glucose. The soft top layer contained 0·6% (w/v) agar. When *str-r* mutants were used as indicators for bacteriocins streptomycin sulphate 1 mg./ml. was added to media.

Ultraviolet radiation source. A 30 W. Hanovia germicidal lamp, which delivered more than 80% of its output at a wavelength of 2537Å, was used at a distance of 12 inches.

Procedures. Streptomycin-resistant mutants of sensitive bacterial strains were selected by plating about 10^{11} organisms on streptomycin agar. After incubation resistant colonies were restreaked on this medium for purification. The optimal temperature for bacteriocin production was determined by incubating 0·1 ml. of overnight cultures of SM 1, 5, 8, 9, 11, 41, 91, 99, ATCC 9986, NCTC 2847 in 7·5 ml. broth for 25 hr at 20°, 23°, 26°, 28°, 30°, 34°, 37°. After centrifugation, dilutions of the clear supernatant fluids were spotted on streptomycin agar which had previously been spread with the streptomycin-resistant indicator in a soft agar overlay; the plates were incubated at 32°.

Induction by ultraviolet radiation. Five ml. samples of broth cultures were u.v.-irradiated in Petri dishes in the dark; 0·1 ml. of the u.v.-irradiated suspensions was then added to 7·5 ml. broth and incubated in the dark at 30° for 24 hr. The general phage techniques used were those of Adams (1959). Turbidity measurements were made with a Fisher Electrophotometer, with a 425 mμ filter. Colonies resistant to inhibitory substances were picked from the zones of inhibition in the lawns of growth. The bacterial action of inhibitory substances was determined by subcultur-

ing from the clear zones to nutrient agar; when no growth occurred it was taken that the substances were bactericidal. Heat inactivation was studied by heating 1 ml. samples of the inhibitory solutions in a water bath at various temperatures for various intervals of time, and then testing for activity.

Inactivation by trypsin was determined by adding 0·2 ml. of a 1 % (w/v) trypsin solution (British Drug Houses) in a 0·1 M-phosphate buffer (pH 7·4) to 1 ml. of bacteriocin solution. The mixture was kept at 37° for 1·5 hr and then tested for activity of the bacteriocin.

Many Serratia strains are lysogenic (Prinsloo & Coetzee, 1964) and bacteriocin preparations may therefore also contain phage. Bacteriocins were demonstrated by their greater diffusibility as follows. Clear supernatant fluids obtained after induction were placed in holes in nutrient agar. After 18 hr at room temperature the surface was covered with soft agar which contained the indicator organism and incubated. Samples were then also taken from the periphery of these zones of inhibition, suspended in 1 ml. broth and retested. The absence of transferable activity confirmed the bacteriocin-like nature of the substances. Bacteriocin antiserum was obtained by injection of 2 ml. bacteriocin broth solution (not necessarily free from phage) subcutaneously into rabbits twice weekly for 6 weeks. Neutralization tests with the antisera were done by mixing 0·1 ml. antiserum with 0·9 ml. bacteriocin solution. The mixture was kept at 37° for 1 hr and then tested for activity. Controls had normal rabbit serum in place of antiserum. Chloroform sensitivity was tested by adding 0·5 ml. chloroform to 2·5 ml. antibiotic solution; the mixture was shaken and left at room temperature for 15 min. After the bacteriocin solution was decanted and bubbled with sterile air at 37° to free it from chloroform, its activity was tested.

Chloroform sterilization of antibacterial solutions was not generally used. Instead, clear supernatant fluids from u.v.-induced suspension were spotted on streptomycin-resistant indicator organisms on streptomycin agar.

Inactivation of the antibacterial solutions by gelatin-liquefying strains was tested by the method of Wahba (1963). Proteolytic strains were grown in nutrient agar for 24 hr at 32°. Bacteriocin solutions were spotted over the proteolytic strains and were incubated overnight. The plates were covered with *str-r* indicator organisms in the top layer containing streptomycin. The presence of bacteriocins in broth cultures of SM 1, 8, 9, 11, 41, 91, 99, NCTC 2847 incubated at 30° were tested after 24, 72, 144 hr for autodestruction by autologous proteloytic enzymes. Bacteriocin solutions were dialysed against distilled water for 24 hr at room temperature.

The lysogeny of Serratia strains was investigated as before (Prinsloo & Coetzee, 1964) and all strains were tested for phage susceptibility. Attempts were made to lysogenize susceptible Salmonella strains with serratia phages. These procedures and also tests for immunity of clones to superinfection with phage were done according to Levine (1957). Dilutions of phage suspension and host bacteria were plated on nutrient agar with phage antiserum. Individual colonies were tested for phage production and resistance to phage. Phage multiplicities of input of 1, 5 and 10 were used; the experiments were done at 32°.

RESULTS

According to the spectrum of activity of the bacteriocins two groups were distinguished.

Group A. These agents resembled bacteriocins in their narrow spectrum of activity and were produced by 71 strains in titres up to 1/100. Some strains were more active in the production of bacteriocins than others. They were all active on serratias and some of them were also active against some salmonella strains and *Escherichia coli.*

Group B. These substances were not active on *Serratia marcescens.* They have a spectrum of activity restricted to *Escherichia coli, Aerobacter aerogenes* and the *Hafnia* species. They are produced by 54 strains. Titres up to 1/100 were obtained. Strain ATCC 9986 differed in that its bacteriocin did not attack *E. coli* strains.

Table 1. *Bacteriocinogenic activity of Serratia marcescens strains*

A group activity	A and B group activity	B group	Non-bacteriocinogenic
		Strain no.	
48, 52, 53, 58, 59, 73, 101, 102, 103, 107, 108, 109, 112, 118, 119, 120, 121, 122, 123, 124, 129, 131, 132, 133, 134, 135, 136, 137, 144, 146, 148, 156, 176, 177, NCTC 8015, 9741	1, 4, 6, 7, 9, 11, 19, 37, 38, 41, 42, 43, 44, 91, 99, 111, 116, 125, 127, 139, 140, 149, 150, 157, 160, 170, 174, 190, 200, 207, ATCC 9986, NCTC 1377, 2847, 9940, 10211	3, 5, 10, 12, 14, 15, 18, 39, 40, 45, 47, 74, 75, 130, 161, 172, NCTC 2446, 9493, 9743	51, 56, 57, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 76, 77, 78, 79, 104, 105, 106, 108, 113, 114, 115, 117, 138, 143, 146, 159, 171, 178, 180, 184, 185, 194, 201, 202, 205, 208, 209, NCTC 8900, 3084, 4618, 8706, 10036, 4619

The agents of groups A and B were produced in broth at an optimum temperature of 30°. Bacteriocins were also produced at 20, 23, 26, 28, 34°, and most strains did not produce bacteriocins at 37°. Group B bacteriocins were produced spontaneously in broth but the concentration was greatly increased by ultraviolet irradiation. Suspensions were u.v.-irradiated for periods of from 5 sec. to 10 min. In no instance was mass lysis observed and turbidity measurements increased steadily with time. This also applied to strains which were both bacteriocinogenic and lysogenic. It was found that 45 sec. of u.v.-irradiation of an overnight broth culture incubated in broth in the dark usually yielded maximal titres. Only 32 of the 73 bacteriocinogenic serratia strains investigated by Hamon & Peron (1961) produced detectable bacteriocins in agar. The remaining 41 strains only liberated bacteriocins after u.v.-induction of broth cultures. As seen in Table 1, 35 strains which produced A agents also produced B agents. This double activity was determined by the use of appropriate indicator organisms. Thus in the case of the A bacteriocins Serratia indicators were used, and for group B agents Hafnia indicators. Overnight as well as exponentially growing cultures were used. With strains which produced both A and B bacteriocins the B agent was usually detectable in supernatant fluids about 1 hr before A activity appeared. In no instance did a strain produce an agent which

was active on itself like the colicin which Ryan, Fried & Mukoi (1955) encountered, and the strains of *Pasteurella pseudotuberculosis* and *P. pestis* which are pestinogenic for pesticin II were also sensitive to it.

The bacteriocins were bactericidal. The bacteriocins of group A were resistant to trypsin and chloroform and withstood 60° for 30 min. They were non-dialysable. These agents were resistant to the action of proteolytic organisms but the bacteriocin liberated by SM 52 was thermolabile, sensitive to trypsin, and completely inactivated by proteolytic organisms. Group B substances were sensitive to trypsin and chloroform and inactivated at 60° for 30 min. They were non-dialysable. These B substances were inactivated by autologous proteolytic enzymes when tested by the method of Wahba (1963). In broth cultures the titre of the B bacteriocins was decreased after 72 hr incubation and could not be demonstrated after 144 hr.

Table 2. *Subdivision of A group bacteriocins of Serratia marcescens*

	Indicator strains of *S. marcescens*				Mutants of indicator strains				
					SM. 42 resistant to		SM. 8 resistant to		
Bacteriocin produced by	SM. 42	8	NCTC 8900	1	41	41+8	11	99	Subdivision
1, 6, 125, 127	+++	.	+++	.	+++	+++	.	.	A_1
9, 91, 199, 122, 139, 177, 200, NCTC 10211	+++	.	.	.	+++	+++	.	.	A_2
8, 44, 48, 101, 116, 121, 123	+++	.	.	.	+++	−	.	.	A_3
11, 38, 43, 58, 73, 99, 137, 144, 146, 148, 157, 176, 190, 207	.	+++	+++	.	.	.	−	−	A_4
41, 136, 140	+++	+++	+++	+++	−	−	.	.	A_5
NCTC 2847, 37	+++	+++	+++	.	+++	+++	.	.	A_6
ATCC 9986	+	.	++	+++	.	.	.	.	A_7
52	+	+	.	+++	.	.	.	.	A_8

+++, clear spots; ++, slightly turbid spots; +, turbid spots; −, negative.

Subdivision of antibiotics

Group A bacteriocins. Resistant mutants of indicator strains were obtained against most of the A bacteriocins apart from those which produced turbid areas of inhibition from which it was not possible to select mutants. Table 2 shows that the A bacteriocins can be differentiated into eight types by their activity on various resistant mutants. This division is supported by the results given in Table 3. Thus A 6 is the only bacteriocin active on salmonellas, A 1 and A 3 are active on *Aerobacter aerogenes* X_1, and bacteriocin A 1 kills *Escherichia coli* ø. The bacteriocins of 40 of the 71 strains which produce the A substances have been subdivided. The remaining strains either produce the bacteriocin in low titres or they do not correspond to any of the eight subtypes.

Group B bacteriocins. Resistant mutants selected from the indicator organism *Hafnia* H_1 to the B bacteriocins produced by SM 5, 6, 9, NCTC 1377 were resistant to

all other bacteriocins of group B. Resistant mutants of *Escherichia coli* ø to bacteriocins produced by SM 2, 5, 11, 43, 44, 45, NCTC 9940, 9743 were also resistant to all the baeteriocins of group B. Strain SM 5 produced only a B bacteriocin; antiserum against this bacteriocin inactivates all the B-group agents. Antiserum was prepared only against the SM 5 bacteriocin. These results indicate possible identity of the group B bacteriocins produced by the 54 strains. There is one exception: the product of ATCC 9986 is similar to the other B bacteriocins by the above criteria, but *E. coli* strains were not susceptible.

Table 3. *Spectrum activity of bacteriocins of Serratia marcescens*

Inhibitory agent	Susceptible strains
A_1	Serratia, *Aerobacter aerogenes* X_1, *Escherichia coli* ø
A_2	Serratia
A_3	Serratia, *A. aerogenes* X_1
A_4	Serratia
A_5	Serratia
A_6	Serratia, Salmonella
A_7	Serratia
A_8	Serratia
B	*E. coli* ø, *E. coli* ca 58, *A. aerogenes* X_2, *Hafnia* H_1–H_6
B (produced by ATCC 9986)	*A. aerogenes* X_2, *Hafnia* H_1–H_6

Distinction between colicins and bacteriocins A, B

Hafnia H_1 was susceptible to the B baeteriocins and not to the various colicins. Serratia strains susceptible to the A baeteriocins were not susceptible to colicins. *Escherichia coli* ø was susceptible to serratia baeteriocins B, A1 and all colicins. Colicin-resistant mutants of *E. coli* ø were still susceptible to the bacteriocins B, A1, except *E. coli* ø mutants resistant to colicin *k*. These mutants were resistant to bacteriocin B, although susceptible, to bacteriocin A1. However, resistant mutants of *E. coli* ø to bacteriocin B were susceptible to colicin K. Colicin K was not neutralized by antiserum to the bacteriocin B liberated by strain SM 5.

Lysogeny

Strains SM 8, 37, 41, 56 liberated phage spontaneously in high titres (1×10^9). Phages derived from the first two strains were active on these strains. These phages may be virulent mutants of prophages carried by these organisms (Jacob & Wollman, 1961) and capable of overcoming homologous immunity. The corresponding temperate phages have, however, not been demonstrated. Thirty-three out of the remaining 139 strains liberated phage spontaneously or after u.v.-induction. As in the case of inducible baeteriocins, mass lysis was never observed and the turbidity readings simply increased with time. Cultures were u.v.-irradiated in various phases of the growth cycle for various periods of time. A period of 1 min. of u.v.-irradiation of an overnight culture usually gave maximal titres after 18 hr incubation. Phages liberated by strains SM 8, NCTC 2847 had similar host ranges, and phages derived from strains SM 9, 41, 52, ATCC 9986 were also similar by this criterion. The results are too unwieldy for presentation but the remaining 10 phages could be differentiated from one another by their host ranges, and 19 phages plated equally well on various serratias and salmonellas (Prinsloo & Coetzee, 1964). The plaques which the 19

phages formed on the 15 Salmonella strains were slightly turbid but attempts to lysogenize three of the Salmonella strains were uniformly negative and the clones remained sensitive to the phages.

Strains which produced phage and an A type bacteriocin often attacked a particular Serratia strain but these agents could invariably be distinguished by using additional indicator organisms. The phage and the bacteriocin type A produced by a single strain could also be distinguished by the selection of mutants of the indicator organism resistant to the bacteriocin. The phage was invariably still active on the mutant.

DISCUSSION

The trypsin-resistant and trypsin-sensitive bacteriocins described previously (Hamon & Peron, 1961) correspond to the A and B bacteriocins described here. The bacteriocins described by Mandel & Mohn (1962) are similar to our B agents. It has been confirmed that the trypsin-sensitive B bacteriocins do not attack *Serratia marcescens* and shown that some of the A subtypes also attack *Escherichia coli*, Aerobacter and Salmonella strains while the B group kill *E. coli*, Aerobacter and Hafnia strains. Mandel & Mohn (1962) showed that a mutant of *E. coli* Row which was resistant to colicin K was also resistant to the substances produced by their Serratia strains. They designated the latter substances sub-species of colicin K. Colicin K resistant mutants of *E. coli* o are also resistant to bacteriocin B but it has been possible to differentiate between the latter and colicin K. The two bacteriocins also have different electrophoretic mobilities (Prinsloo, Maré & Coetzee, 1965). Hamon & Peron (1961) demonstrated that 86% of their 85 Serratia strains were bacteriocinogenic. The investigation of Mandel & Mohn (1962) showed 58% of 31 strains of *S, marcescens* to be bacteriocinogenic for *E. coli* strains. The figure found in the present work is 64·7% for 139 strains of *S. marcescens*. Our strains do not undergo clearing after u.v.-induction and resemble the colicinogenic strains of *E. coli* (Fredericq, 1955), a pyocin-producing strain of *Pseudomonas aeruginosa* (Hamon, 1956), and pestocinogenic strains of *Pasteurella pestis* (Ben-Gurion & Hertman, 1958) in this respect.

Like the strains of Hamon & Peron (1961) many of our strains produce more than one antibiotic material. This has been confirmed (Prinsloo *et al.* 1965) by the demonstration that the A bacteriocins are electrophoretically mobile, while B agents produced by the same strain are stationary. A particular *Escherichia coli* strain may produce two or more distinct colicins (Fredericq, 1958), individual strains of *Enterobacter cloaca* (Hamon & Peron, 1962) are also believed to be multiple cloacinogenic, and strains of *Salmonella typhimurium* have been rendered doubly colicinogenic (Smith & Stocker, 1962).

The mole % guanidine + cytosine of the genera *Serratia* and *Salmonella* are 58 and 50%, respectively (Marmur, Falkow & Mandel, 1963). Despite this difference, 19 of the *Serratia marcescens* phages multiplied in various Salmonella species, but lysogenization by the serratia phages could not be demonstrated in salmonellas. Phages may multiply in bacteria with base compositions very different from their own (Lanni, 1960), and the present examples may only indicate that some Serratia and Salmonella strains share phage receptors (Meynell, 1964).

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Diurnal Changes in the Concentrations of Micro-organisms in the Rumens of Sheep Fed Limited Diets Once Daily

WITH AN APPENDIX ON THE KINETICS OF RUMEN MICROBES AND FLOW

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SUMMARY

The pattern of change of concentration of different groups of micro-organisms in the rumen was found to be characteristic of the group and little affected by the time of day, the nature of the diet, or the host animal. The dilution rate of rumen liquor and the rate of change of concentration of several groups of micro-organisms were measured at intervals following feeding; this allowed calculation of the apparent specific growth rate. The changes in concentration of the ophryoscolecid ciliate protozoa, the selenomonads and the oval organism described by Eadie could be explained as the resultant of the changes in dilution rate due to the act of eating and in growth rate in response to incoming nutrients. Rates of change in concentration greater than could be accounted for on the basis of growth and dilution alone were found with the peptostreptococci, the polymastigate flagellate protozoa and the holotrich ciliate protozoa. It is suggested that the peptostreptococci underwent lysis or engulfment by other organisms and that the polymastigates became sequestered, probably close to the rumen wall. The concentration changes of the holotrichs were more difficult to understand, but it would appear that little division took place for some 18 hr after feeding, followed by several divisions in quick succession. The oscillospirae showed two peaks in concentration; no explanation can be offered for this.

INTRODUCTION

Warner (1962*b*) found that the concentrations of different groups of micro-organisms in the rumen of a single sheep fed once daily changed with time in different ways, with different amplitudes and reaching maxima and minima at different times. The present studies extend this work to other sheep, diets and times of feeding. The rates of change of concentration are compared with the dilution rate of the rumen liquor, allowing calculation of the growth rate of the micro-organisms. Some tentative explanations of the various patterns of concentration change are offered. Subsequent papers describe studies made of sheep offered limited quantities of feed at frequent intervals throughout the day (Warner, 1966*a*) and of sheep with unlimited quantities of feed available, either in pens or at pasture (Warner, 1966*b*).

METHODS

Sheep. In the main experiments English Leicester–Merino crossbred ewes or wethers were used; in the minor experiments other Merino ewes were used. The sheep were trained to eat their ration within 1 hr.

Enumeration of micro-organisms. The standard counting techniques of Warner (1962*a*) were used.

Identification of organisms. Genera of ophryoscolecid protozoa were identified and named following Lubinsky (1957). It should be noted that the organisms described as *Metadinium* sp. by Warner (1962*b*) were misnamed; following Eadie (1962*a*), they should have been described as *Polyplastron* sp.

Polymastigates and selenomonads were identified as previously (Warner, 1962*b*).

Peptostreptococci were the organisms given the number 27 by Moir & Masson (1952) and described simply as streptococci by Warner (1962*b*). They are here renamed to avoid confusion with *Streptococcus bovis*, believed to be present in some of these specimens, but not positively identified or counted.

Table 1. *Major experiments*

Expt. no.	Sheep no.	Protozoal type*	Diet†	Time of feeding (hr)
1	SO74	A	700 g. R9	09.00
2	SO74	A	700 g. R9	15.30
3	SO74	A	700 g. F19	09.00
4	SO74	A	700 g. F19	15.30
5	1319	B	700 g. R9	09.00
6	B586	A	700 g. M2	09.00

* Ophryoscolecid protozoal population type (Eadie, 1962*a*, *b*); polyplastrons and diploplastrons were seen in type A populations, epidinia, diplodinia and eudiplodinia in type B.

† Diet R9 consisted of 50 % wheaten chaff, 50 % lucerne chaff. Diet F19 consisted of 50 % lucerne chaff, 20 % wheat, 10 % linseed meal, 10 % coconut meal, 10 % oats. Diet M2 consisted of 50 % lucerne chaff, 50 % oats.

The organisms described here as Eadie's ovals were described by Eadie (1962*a*) in Scotland and independently as 'flagellates' by Warner (1962*b*) in Australia; they have since been described in Egypt by Abou Akkada & El-Shazly (1964).

Experimental design. Six major experiments were carried out in which samples of rumen contents were collected at intervals of about 2 hr for 24 hr or more, and examined thoroughly for all important groups of organisms. These experiments are described in Table 1.

Other sheep were fed 800 g. of diet R9 with or without 1·25 % added NaCl at either 09.00 hr or 14.00 hr, and were used in minor experiments as described later, only one group of micro-organisms being examined at a limited number of times.

In another experiment, the dilution rate of the rumen liquor was determined. The ^{51}Cr complex of ethylenediaminetetra-acetic acid ($^{51}CrEDTA$) was added to the rumen of sheep SO74 and the concentration of ^{51}Cr determined from time to time as described by Downes & McDonald (1964). The dietary regimen was 700 g. diet R9 fed at 14.00 hr.

In all these experiments, access to water was allowed at all times. Animals were fed according to the experimental schedule for at least 3 weeks before sampling, and were thoroughly accustomed to the routine.

EXPRESSION OF RESULTS

It is shown in the appendix to this paper that plots of the logarithm of the concentration of a reference solute such as ^{51}CrEDTA and of the micro-organisms against time can be used to calculate the dilution rate of the rumen liquor and the growth rate of the micro-organisms. Monod (1949) pointed out the convenience of using logarithms to the base 2 for graphical presentation of work with bacterial cultures; similar considerations apply to flowing systems and hence to work with organisms in the rumen.

It was found previously (Warner 1962*b*) that under apparently constant conditions of nutrition the concentration of any one species of micro-organism in the rumen at a specified time after feeding can stabilize at any of a wide range of concentration values. However, it was expected that the pattern of growth behaviour of a given organism over the 24 hr feeding cycle would be largely independent of its initial concentration, so to compare concentration curves at different concentration levels the following procedure was adopted to plot the results of any one experiment.

(i) The concentration of each group of organisms was estimated for each sample of rumen liquor, and the $\log_2$ concentration was computed, using the loglog scales of a slide rule and units chosen to give a minimum concentration between 1 and 10.

(ii) The mean of these $\log_2$ concentrations for each group of organisms was calculated; the concentration corresponding to this mean was called the basal concentration, and is recorded in the legends to the figures.

(iii) The deviations of the $\log_2$ concentrations from the mean were computed and plotted as the relative $\log_2$ concentrations about the mean as arbitrary zero.

Examples of sets of individual curves plotted in this way are shown in Figs. 1, 11 and 13. A mean curve for each organism was then constructed from the corresponding set of individual curves. It had been inconvenient to collect specimens in the various experiments at identical times after feeding. Consequently, these mean curves were plotted by computing values for 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 23 hr after feeding, from each individual curve, and then taking the mean for each time. Thus Fig. 2 contains the mean curve for the individual curves plotted in Fig. 1. Other mean curves were plotted similarly. It should be noted that the zero line of this mean curve has not a simple meaning.

These curves were then used to calculate the relative rates of increase of concentration, r, as described in the appendix. The sum of this rate and the dilution rate D equals the net rate of formation of microbial cells ρ; for a randomly dividing population when the death rate λ is negligible, this is the same as the specific growth rate ν.

An alternative calculation of ν can also be made from the division index, the proportion of dividing organisms. Warner (1962*b*) used an equation of Crick (1948) as cited by Hughes (1952) to determine the doubling time, g, of entodinia in the rumen. Smith & Dendy (1962) showed that the equation should have been $g = T \ln 2/\ln (1+I)$ instead of that misquoted by Hughes (1952),

$$g = T \ln 2/\ln [(1+2I)/(1+I)],$$

where T is the time needed to complete division and I is the proportion of dividing cells; for small values of I the two formulae give the same result. Hungate, Bryant & Mah (1964) correctly criticized the use of this formula to calculate genera-

tion times of entodinia at various times of the day, since some degree of synchronicity was present; however, the equation can be used to give a simple measure of the rate of increase of numbers at any instant, without any implications about the time between successive divisions. From the equation of Smith & Dendy (1962) above, it can be seen that $\nu = \ln 2/g = \ln (1+I)/T = I/T$ approximately for small values of I. In the work reported here, the observer who estimated the proportion of dividing cells used different criteria from those used in the studies of Warner (1962*b*) and it is believed that $I' = 0{\cdot}75I$, where I' is the proportion of cells recognizable as dividing in the living preparations used to determine the time to complete division T, and I is the proportion of cells recognizable as dividing in fixed preparations, as recorded in this paper. For entodinia, therefore, $\nu = I'/T = 3I$ hr^{-1}, since $T = 0{\cdot}25$ hr (Warner, 1962*b*).

The time of day is expressed on the 24 hr clock as 01.00 hr, etc.; the time after presentation of feed, briefly the time after feeding, is expressed as 1 hr, etc.

RESULTS

The concentrations of micro-organisms were found to change with time in patterns characteristic of the species, as shown by Figs. 1–14. Closely similar curves were obtained despite variations in basal concentrations, as shown by the curves for entodinia in Expts. 3 and 4 (Fig. 1), dasytrichs in Expts. 1 and 5 (Fig. 11) and oscillospirae in Expts. 2 and 4 (Fig. 13).

Altering the time of feeding shifted the phase of these patterns by an equivalent amount, as is seen by comparing pairs of curves for Expts. 1 and 2 (Figs. 1*a*, 11*a*, and 13*a*) and for Expts. 3 and 4 (Figs. 1*b*, 11*b* and 13*b*). No evidence was seen of the influence of any diurnal rhythm other than that of feeding.

No effect on the patterns of concentration change due to the host animal could be detected in comparing the results of Expts. 1 and 5; examples may be seen in Figs. 1 and 11. The patterns obtained with the three different diets, R9 (Expts. 1, 2 and 5; Figs, 1, 11 and 13), F19 (Expts. 3 and 4; Figs. 1, 11 and 13) and M2 (Expt. 6, Figs. 1 and 11) were very similar, though small differences in magnitude (Figs. 1*b*, 1*c*, 11) or in timing (Figs. 1*c* and 13) can be seen.

Similar findings to the above were made with the other micro-organisms, suggesting that the pattern of concentration change was primarily determined by the species of micro-organism under consideration, and little affected by its initial concentration, the time of day, the nature of the diet, or the host animal. Consequently, it was felt to be justifiable to construct the mean curve as described above. It should be noted, however, that the minor differences in timing previously mentioned tend to make the mean curve flatter than the individual curves, with lower maxima and higher minima (compare, particularly, individual curves in Figs. 11 and 13 with the mean curves in Figs. 12 and 14). Consequently, in reporting the results of many experiments, data from the unpublished individual curves as well as the published mean curves are given.

Entodinia. Immediately following feeding, the concentration of entodinia decreased at a rate $r = -0{\cdot}16$ hr^{-1} (Fig. 2) and continued to decrease for the first 2–4 hr. However, the proportion of dividing organisms and hence the growth rate started to increase immediately and increased to a maximum of some four times the

initial rate at about 8 hr after feeding, after which it decreased gradually over the remaining 16 hr. The concentration increased from its minimum at 2–4 hr to a maximum at 16–20 hr at a rate of about $r = 0{\cdot}05$ hr^{-1} (range, from Fig. 1, about 0·03–0·12 hr^{-1}). During the last 2–6 hr before feeding, when the growth rates were minimal, the concentration decreased at a mean rate of $r = -0{\cdot}07$ hr^{-1}.

Other ophryoscolecid protozoa. The diploplastrons (Fig. 3), polyplastrons (Fig. 4) and epidinia (Fig. 5) all showed patterns of concentration change similar to the entodinia, a rapid decrease in concentration after feeding, then a slower increase to

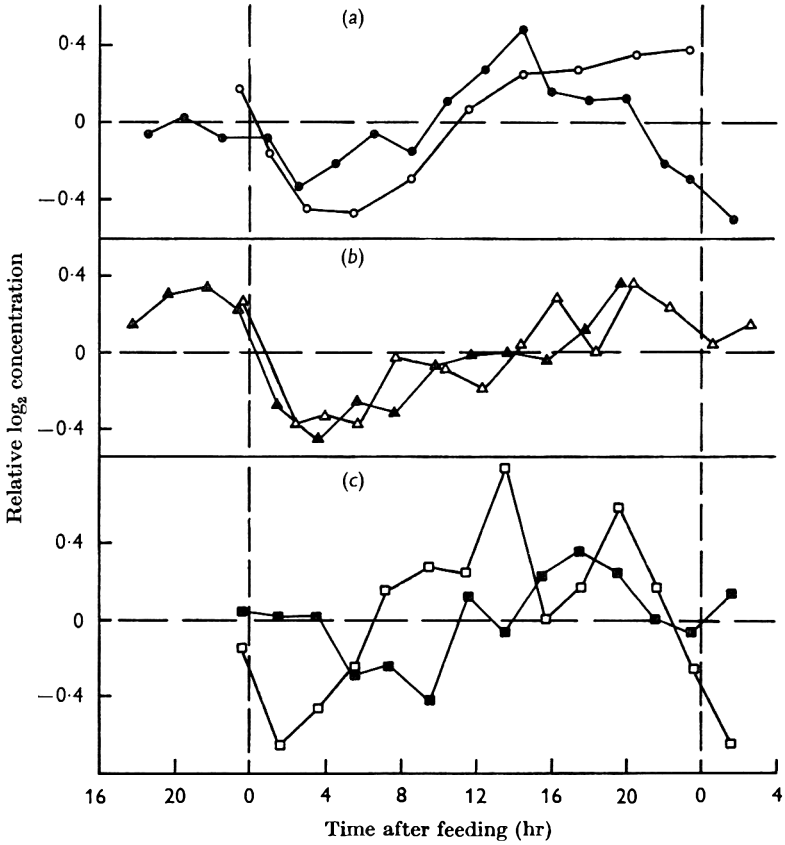

Fig. 1. Changes in concentration of entodinia in individual experiments (basal concentrations of entodinia in numbers/ml. are given in brackets):

(*a*) ○ Expt. 1 sheep SO74 fed diet R9 at 09.00 hr ($1{\cdot}3 \times 10^5$).
● Expt. 2, sheep SO74 fed diet R9 at 15.30 hr ($2{\cdot}0 \times 10^5$).
(*b*) △ Expt. 3, sheep SO74 fed diet F19 at 09·00 hr ($7{\cdot}3 \times 10^5$).
▲ Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($4{\cdot}4 \times 10^5$).
(*c*) □ Expt. 5, sheep 1319 fed diet R9 at 09.00 hr ($3{\cdot}9 \times 10^5$).
■ Expt. 6, sheep B586 fed diet M2 at 09.00 hr ($2{\cdot}6 \times 10^6$).

a maximum a few hours before the next feed, followed by a slow decline. The diplodinia (Fig. 5) were examined in only one experiment; this seemed to indicate an absence of the rapid initial decrease in concentration. The organisms were, however, present in very low concentration and this seems to lead to increased counting error, even though the total number of organisms counted was similar to that counted in

the other experiments. It seems possible that, in fact, the diplodinia suffer similar concentration changes to the other ophryoscolecids. The polyplastrons, diplodinia and epidinia (Figs. 4, 5) changed in concentration over a wider range than the entodinia or diploplastrons (Figs. 2, 3).

In Expt. 5 (Fig. 5), the epidinia and diplodinia were present in low concentration, making the estimation of the proportion of dividing organisms difficult and inaccurate, but for both organisms peak proportions exceeding 0·10 were noted.

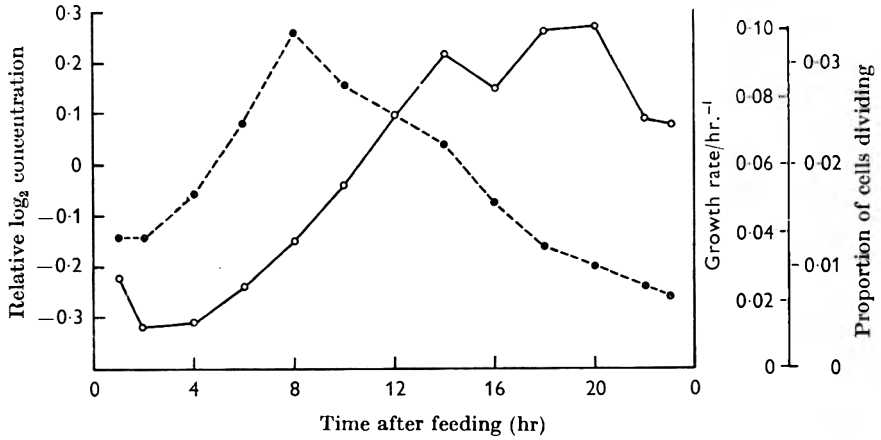

Fig. 2. Mean curves for concentration (○), and proportion of dividing organisms and specific growth rate ν (●) for entodinia; data from the experiments described in Fig. 1 were used.

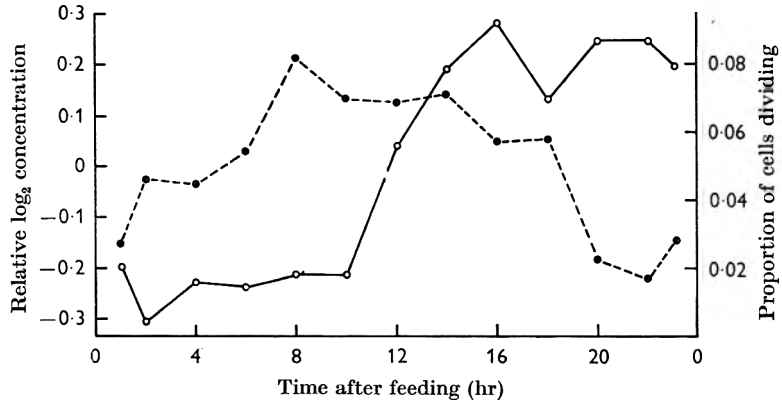

Fig. 3. Mean curves for concentration (○) and proportion of dividing organisms (●) for diploplastrons; data used were from the following experiments (basal concentrations of diploplastrons in numbers/ml. are given in brackets): Expt. 2, sheep SO74 fed diet R9 at 15.30 hr ($5{\cdot}1 \times 10^3$); Expt. 3, sheep SO74 fed diet F19 at 09.00 hr ($3{\cdot}6 \times 10^4$); Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($8{\cdot}7 \times 10^3$); Expt. 6, sheep B586 fed diet M2 at 09.00 hr ($5{\cdot}6 \times 10^4$).

The mean proportion of dividing organisms found for all the higher ophryoscolecids was greater than that noted for the entodinia (Fig. 2).

Selenomonads. The concentration of selenomonads decreased after feeding (Fig. 6) at a mean rate of about $r = -0{\cdot}22\ \text{hr}^{-1}$ (range $-0{\cdot}1$ to $-0{\cdot}5\ \text{hr}^{-1}$) to a minimum

usually within the first 2 hr. Concentrations then remained fairly steady for 8–12 hr and then increased more rapidly (r = 0·03–0·11, mean 0·05 hr^{-1}) to a peak 16–24 hr after feeding, then perhaps slowly declining.

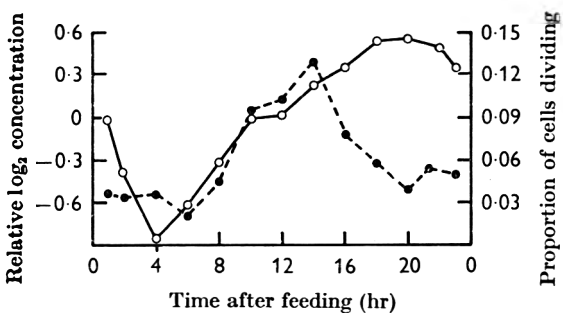

Fig. 4

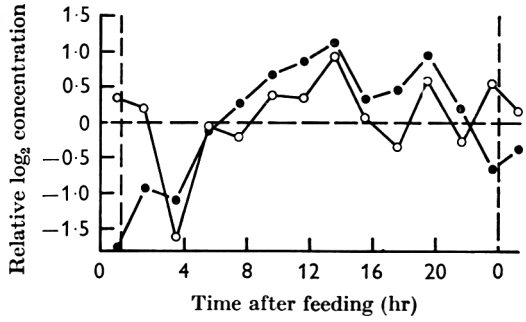

Fig. 5

Fig. 4. Mean curves for concentration (○) and proportion of dividing organisms (●) for polyplastrons; data used were from the following experiments (basal concentrations of polyplastrons in numbers/ml. are given in brackets): Expt. 3, sheep SO74 fed diet F19 at 09·00 hr ($3·5 \times 10^3$); Expt. 4, sheep SO74 fed diet F19 at 15·30 hr ($4·0 \times 10^3$).

Fig. 5. Changes in concentration of micro-organisms in Expt. 5, sheep 1319 fed diet R9 at 09.00 hr (basal concentrations of micro-organisms in numbers/ml. are given in brackets): (○) epidinia ($4·2 \times 10^3$); (●) diplodinia ($2·9 \times 10^3$).

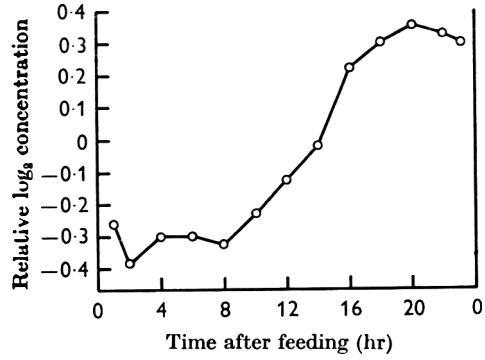

Fig. 6

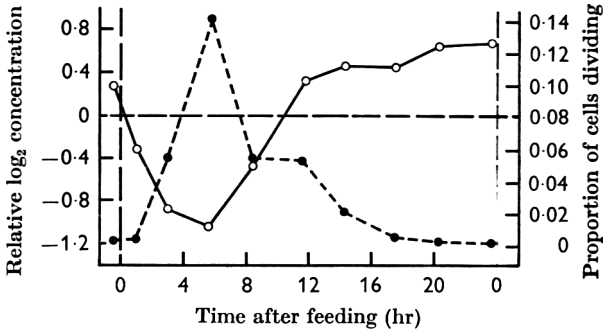

Fig. 7

Fig. 6. Mean curve for concentration of selenomonads; data used were from the following experiments (basal concentrations of selenomonads in numbers/ml. are given in brackets): Expt. 1, sheep SO74 fed diet R9 at 09.00 hr ($2·8 \times 10^7$); Expt. 2, sheep SO74 fed diet R9 at 15.30 hr ($5·5 \times 10^7$); Expt. 3, sheep SO74 fed diet F19 at 09.00 hr ($3·4 \times 10^8$); Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($5·8 \times 10^7$); Expt. 5, sheep 1319 fed diet R9 at 09·00 hr ($7·6 \times 10^6$).

Fig. 7. Changes in concentration (○) and proportion of dividing organisms (●) for Eadie's ovals in Expt. 1, sheep SO74 fed diet R9 at 09·00 hr (basal concentration $4·1 \times 10^6$/ml.)

Eadie's ovals. These organisms were observed in only a few experiments. While the concentration curves were generally similar, the maxima and minima occurred at somewhat different times and this made the mean curve misleading; consequently, figures for one experiment only are given in Fig. 7. The concentration decreased

after feeding at a rate of about $r = -0{\cdot}2\ hr^{-1}$ to a minimum at 6–12 hr, then rising at a rate of about $0{\cdot}17\ hr^{-1}$ to a plateau at 12–20 hr, remaining fairly constant thereafter, at a level about four times the minimum. The proportion of dividing organisms was very low in the fasting animal, rising very rapidly after feeding to a maximum at about the time of minimum concentration, declining again rather more slowly.

Total bacterial counts. There was a decrease in concentration (Fig. 8) after feeding at a mean rate $r = -0{\cdot}14\ hr^{-1}$ (range 0·0 to $-0{\cdot}19$) to a minimum at 1–4 hr, then a slow increase at a mean rate $r = 0{\cdot}03\ hr^{-1}$ to a maximum at 12–20 hr; from about 20 hr after feeding, the concentration usually decreased at a rate about $-0{\cdot}03\ hr^{-1}$. For Expts. 1 and 6, the curves were very flat, the maxima being only some 15% greater than the minima; in Expt. 5, however, the range was twofold.

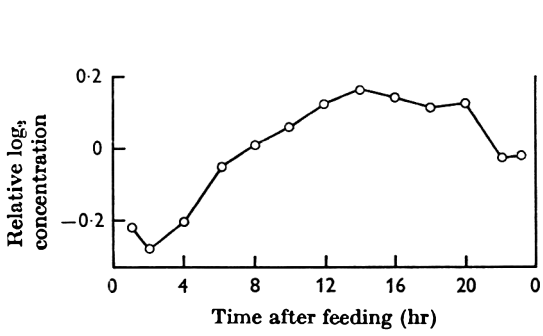

Fig. 8

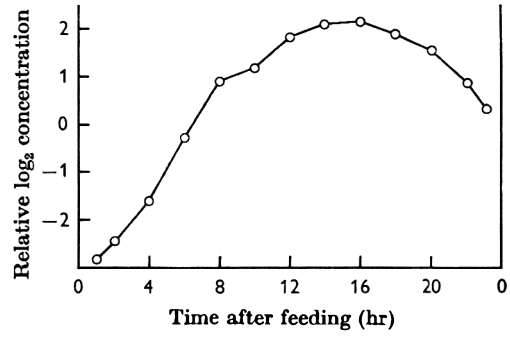

Fig. 9

Fig. 8. Mean curve for concentration of total bacteria; data used were from the following experiments (basal concentrations of bacteria in numbers/ml. are given in brackets): Expt. 1, sheep SO74 fed diet R9 at 09.00 hr ($2{\cdot}0 \times 10^{10}$); Expt. 2, sheep SO74 fed diet R9 at 15.30 hr ($1{\cdot}8 \times 10^{10}$); Expt. 3, sheep SO74 fed diet F19 at 09.00 hr ($2{\cdot}7 \times 10^{10}$); Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($2{\cdot}3 \times 10^{10}$); Expt. 5, sheep 1319 fed diet R9 at 09.00 hr ($2{\cdot}6 \times 10^{10}$); Expt. 6, sheep B286 fed diet M2 at 09.00 hr. ($2{\cdot}6 \times 10^{10}$).

Fig. 9. Mean curve for concentration of peptostreptococci; data used were from the following experiments (basal concentrations of peptostreptococci in numbers/ml. are given in brackets): Expt. 1, sheep SO74 fed diet R9 at 09.00 hr (8×10^4); sheep 1184 fed diet R9 at 14.00 hr (4×10^4); sheep 2714 fed diet R9 at 14.00 hr (1×10^4); sheep MA94 fed diet R9 at09.00 hr (2×10^4).

Peptostreptococci. This organism is multicellular, and was counted as the number of chains. There was a great decrease in concentration after feeding (Fig. 9) at a mean rate $r = -1{\cdot}7\ hr^{-1}$ (range $-0{\cdot}7$ to $-2{\cdot}8\ hr^{-1}$) to a minimum 1–4 hr after feeding, then a steady increase at a mean rate of about $0{\cdot}4\ hr^{-1}$ to a maximum at about 12–18 hr, and finally a decrease at a slowly increasing rate up to about $r = -0{\cdot}3\ hr^{-1}$. The number of cells per chain appeared to be relatively constant. In Expt. 1, 40 chains were examined in each specimen. In all specimens the mean number of cells per chain was between 5 and 6, with standard deviations about 2. More than half the chains had four cells, more than a quarter eight; other numbers found in this series were, in decreasing order of frequency, 6, 2, 3, 5 and 12. Accurate counts have not been made, but it appears that, in other experiments, the mean chain length may have been somewhat different; in some experiments, 16 or more

cells per chain were not rare. Chain length may be a characteristic of a particular strain of peptostreptococcus, or perhaps of particular environmental conditions.

Polymastigates. These flagellates showed a large increase in concentration 1–2 hr after feeding (Fig. 10) at a mean rate $r = 1{\cdot}4\ hr^{-1}$; in one experiment, $r = 3{\cdot}8\ hr^{-1}$, and the concentration increased 50-fold in 3 hr. This increase was followed by an almost equally rapid decrease in concentration to a minimum at 6–12 hr after feeding, followed by a fluctuating concentration value. In most experiments, the ratio of maximum to minimum concentration exceeded 25; the averaging process used to draw the mean line masked this, showing only an eightfold range, due mainly to differences in timing of the minima and the subsequent four- to eightfold fluctuations.

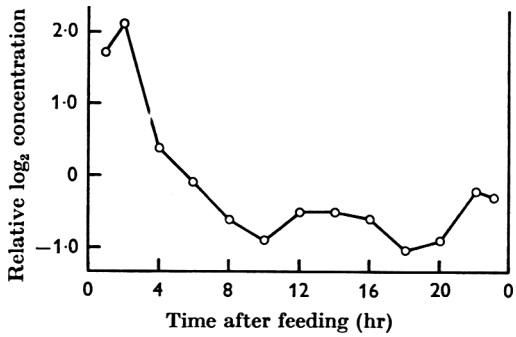

Fig. 10. Mean curve for concentration of polymastigates; data used were from the following experiments (basal concentrations of polymastigates in numbers/ml. are given in brackets): Expt. 1, sheep SO74 fed diet R9 at 09.00 hr (7×10^3); Expt. 2, sheep SO74 fed diet R9 at 15.30 hr (3×10^3); Expt. 3, sheep SO74 fed diet F19 at 09.00 hr (1×10^4); Expt. 4, sheep SO74 fed diet F19 at 15.30 hr (5×10^3); Expt. 5, sheep 1319 fed diet R9 at 09.00 hr (1×10^3); Expt. 6, sheep B586 fed diet M2 at 09.00 hr (2×10^4).

This great increase in concentration after feeding suggested that organisms might perhaps be entering the rumen with the feed or the drinking water. However, no polymastigates were seen in any of the following preparations: the deposit from centrifuged water from the drinking troughs; unfiltered aqueous extracts of the feed; cultures of feed incubated with water or centrifuged rumen liquor for 24 hr at 39° in completely filled glass-stoppered bottles, presumed to be anaerobic; cultures of water from the drinking troughs incubated as above either alone or mixed with an equal volume of centrifuged rumen liquor. No organisms suggesting cysts of these polymastigates were noticed, confirming Braune (1913).

In an attempt to identify the stimulus for the increase in concentration, four extracts of the feed were prepared as follows:

(1) 500 g. coarsely ground diet R9 mixture was extracted for 45 min. with water in a shaking machine, and the extract was filtered.

(2) A similar extract was dialysed against running tap water overnight, then against several changes of distilled water in the refrigerator.

(3) Following the procedure of Smith & Benitez (1955), 500 g. finely ground diet R9 mixture was extracted with 1500 ml. 80% (v/v) acetone in water, filtered on a Buchner funnel and washed with 3×150 ml. 80% (v/v) acetone in water. To the filtrate (about 1000 ml.), 320 g. talc and 1000 ml. water were added and the mixture

filtered through a layer of talc on a Buchner funnel. The filtrate was concentrated in a rotary evaporator under reduced pressure, using a silicone oil anti-foam. The concentrate was divided into two portions and each was evaporated to dryness to remove acetone, taken up in 100 ml. water and centrifuged. The supernatant fluid in each portion contained about 8·0 g. dry matter (about 150 mg. N). One portion was used without further treatment.

(4) The second portion was mixed with 250 ml. 6 N-HCl and hydrolysed overnight. The HCl was removed by repeated evaporation under reduced pressure, the mixture brought to 100 ml. with water and filtered. It was found to contain about 3·8 g. dry matter (about 135 mg. N).

Table 2. *Effect of intra-ruminal administration of feed extracts on the concentration of polymastigates in the rumen*

The normal feed was given immediately after the 2 hr sample

Sample	Concentration of polymastigates (thousands/ml.)			
	Water extract	Dialysed water extract	Acetone extract	Hydrolysed acetone extract
Pre-dosing	6	4	6	10
1 hr post-dosing	43	11	120	13
2 hr post-dosing	32	7	90	23
1 hr post-feeding	12	29	15	35

These four extracts were injected into the rumens of sheep held 24 hr without feed; 2 hr later, feed was given. Samples of rumen liquor were taken before, and 1, 2 and 3 hr after, the extracts were injected, and examined for polymastigates. The results are given in Table 2. Both aqueous and acetone extracts of feed elicited the response of rapid increase in concentration of the polymastigates followed by a slower decline; subsequent feeding did not reverse the decline. The dialysed (2) and hydrolysed (4) extracts elicited little if any response and subsequent feeding produced a small increase in concentration. No response was elicited by the injection of solutions of glucose or of bacteriological peptone.

Holotrich protozoa. The dasytrichs (Figs. 11, 12) reached a peak concentration at or shortly after the time of feeding, and then fell steadily in concentration (mean rate of fall $r = -0{\cdot}11$ hr^{-1}) to a minimum around 12–20 hr. This was followed by a rapid increase in concentration at a mean rate of 0·30 hr^{-1}, range 0·14–0·70 hr^{-1}. The isotrichs (Fig. 12) behaved similarly, though moving through a somewhat smaller range.

In several supplementary experiments in which one day's feed was withheld, it was found that concentrations of holotrichs increased rapidly from the minimum at about 20 hr to maxima at 22–28 hr after feeding. The subsequent rate of decrease appeared to be slightly less than in the main experiments, but sampling was terminated before this was determined with certainty.

Throughout the first 16 hr after feeding, the proportion of dividing holotrichs was very low, usually 0·001 or less. This proportion then rose to a maximum at or just after the time of minimum concentration, falling rapidly again. The maximum proportion of dividing holotrichs was usually about 0·10, but in a few instances even higher proportions, up to 0·28, of dividing dasytrichs have been seen.

Sporadically in this work, the dasytrichs have been seen in conjugation. The proportion of conjugating organisms has usually been 0·01 or less, but on a few occasions it has been over 0·10. So far, no pattern has been noted in this phenomenon, and it appears to be unrelated to normal division.

Oscillospirae. These multicellular organisms were counted as the number of trichomes, not the number of cells. Two peaks in concentration were found (see Figs. 13, 14), the first at about the time of feeding. Following this, the concentration declined at a mean rate $r = -0{\cdot}09$ hr^{-1} to a minimum 6–8 hr after feeding, increased again at a mean rate $r = 0{\cdot}04$ hr^{-1} to a second peak 6–14 hr after the first, and then

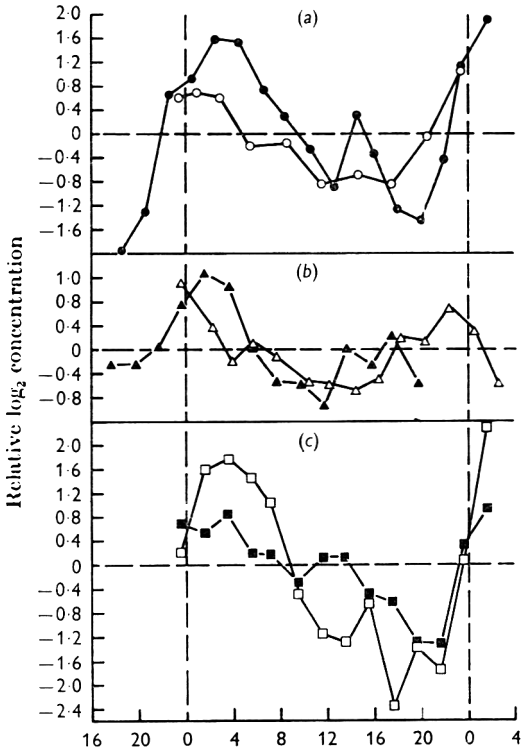

Fig. 11

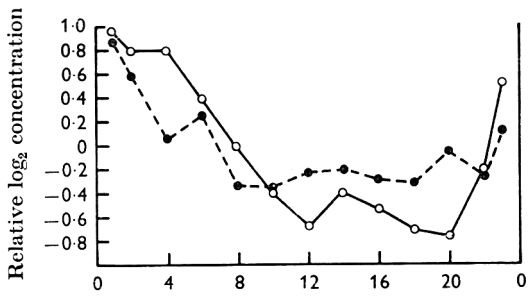

Fig. 12

Fig. 11. Changes in concentration of dasytrichs in individual experiments (basal concentrations of dasytrichs in numbers/ml. are given in brackets):

(*a*) ○ Expt. 1, sheep SO74 fed diet R9 at 09.00 hr ($2{\cdot}2 \times 10^4$);
● Expt. 2, sheep SO74 fed diet R9 at 15.30 hr ($1{\cdot}2 \times 10^4$);

(*b*) △ Expt. 3, sheep SO74 fed diet F19 at 09.00 hr ($3{\cdot}2 \times 10^3$);
▲ Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($3{\cdot}0 \times 10^3$);

(*c*) □ Expt. 5, sheep 1319 fed diet R9 at 09.00 hr ($6{\cdot}3 \times 10^3$);
■ Expt. 6, sheep B585 fed diet M2 at 09.00 hr ($1{\cdot}4 \times 10^4$).

Fig. 12. Mean curves for concentration of (○) dasytrichs (data from Fig. 11) and of (●) isotrichs, where the data used were from the following experiments (basal concentrations of isotrichs in numbers/ml. are given in brackets): Expt. 2, sheep SO74 fed diet R9 at 15·30 hr ($2{\cdot}2 \times 10^3$); Expt. 3, sheep SO74 fed diet F19 at 09.00 hr ($1{\cdot}2 \times 10^4$); Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($6{\cdot}8 \times 10^3$); Expt. 5, sheep 1319 fed diet R9 at 09.00 hr ($1{\cdot}2 \times 10^3$); Expt. 6, sheep B586 fed diet M2 at 09.00 hr ($1{\cdot}3 \times 10^4$).

declined once more at a mean rate $r = -0{\cdot}03$ hr^{-1} to a second minimum about 18 hr after feeding, beginning to increase again at a mean rate about 0·07 hr^{-1} before the next feeding period.

It was not found practicable to count the number of cells per trichome, but the length of the trichomes was measured. This length was highly variable, with a coefficient of variation of 20–40 %, so 100 cells were measured in each sample; an ocular micrometer was used. The minimum length seen was about 6 μ, the maximum over 100 μ. After feeding, there was a rapid increase of about 20 % in the mean length, the cells became highly refractile and strongly iodophilic. The length then decreased, at first rapidly, then more slowly, while the trichomes gradually lost their

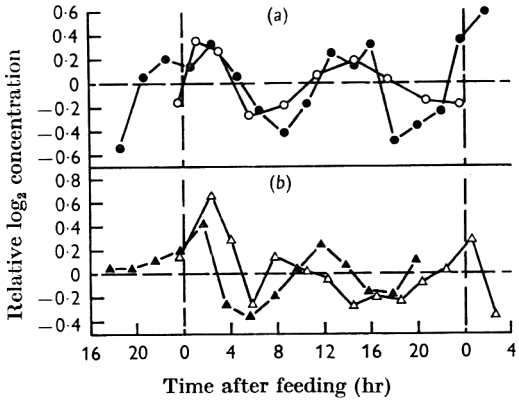

Fig. 13

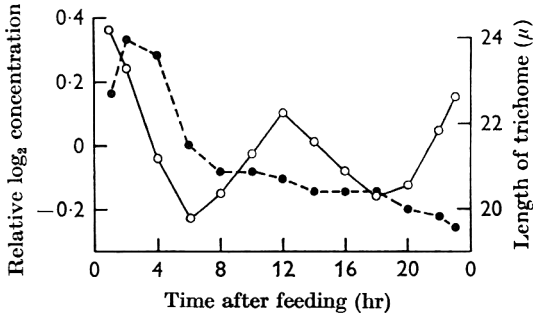

Fig. 14

Fig. 13. Changes in concentration of oscillospirae in individual experiments (basal concentrations of oscillospirae in numbers/ml. are given in brackets):
(*a*) ○ Expt. 1, sheep SO74 fed diet R9 at 09.00 hr ($3{\cdot}4 \times 10^5$);
● Expt. 2, sheep SO74 fed diet R9 at 15.30 hr ($3{\cdot}1 \times 10^5$);
(*b*) △ Expt. 3, sheep SO74 fed diet F19 at 09·00 hr ($1{\cdot}1 \times 10^5$);
▲ Expt. 4, sheep SO74 fed diet F19 at 15.30 hr ($6{\cdot}9 \times 10^4$).

Fig. 14. Mean curves for concentration (○) and mean length of trichome (●) for oscillospirae; data from the experiments described in Fig. 13 were used.

refractility and internal polysaccharide. In Expt. 4 only, a statistically significant second peak in length was seen at the time of the second peak in concentration; no such second peak was seen in any other experiment and it may have been a chance effect.

Marker dilution. The results of the experiment using ^{51}CrEDTA as a marker of rumen dilution are shown in Fig. 15. Before feeding, the dilution rate was 0·063 hr^{-1}, and during the hour that the animal spent eating, the rate increased considerably to 0·234 hr^{-1}. Nearly all the 1800 ml. water drunk during the experiment was consumed within the times 14.30–15.30 hr, i.e. in the last portion of the feeding period and shortly thereafter. During the period 1–3 hr after feeding, a decreased dilution rate, 0·039 hr^{-1}, was observed; the rate then became fairly steady at 0·052 hr^{-1} for the remainder of the experiment. The overall average dilution rate was 0·059 hr^{-1}. The prefeeding volume of rumen liquor was calculated as about 7·5 l.; the sheep weighed about 42 kg.

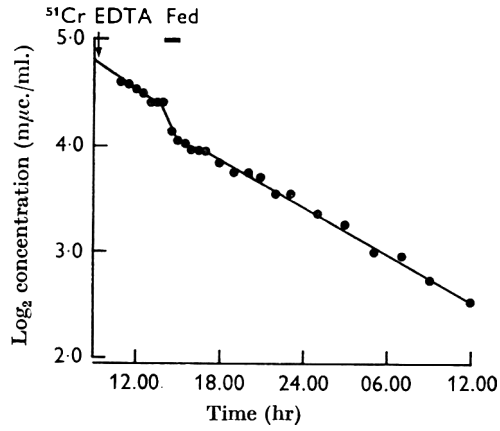

Fig. 15. Dilution curve for the rumen marker ^{51}Cr EDTA in sheep SO74 fed diet R9 at 14.00 hr.

DISCUSSION

The nutritional economy of the ruminant animal depends on the absolute numbers of micro-organisms present in the rumen and flowing to the omasum. But the volume of rumen contents and the rate of flow are not constant (see Warner, 1964), so that the significance to the host of the concentration figures as presented here is uncertain (Clarke, 1965). However, the biology and ecology of the micro-organisms of the rumen are comparatively unaffected by changes in rumen volume and their discussion requires knowledge only of relative flow rates, i.e. dilution rates, and of concentrations.

The volume of rumen contents found for sheep SO74 was greater and the resting dilution rate less than in the sheep of similar breed and weight described by Stacy & Warner (1966). However, all the latter animals were relatively slow eaters and there is some (unpublished) evidence from these laboratories and elsewhere that more rapid eaters tend to have large rumen volumes. There is no evidence that the rate of secretion of saliva in the resting state, 4–7 ml./min. (Stacy & Warner, 1966) depends on this factor, so that resting dilution rates would be less for the more rapid eaters; Stacy & Warner (1966) found rates of 0·07–0·11 hr^{-1}, as compared with 0·05–0·06 hr^{-1} for sheep SO74 in this work. During feeding, the amount of saliva secreted per g. feed consumed seems to be independent of the animal, and about 2·5–3·0 ml./g. for hay-type diets (Bailey, 1961; Meyer, Bartley, Morril & Stewart, 1964; Stacy & Warner, 1966), so that the rapid eaters would secrete more saliva per unit time while eating than the slow eaters, and hence the dilution rates would be similar; Stacy & Warner (1966) found rates of 0·19–0·28 hr^{-1}, as compared with 0·23 hr^{-1} found in the present work. For a short while after feeding, the flow of saliva and the dilution rate were diminished below the normal resting rate (Bailey & Balch, 1961; Stacy & Warner, 1966). Consequently it is believed that the results of the experiment described in Fig. 15 can be taken as typical for sheep SO74 and, on the basis of feeding rates, as probably typical for sheep 1319 and B586 in Expts. 1–6.

Warner (1962*a*) defined the standard count as the concentration of micro-

organisms found in strained rumen liquor, and the total count as the concentration found in whole rumen contents after the feed particles had been washed and disintegrated. The concentration of organisms as measured by the standard count can be affected by dilution of the rumen liquor, by division or death and lysis of organisms, by engulfment by other organisms, and by sequestration of organisms in and return from places not sampled at examination, such as close to the rumen wall, or in close contact with the solid mass of feed found with some diets.

A measure of sequestration is given by comparison of the standard count with the total count of micro-organisms. Results of such studies by Warner (1962*a*, *b*), with other unpublished data collected at the same time, showed conclusively that sequestration among the feed particles was of major importance only for the small bacteria included in the total bacterial counts. Individual bacterial groups could be represented in the standard count at only 12% or less of their true concentration (Warner, 1962*a*); this proportion varied considerably with different groups. Sequestration among feed particles was of only minor significance for the larger organisms. A small increase in sequestration was noted shortly after feeding and again some 12–16 hr later (Warner, 1962*b*) for several groups of organisms, including the entodinia, oscillospirae and dasytrichs, while the peptostreptococci showed increased sequestration for some 8 hr after feeding; however, at most times the ratio of total to standard count was fairly constant and close to 1, that is, the standard counts approximated fairly well to the true counts for all the organisms discussed in this work, except for the small bacteria. Clarke (1965), who used a sampling technique expected to give counts corresponding to the total counts of this laboratory, and Johnson, Hamilton, Robinson & Garey (1944), Purser & Moir (1959) and Purser (1961), who used techniques similar to the standard count of this laboratory, all found patterns of concentration change for the ciliate protozoa similar to those found here. However, it should be noted that organisms sequestered among the papillae of the rumen wall would probably escape counting by any of these techniques.

Inspection of Figs. 1–14 shows a number of qualitatively different patterns of concentration change, and evidence is presented that this pattern depended primarily on the nature of the organism and the feeding regimen and was little affected by differences in the host animal or, within the range studied, the diet. This is confirmed by the finding of similar patterns for a different sheep fed on diet R9 (Warner, 1962*b*) and for grazing sheep where the grazing behaviour resembled the feeding behaviour of these pen-fed sheep (Warner, 1966*b*). Also, Johnson *et al.* (1944), Purser & Moir (1959) and Clarke (1965) found similar patterns for the ophryoscolecid protozoa, and Purser (1961) and Clarke (1965) found similar patterns for the holotrichs, with animals fed rather different diets. Total bacterial counts, where many very different species may be confounded, may be expected to be more affected by the nature of the diet, since the different diets would allow different species to dominate and these species might well have different growth behaviours. Such differences due to diet can be seen in the work of Nottle (1956), El-Tabey Shehata (1958) and Bryant & Robinson (1961).

Probably the simplest concentration pattern to interpret is where feeding is accompanied by a rapid decrease in concentration of micro-organisms, followed by a slow increase for some time and perhaps by a slow decrease just before the next feeding period. Such curves are seen with the ophryoscolecid protozoa, the entodinia

(Fig. 2), diploplastrons (Fig. 3), polyplastrons (Fig. 4), epidinia (Fig. 5) and possibly diplodinia (Fig. 5); the selenomonads (Fig. 6); Eadie's ovals (Fig. 7) and the total bacterial count (Fig. 8). The data are summarized in Table 3, and for the entodinia in Table 4.

The data for the entodinia (Table 4) are more detailed and probably more accurate than for the other groups of organisms. The rate of increase of concentration, r, as actually measured closely approximated to the value calculated from the difference between the growth rate ν and the dilution rate D. The calculation of ν assumes that T, the time needed to complete division, is independent of the species of entodinia

Table 3. *Mean rates of change of concentration of rumen micro-organisms*

Organism	Post-feeding rate (hr^{-1})	Time at minimum* (hr)	Rate during phase of increase (hr^{-1})	Time at maximum* (hr)	Pre-feeding rate (hr^{-1})
Entodinia	−0·16	2–4	0·05	14–20	−0·05
Diploplastrons	−0·16	2–10	0·06	16–22	−0·04
Polyplastrons	−0·18	4	0·07	18–20	−0·09
Epidinia	−0·35	4	0·17	14	−0·05
Diplodinia	0	0	0·15	14	−0·10
Selenomonads	−0·22	2–8	0·05	18–22	−0·01
Eadie's ovals	−0·21	6	0·17	14–24	0
Total bacteria	−0·14	2	0·03	12–20	−0·03
Peptostreptococci	−1·7	1	0·4	12–18	−0·3

* A single figure indicates a sharp minimum or maximum at the stated time after feeding; a range indicates the period during which the concentration was approximately constant.

Table 4. *Rates of increase of concentration of entodinia*

Values of the rate of increase r and the dilution rate D were calculated for the periods shown from Figs. 2 and 15 respectively. The values for the growth rate ν were averages calculated from the data of Fig. 2.

Time after feeding (hr)	r (hr^{-1})	ν (hr^{-1})	D (hr^{-1})	$\nu - D$ (hr^{-1})
0–24	0·000	0·053	0·059	−0·006
0–2	−0·10	0·04	0·15	−0·11
2–4	0·00	0·04	0·04	0·00
4–14	0·04	0·07	0·05	0·02
14–20	0·01	0·04	0·05	−0·01
20–24	−0·05	0·02	0·06	−0·04

and unvarying with time (i.e. changes in environment), which may not be completely true (James, 1960), though Warner (1962*b*) was unable to detect any differences in a limited series of experiments. The technique used to estimate T probably yields an overestimate because of unphysiological conditions on the microscope slide (cf. Browning, Varnedoe & Swinford, 1952); this would give an underestimate of ν and would perhaps account for the differences between r as measured and the calculated $\nu - D$. The closeness of these estimates of r nevertheless suggests that there was little sequestration or death of these organisms, so that $\lambda = 0$ and $\nu = \rho$. This confirms the statements made above based on the findings of Warner (1962*b*); dead entodinia were seen only very rarely in this work.

The simplest interpretation of the data appears to be that the initial decrease in concentration was caused by dilution due to the rapid influx of saliva into the rumen that accompanies feeding, and to the concurrent consumption of water. Clarke (1965) showed that this initial drop in concentration did not indicate destruction of organisms, since the total number of ophryoscolecid protozoa in the rumen was practically unchanged over the feeding period. The growth rate then increased in response to the incoming nutrients and soon exceeeded the dilution rate, thus allowing an increase in concentration. With the exhaustion of nutrients, the growth rate declined until it was less than the dilution rate, so that shortly before the next feeding period there was a decrease in concentration.

Both the rate of change of concentration and the ratio of maximum to minimum concentration were similar for the entodinia and the diploplastrons (Table 3, Figs. 2, 3), but the proportion of dividing organisms was at all times greater for the latter than for the former. This suggests that T, the time needed to complete division, was greater for the diploplastrons than for the entodinia. The ratio of maximum to minimum concentration for the polyplastrons (Fig. 4) was slightly greater and for the diplodinia and epidinia (Fig. 5) considerably greater than for the entodinia (Fig. 2) or diploplastrons (Fig. 3). This is reflected in the rates of change of concentration (Table 3) and indicates that the growth rates varied over a wider range; this is confirmed by the wider range for the proportion of dividing cells.

Purser & Moir (1959) claimed that the diurnal changes in concentration of the ophryoscolecid protozoa could be largely explained by dilution following feeding and an inhibition of division due to the decrease in pH value. If they had calculated the proportion, rather than the absolute concentration, of dividing organisms as a measure of growth activity, they would have found that minimal growth occurred in the period 16–24 hr after feeding, when the pH value was increasing to its maximum, and maximal growth occurred at 8 hr after feeding when the pH value was still moderately low. However, they would also have found that the proportion of dividing organisms increased immediately after feeding and then decreased somewhat before rising to its maximum; this decrease was associated with the period of minimal pH value and may have been caused by it. No such phenomenon was noted in the present work, but it is likely that the pH value did not decrease much below pH 5·9 in these experiments, as compared with decreases to pH 5·4–5·7 in the work of Purser & Moir (1959). A puzzling feature of this latter work is revealed when the dilution rate is calculated from the equation $D = \rho - r$, making any reasonable assumption about the relation between $\rho = \nu$ and the proportion of dividing organisms as measured by these authors. High values for the dilution rate, exceeding 0·4 hr^{-1}, are found for the first hour after feeding, followed by low figures for a few hours, increasing again to a maximum exceeding 0·2 hr^{-1} at 6–8 hr after feeding, and finally decreasing to a very low value in the last period of the 24 hr. No such phenomenon was noted in the present work.

A superficially similar curve was shown by the peptostreptococci (Fig. 9), but the amplitude of the fluctuations (Table 3) was much greater. The considerable rate of decrease before feeding, and the very rapid decline following feeding, where in both cases the sum of r and D, i.e. the net growth rate ρ, was negative, indicate some means of removing the organisms in addition to dilution. The figures of Warner (1962*b*) indicated some sequestration on feed particles during most of the period

of low concentration, but this could not have accounted for all the decline in concentration; dissolution or engulfment must have occurred. The rate of increase found a short time after feeding, $r = 0{\cdot}4\ \mathrm{hr}^{-1}$, is readily compatible with the growth capabilities of the organism, as known from cultures, for doubling times of about 2 hr are all that would be required.

The polymastigate flagellate protozoa appeared to undergo sequestration, probably on the rumen wall, and return to the general rumen liquor, since otherwise 2–5 or more divisions would have needed to occur within the hour after feeding, followed by an almost equally rapid death rate, which seems unlikely. Dividing or dead forms have been positively identified only rarely. The stimulus for return was definitely associated with the feed, and appeared to be some substance of low molecular weight, soluble in acetone as well as water, and destroyed by acid treatment. Perhaps, during the fasting period, some similar substance diffused across the rumen wall from the blood in sufficient concentration to attract the organisms there. The normal mixing of the rumen and the rapid rate of locomotion of the organism would seem sufficient to account for the observed fluctuations in concentration.

The holotrich ciliate protozoa showed more puzzling behaviour (Figs. 11, 12). After reaching a maximum near feeding time, the concentration of dasytrichs decreased until 12–20 hr after feeding, at a mean rate of about $r = -0{\cdot}11\ \mathrm{hr}^{-1}$ (Fig. 12); in some experiments, rates of $-0{\cdot}3\ \mathrm{hr}^{-1}$ or lower were maintained for some hours. This was much greater than can be accounted for by dilution, even though the growth rate was effectively zero. Clarke (1965) suggested that this decline was due to bursting of the ciliates when overfull of synthesized polysaccharide (Sugden & Oxford, 1952). Use can be made of an expression analogous to that used in the calculation of growth rate, i.e. $\lambda = -\rho = -(r+D) = I'/T'$, where I' is the proportion of organisms showing signs of dissolution, and T' the time needed to complete this process, i.e. the time required for an apparently normal organism to become unrecognizable as a holotrich. In this work, very few bursting organisms were seen, and I' was of the order of 0·001 or less. This suggests that T' may have been considerably less than 1 min; there is no experimental evidence about this, but it would seem to be a very short time.

The alternative hypothesis of sequestration (Purser, 1961) does not seem to be any more likely. Previous work in this laboratory showed no evidence of sequestration amongst feed particles, and if it occurred amongst the papillae of the rumen wall, accumulations would be found in such concentrations as should be obvious to the naked eye; in other work, the rumen wall of sheep inspected at various times after feeding showed no such appearance.

During the period 20–28 hr after feeding, a sustained rapid rate of increase of concentration of dasytrichs was noticed, at a mean rate of about $0{\cdot}3\ \mathrm{hr}^{-1}$, while the concentration increased by some 4 to 20-fold or more within a few hours. This would imply a doubling time of 1·5–2·0 hr sustained over 2–4, or more, divisions. Similar large rapid increases were recorded by Purser (1961) and by Clarke (1965), though the latter author found the increase to occur slightly later. Now, the number of divisions undergone by a micro-organism per day is $24/g$, i.e. $24\nu/\ln 2$; since, considered over the 24 hr feeding cycle, the rumen approximates a steady-state system, this equals $24\ D/\ln 2$ (see appendix), i.e. about 35 D divisions occur per day provided the death rate λ is negligible and no sequestration occurs. Hence, to main-

tain numbers against an overall mean dilution rate of 0·06–0·11 hr^{-1}, each organism must divide on average 2–4 times per day; perhaps an extra 1–2 divisions would be needed to make up numbers if the earlier decline were due to dissolution of organisms. It appears that nearly all the division in the holotrichs occurs within a few hours, since very few dividing organisms were seen at other times. Campbell (1930) noted that in cattle normally very few dividing isotrichs were seen but that on one occasion 0·6 of the population were dividing. Hence it would appear that the dasytrichs undergo 2–7 divisions within 4–8 hr, and then divide no more for about 18 hr; and the isotrichs behave similarly. It can be calculated that the time needed to complete division is of the same magnitude as for the entodinia.

It was noticed that dividing holotrichs never contained obvious feed particles (starch grains, etc.), but were never void of internal polysaccharide. It may therefore be postulated that nutrients must be metabolized to some particular state, a process requiring perhaps 18 hr, before division can take place, and that several divisions then occur in rapid succession. No analogous phenomenon is known to the author.

No evidence on the nature of the stimulus for conjugation among the holotrichs was found in the present work. No detailed cytological studies were made, but the process appeared to be identical with that described by Dogiel & Fedorowa (1925).

The oscillospirae (Figs. 13, 14) showed a pattern of concentration change difficult to interpret, there being two maxima in the 24 hr feeding cycle. The first peak, shortly after feeding, was associated with the maximum length of the trichomes. The second peak, 8–16 hr later, occurred with slowly decreasing trichome length. An interesting feature was that the concentration was rising before feeding, though the length was slowly decreasing. It was hoped that the trichome length would have given an indication of the number of cells per trichome, so that the concentration of trichomes would have increased when the length decreased and vice versa. However, this was not the case, and it would appear that the length of the trichomes reflected their nutritional rather than their reproductive status. Previous work (Warner, 1962*b*) indicated that much, but not all, of the variation in concentration of oscillospirae, as measured by the standard count, disappeared when the total count was used, suggesting that sequestration and return accounted for some part of the changes in concentration. This might account for the fact that the initial rate of decline in concentration exceeded the dilution rate. However, the double peak was still obvious in the total count and remains inexplicable.

It became apparent during this work that at least two strains or species of organisms were included in the group 'oscillospirae'. Only one strain was present in most animals, but in some a mixture occurred. The strains differ in diameter; one has a diameter near 7 μ, one near 4 μ, and there may be a third still thinner. Spores, when formed, appeared more uniform in size, being 6–7 μ in diameter in all strains. The tendency to form spores also differed considerably; some strains formed spores very rarely, others more frequently. The number of spores per trichome also varied, some commonly forming one and only rarely two spores, others, particularly the thinner strains, forming up to six spores. This character is not mentioned for *Oscillospira guillermondii*, the only species described in *Bergey's Manual*. The behaviour of all these oscillospira strains, both in concentration and in trichome length, appeared to be similar, and they have not been differentiated in the present work.

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APPENDIX

Kinetic study of particles, solutes and micro-organisms in the rumen

Previous studies of the kinetics of feed particles in the rumen (Blaxter, Graham & Wainman, 1956; Brandt & Thacker, 1958; Garner, 1964) have made the basic assumption of steady-state flow, i.e. it has been assumed that movements of water into and out of the rumen were constant and equal, so that the volume remained constant. This assumption is at best a crude approximation under most circumstances (see Warner, 1964). Hydén (1961*a*) made use of an inert reference solute, polyethylene glycol, as a marker for water movements in the rumen. He also assumed steady-state flow for his main mathematical treatment, but showed how to calculate outflow of water from the rumen when the volume varied. Hydén (1961*b*) applied his treatment to estimate the net flux of water across the rumen wall, while Engelhardt (1963), who used a similar approach, endeavoured to measure the total flux by using tritiated water. No kinetic study of the micro-organisms in the rumen is known, but a considerable literature exists for continuous-flow cultures (see James, 1961), which are in many ways analogous to the rumen but again many parameters are constant. The present treatment has been developed without making any assumptions about the constancy of parameters pertaining to events in the rumen. The symbols agreed by Andreyev *et al.* (1964) have been used where applicable.

Fundamental kinetic equation

Consider particles, solute molecules, or micro-organisms that cannot cross the rumen wall, that move at the same rate as water into the omasum, and that may be formed or destroyed in the rumen but only at rates directly proportional to the numbers present. Instantaneous and complete mixing is assumed.

At the instant t, let J be the inflow rate of water down the oesophagus, H the concentration of the substance in this inflowing water, W the net rate of entry of water across the rumen wall (the concentration of the substance in this water is nil), F the net inflow rate of water into the rumen ($F = J + W$), V the volume of water in the rumen, C the concentration of the substance in the rumen, E the outflow rate of water to the omasum (the concentration of the substance in this water is C, due to the assumed perfect mixing), and k the net rate constant for formation and destruction of the substance in the rumen. During the interval from time t to time $t + \delta t$, a simple balance equation holds:

$$\begin{aligned} &\text{Input} + \text{Formation} - \text{Output} = \text{Accumulation} \\ &J\,\delta t H + k\,\delta t\,VC - E\,\delta t C = (V+\delta V)(C+\delta C) - VC \\ &= \delta V C + V\delta C + \delta V \delta C = (F-E)\,\delta t C + V\delta C + \delta V\ \delta C \end{aligned}$$

Divide by δt and take to the limit:

$$JH + kVC = FC + V\frac{\mathrm{d}C}{\mathrm{d}t}. \tag{1}$$

Special cases

Single-shot marker experiment. An inert reference substance such as polyethylene glycol is introduced into the rumen at time $t = 0$, and the concentration measured at intervals thereafter. In this case, $H = 0$, $k = 0$; let $C = c$. Then equation (1) becomes $0 = Fc + V\mathrm{d}c/\mathrm{d}t$, or

$$\frac{1}{c}\frac{\mathrm{d}c}{\mathrm{d}t} = -\frac{F}{V} = -D, \tag{2}$$

where $D = F/V$ is the relative net inflow rate of water or dilution rate.

The curve of $\ln c$ plotted against t has an instantaneous gradient of

$$\frac{\mathrm{d}\ln c}{\mathrm{d}t} = \frac{1}{c}\frac{\mathrm{d}c}{\mathrm{d}t}$$

and hence, from equation (2), $-D$. This curve is usually approximately linear, i.e. D is approximately constant, for substantial periods of time, and hence

$$\ln c = \ln c_0 - Dt \tag{3}$$

equivalent to

$$c = c_0 \mathrm{e}^{-Dt}, \tag{4}$$

where the subscript o refers to the instant $t = 0$. It is usually convenient to plot c against t on semi-logarithmic paper, or alternatively to plot against t the logarithm of c taken to some convenient base such as 2, and to fit a straight line by eye. An estimate is then made of the half time, $t_{\frac{1}{2}}$, which is the time needed for the concentration to fall by one half or for the $\log_2$ concentration to fall by 1. Then

$$D = (\ln 2)/t_{\frac{1}{2}}. \tag{5}$$

Since the problem appears to involve the functional relationship between the variables rather than the estimation of the mean of one as a function of the other, it is doubtful whether the fitting of a regression line would lead to a more accurate estimate of D.

Ulyatt (1964), who worked with grazing sheep, fitted a quadratic rather than a linear expression to the plot of $\ln c$ against t, but he made no attempt to correlate departures from linearity with patterns of eating, drinking or ruminating.

Continuous infusion of marker. The reference substance is infused continuously into the rumen. Here, $k = 0$; let $H = h$, $C = c$. Then equation (1) becomes

$$Jh = Fc + V\mathrm{d}c/\mathrm{d}t. \tag{6}$$

This expression is difficult to handle without further information.

Micro-organisms. Micro-organisms that are 'fixed' to the feed particles or sequestered close to the rumen wall would not in general move with water to the omasum and are therefore beyond the scope of this treatment. For the remaining organisms, $H = 0$; let $C = n$, the concentration in numbers of live cells per unit volume, and $k = \rho = \nu - \lambda$, where ν and λ are specific growth and death rates and ρ the net specific growth rate. Then equation (1) becomes $\rho Vn = Fn + V\,\mathrm{d}n/\mathrm{d}t$, that is

$$\frac{1}{n}\frac{\mathrm{d}n}{\mathrm{d}t} = \rho - D = r, \tag{7}$$

where $r = \rho - D$, the relative rate of change of concentration. When r is constant, it can be determined as for D above, from the equation

$$\ln n = \ln n_0 + rt. \tag{8}$$

Experimental determination of r and D allows the calculation of ρ which, if the death rate λ is negligible, equals the specific growth rate ν.

Outflow. There is no outflow term in the general equation (1), but the outflow of water and of the substances that move with the water is of considerable importance to the economy of the animal. The amount of a substance of the type considered in this study that leaves the rumen is equal to the difference between the sum of the initial amount and what arrives there by inflow or formation, and the final amount in the rumen, that is

$$\int_0^t EC\,\mathrm{d}t = C_0V_0 + \int_0^t JH\,\mathrm{d}t + \int_0^t kCV\,\mathrm{d}t - CV. \tag{9}$$

In a single-shot marker experiment as described above, if E can be considered constant, equation (9) reduces to

$$E = \frac{c_0V_0 - cV}{\int_0^t c\,\mathrm{d}t}. \tag{10}$$

The integral $\int_0^t c\,\mathrm{d}t$ could be estimated graphically, or, if D was also constant, from

$$\begin{aligned}\int_0^t c\,\mathrm{d}t &= \int_0^t c_0\mathrm{e}^{-Dt}\,\mathrm{d}t \text{ (from equation (4))}\\ &= c_0(1-\mathrm{e}^{-Dt})/D = (c_0-c)/D \quad \text{(from equation (4))}\\ &= (c_0-c)t/(\ln c_0 - \ln c) \quad \text{(from equation (3)).}\end{aligned}$$

Hence

$$E = \frac{(c_0V_0 - cV)(\ln c_0 - \ln c)}{(c_0-c)\,t}. \tag{11}$$

The relative outflow rate or production rate M may be defined as E/V, analogous to D.

Steady state. Flow through the rumen approximates to a steady state if considered over a complete feeding cycle or for short periods of time that are well separated from times of eating, drinking or ruminating. Then F and E are equal and constant, V is constant; hence $D = M$.

If marker is infused at a constant rate, equation (6) reduces to $Jh = Fc$ = rate of infusion of marker. Experimentally this technique often gives a better estimate of F than can be obtained from calculations of V and D in a single-shot marker experiment.

For micro-organisms in a steady-state system, $r = 0$ and $\rho = D = M$.

Mean residence time. The average time spent by a particle in the rumen, the mean residence time θ, may be calculated as the time needed for the number of particles leaving the rumen to equal the average number in the rumen during the period of observation, provided there is no destruction of particles within the rumen. It can be readily calculated only for steady-state conditions, when $E\theta = V$, or

$$\theta = V/E = 1/M = 1/D. \tag{12}$$

This term is formally analogous to turnover time, as used in metabolic studies.

Dimensions and units

Note that k, D, M, ρ, ν, λ, and r are all rate constants, with physical dimensions of $[T^{-1}]$ and their units should be in conformity and the same for all, such as hr^{-1} or day^{-1}. The commonly applied unit for D, per cent per hour (Hydén, 1961*a*) can give a misleading impression if applied to, say, ρ, and the value cannot be used unchanged in expressions of the form of equation (4).

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Periodic Changes in the Concentrations of Micro-organisms in the Rumen of a Sheep Fed a Limited Ration every Three Hours

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SUMMARY

A sheep was given small amounts of feed at 3-hr intervals and the concentrations of several groups of micro-organisms in the rumen were measured at intervals over 28 hr. A 3-hr rhythm of concentration change was seen for most micro-organisms. Superimposed on this was a gradual decline in concentration of several groups of micro-organisms, presumably due to removal of nutrients by repeated sampling from the rumen.

INTRODUCTION

In the preceding paper (Warner, 1966) the pattern of changes in concentration of the micro-organisms in the rumen of sheep fed a limited ration once daily was shown to depend primarily on the nature of the micro-organisms and the time of feeding. No evidence for any other diurnal rhythm was found. The present paper reports a study of the changes in concentration of the rumen microbes in a sheep fed at 3-hr intervals. The aim was to determine whether nearly constant conditions in the rumen could be achieved. The frequently repeated, regular sequence of observations also allowed a study of the effects of repeated sampling on the micro-organisms.

METHODS

An English Leicester–Merino crossbred ewe S074 was given 89 ± 6 g. of the diet R9 (equal parts of lucerne and wheaten chaffs) every 3 hr by means of an automatic dispenser. The animal nearly always consumed this feed within 5 min. Water was available at all times. Before the start of the experiment, the animal had spent 6 weeks on this routine, and before that had been fed 700 g. diet R9 once daily.

On the day of the experiment, samples (about 50 ml.) of rumen contents were removed through a fistula; care was taken to collect the material from several sites within the rumen. The pH value was determined without delay. The remainder of the specimen was strained and sampled for microbiological examination by the standard technique of Warner (1962*a*); nomenclature of the organisms followed Warner (1966). A further portion of the strained rumen liquor was diluted with twice its volume of 0·1 N–HCl, allowed to stand at least 10 min., and filtered. Estimations of ammonia and volatile fatty acids were made on the filtrate by the

methods of Warner (1964) and Warner & Stacy (1965), respectively. Rumen samples were removed at intervals throughout 28 hr, at one of 3 times, 0·5, 1·5, 2·5 hr after the time of feeding.

RESULTS

When the results concerning a particular micro-organism were considered within three groups, namely those for 0·5, 1·5 and 2·5 hr after feeding, respectively, it was found that for some species the concentrations showed overall decreases with increasing time measured from the beginning of the experiment. For micro-organisms for which the data suggested the possibility of such a decrease, instead of an ordinary analysis of variance to compare results of the three groups of observations, a simple linear regression analysis against time from the beginning of the experiment was carried out for each group. The three corresponding regression coefficients and

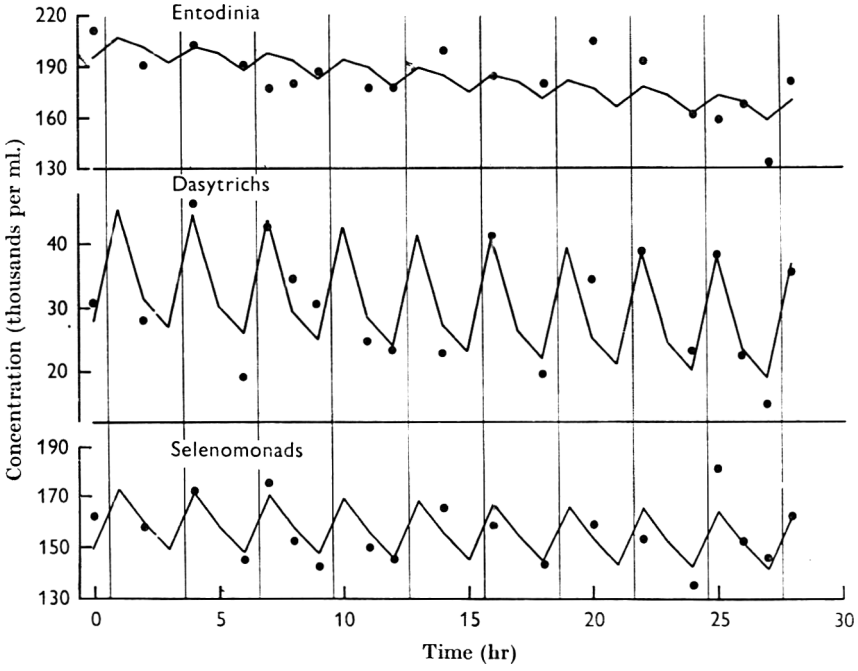

Fig. 1. Concentration of entodinia, dasytrichs and selenomonads in the rumen of a sheep fed at 3-hr intervals. The lines connect points plotted for the calculated mean values, allowing for the regression due to time (see text). The vertical lines indicate the time of feeding.

their standard errors were computed and then assuming that the three lines were in fact parallel, a common gradient and its standard error was then found by pooling regression results from the groups. By removal of the appropriate regression sums of squares, an analysis of covariance was then made to discover whether there was any difference between the three groups not due to the time after the beginning of the experiment.

Where no overall decline as described above was suspected, the means of values found for each time after feeding were entered in Table 1. Where such a decline

Table 1. *Micro-organisms and metabolites in the rumen of a sheep fed at 3-hr intervals*

	Mean, calculated to zero time, ± S.D. at indicated times after feeding			Significance of difference* (*F* test)	Regression gradient per hr
	0·5 hr	1·5 hr	2·5 hr		
pH value	6·39 ± 0·08	6·45 ± 0·03	6·53 ± 0·03	***	—
Volatile fatty acids (m-equiv./l.)	95 ± 6	93 ± 8	84 ± 12	**	—
Ammonia (m-equiv./l.)	14·1 ± 1·2	12·7 ± 1·2	11·9 ± 1·0	**	—
Entodinia (concn. × 10^{-3}/ml.)	208 ± 16	205 ± 17	196 ± 14	n.s.	−1·4 ± 0·4
Proportion of entodinia dividing	0·032 ± 0·005	0·023 ± 0·003	0·021 ± 0·004	***	−0·005 ± 0·001
Diploplastrons (concn. × 10^{-3}/ml.)	15·7 ± 2·2	15·7 ± 2·2	14·6 ± 2·6	n.s.	−0·03 ± 0·06
Proportion of diploplastrons dividing	0·034 ± 0·006	0·030 ± 0·006	0·035 ± 0·011	n.s.	0·0001 ± 0·0002
Dasytrichs (concn. × 10^{-3}/ml.)	46 ± 1	32 ± 6	28 ± 5	***	−0·3 ± 0·1
Proportion of dasytrichs dividing	0·005 ± 0·001	0·005 ± 0·003	0·011 ± 0·003	*	—
Proportion of dasytrichs conjugating	0·001	0·002	0·002	n.s.	—
Isotrichs (concn. × 10^{-3}/ml.)	15 ± 4	9 ± 2	9 ± 3	**	−0·2 ± 0·1
Proportion of isotrichs dividing	0·003 ± 0·002	0·005 ± 0·003	0·008 ± 0·003	*	—
Polymastigates (concn. × 10^{-3}/ml.)	35 ± 6	18 ± 5	10 ± 6	***	—
Oscillospirae (concn. × 10^{-3}/ml.)	115 ± 9	94 ± 7	91 ± 6	***	−0·8 ± 0·2
Selenomonads (concn. × 10^{-6}/ml.)	173 ± 11	161 ± 7	151 ± 7	***	−0·3 ± 0·2
Total bacteria (concn. × 10^{-8}/ml.)	177 ± 7	166 ± 10	167 ± 11	n.s.	0·3 ± 0·2

* When a figure is given for the gradient in the next column, significance was calculated as described in the text, otherwise a normal analysis of variance was used. n.s. = not significantly different; *, **, *** = significantly different at levels of probability $P < 0·05$, 0·01, 0·001 respectively.

seemed possible, the experimental values were corrected to zero time by using the calculated regression coefficient and the means then computed. In Fig. 1 the actual, experimental values are plotted, together with a line joining points calculated from the means described above and the regression coefficient. It can be seen that, even when fed as frequently as at 3-hr intervals, considerable rhythmical fluctuations in concentration of both micro-organisms and metabolites occurred due to the feeding regime, though these were much less marked than when the animal was fed only once daily (Warner, 1966). No evidence was seen in Fig. 1 or in the other unpublished curves for any other diurnal rhythms.

DISCUSSION

Immediately following feeding there was an increase in metabolic activity, shown by the increase in ammonia and volatile fatty acid concentration and the decrease in pH value. All micro-organisms showed some degree of increase in concentration immediately after feeding, presumably due to the increase in nutrients, but the relative magnitude of this increase and the subsequent pattern of decline were characteristic of the micro-organism (Fig. 1).

The entodinia (Table 1) showed a small but statistically non-significant increase in concentration after feeding, and a considerable and highly significant increase in the proportion of dividing organisms. It is presumed that the reason why the considerably increased growth rate caused little increase in concentration was that the dilution rate also increased due to the increased flow of saliva accompanying feeding (Warner, 1966). The diploplastrons also showed a small but non-significant increase in concentration after feeding but, perhaps owing to greater variability, no significant difference in the proportion of dividing organisms was seen. The post-feeding increase in total bacterial concentration was also non-significant.

The concentration of selenomonads declined steadily, that of the oscillospirae at a decreasing rate, following the highly significant post-feeding rise. As in the sheep fed once daily (Warner, 1966) the polymastigates showed the greatest increase in concentration of all micro-organisms.

The holotrich ciliates showed minimal concentration and maximal proportion of dividing organisms immediately before feeding, and vice versa immediately after. In view of the previous work (Warner, 1966) one may speculate whether the stimulus to division was the immediately preceding feed or one considerably earlier. A few conjugating dasytrichs were seen in many specimens.

Over the 28 hr of the experiment there was a slow decline in the concentration of entodinia, holotrichs, oscillospirae and selenomonads. The proportion of dividing entodinia also declined, indicating inhibition of reproduction. During this 28 hr some 1000 ml. rumen contents were removed in sampling; this material would contain feed particles in various stages of degradation, soluble feed constituents, metabolic products and microbial cells; that is, nutrients for many of the rumen micro-organisms.

No overall decline in concentration was seen with the diploplastrons, but counting accuracy was comparatively low. The polymastigates maintained their numbers; if the suggestion of Warner (1966) is correct, then these organisms spend a considerable time sequestered close to the rumen wall, presumably for nutritional reasons,

so that the sampling procedure would remove proportionately less of these organisms, and the nutrition of the remainder would be little affected. The total bacterial count also did not decline, indeed there may have been a small increase. It is probable that many of the organisms counted here were normally 'fixed' species, in transit between one feed particle and another; since the larger feed particles were not removed at sampling, a considerable reservoir of organisms and their substrates would remain.

The holotrichs decreased in concentration more than the ophryoscolecids. A similar finding was made in starving cattle by Meiske, Salsbury, Hoefer & Lueckel (1958). This marked decrease in protozoa may have allowed a compensatory increase in the bacteria; defaunated animals commonly have high bacterial counts (Eadie, Hobson & Mann, 1959; Bryant & Small, 1960; Eadie & Hobson, 1962; Walker & Hope, 1964; see also Warner, 1962*b*).

Purser & Moir (1959) collected from sheep 8 specimens of rumen liquor of at least 100 ml. each, on each of 3 consecutive days. They found that the ophryoscolecid protozoa decreased considerably in mean daily concentration over the 3 days. This was asssociated with a decrease in minimal pH value. In the work reported here there was no change in pH value, while in the starvation experiments of Meiske *et al.* (1958) the pH value increased. While the causes are still uncertain, this work emphasizes that removal of a significant amount of rumen contents affects the remaining micro-organisms and their metabolism.

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Diurnal Changes in the Concentrations of Micro-organisms in the Rumens of Sheep Fed to Appetite in Pens or at Pasture

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SUMMARY

The concentrations of the various groups of micro-organisms in the rumens of sheep when feeding on a roughage diet to appetite or grazing a mixed pasture, changed with time in patterns very similar to those previously found in sheep fed restricted diets once daily. It is postulated that these patterns will be present wherever the daily eating behaviour of the animal includes a major fairly continuous period during which all or most of the ration is eaten, and long periods during which little or no feed is consumed.

INTRODUCTION

In the previous papers in this series (Warner, 1966*a*, *b*), I examined the changes in concentration of the micro-organisms in the rumens of sheep following the giving of restricted amounts of feed which were consumed promptly. However, in natural circumstances and under farming conditions, most ruminants have virtually unlimited amounts of feed available and may choose their own times and rates of eating. To gain information about the rumen microbial population under more natural conditions, studies were made of the micro-organisms from the rumens of two sheep grazing with unrestricted access to a mixed pasture and two sheep offered a dry roughage diet *ad libitum*. The patterns of change of concentration for the various groups of micro-organisms were compared with the patterns found previously.

METHODS

Two English Leicester–Merino crossbred wethers (numbers 1330 and 2985) were fed wheaten chaff to appetite. Feed residues were removed at about 08.30 hr and at 10.30 hr a fresh batch of feed was presented; water was available at all times. At approximately 2-hr intervals, rumen liquor samples were collected and the feed and water consumptions measured.

Two other sheep (numbers 1593 and 2750) were grazing in a flock of 8 sheep on a pasture consisting predominantly of *Lolium perenne* and *Trifolium subterraneum*. They were inspected at approximately $\frac{1}{2}$ hr intervals and their grazing behaviour noted (Hull, Lofgreen, & Meyer 1960). Water was available to the sheep, but they were not seen to drink; the pasture was fairly wet following recent spring rain. Rumen liquor samples were collected at approximately 2-hr intervals.

The experiments were concurrent, i.e. all the samples were collected within one day.

Micro-organisms were identified and counted as described by Warner (1966*a*). The organisms described here as lampropedias were organism no. 25 of Moir & Masson (1952), the 'window-pane sarcina', which is almost certainly related to *Lampropedia hyalina* as described by Pringsheim (1955) and Chapman, Murray &

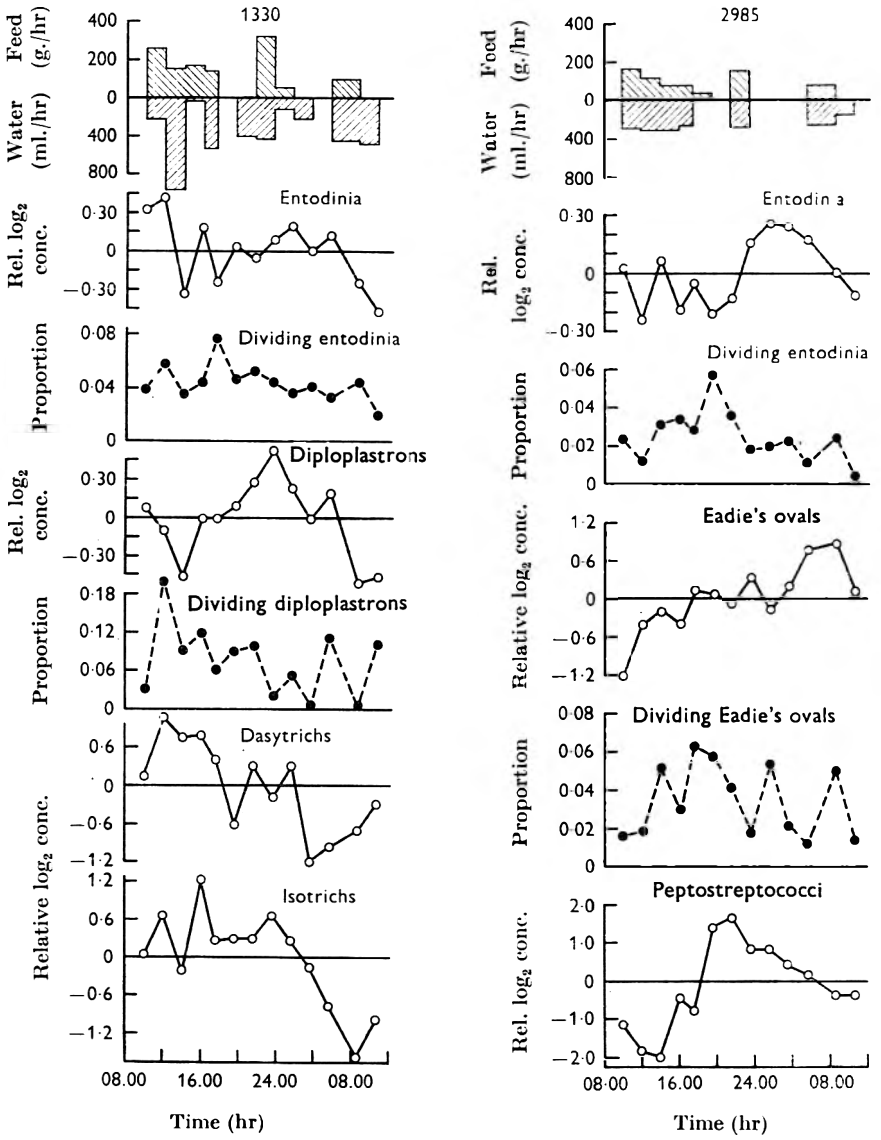

Fig. 1. Feeding and drinking behaviour and changes in rumen microbial population in sheep nos. 1330 and 2985, fed wheaten chaff to appetite. Basal concentrations in numbers/ml. were as follows (data for sheep nos. 1330 and 2985, respectively): entodinia, $2{\cdot}3 \times 10^5$, $3{\cdot}0 \times 10^5$; diploplastrons, $4{\cdot}0 \times 10^3$, 0; dasytrichs, $2{\cdot}3 \times 10^3$, 0; isotrichs, $2{\cdot}4 \times 10^3$, 0; Eadie's ovals, 0, $7{\cdot}7 \times 10^6$; peptostreptococci, 0, $6{\cdot}0 \times 10^4$.

Salton (1963). These organisms were arranged in plates or sheets of moderately uniform size, and were counted as numbers of plates.

The graphical presentation of the data as relative $\log_2$ concentrations, with the basal concentrations given in the legends to the figures, has been described previously by Warner (1966*a*).

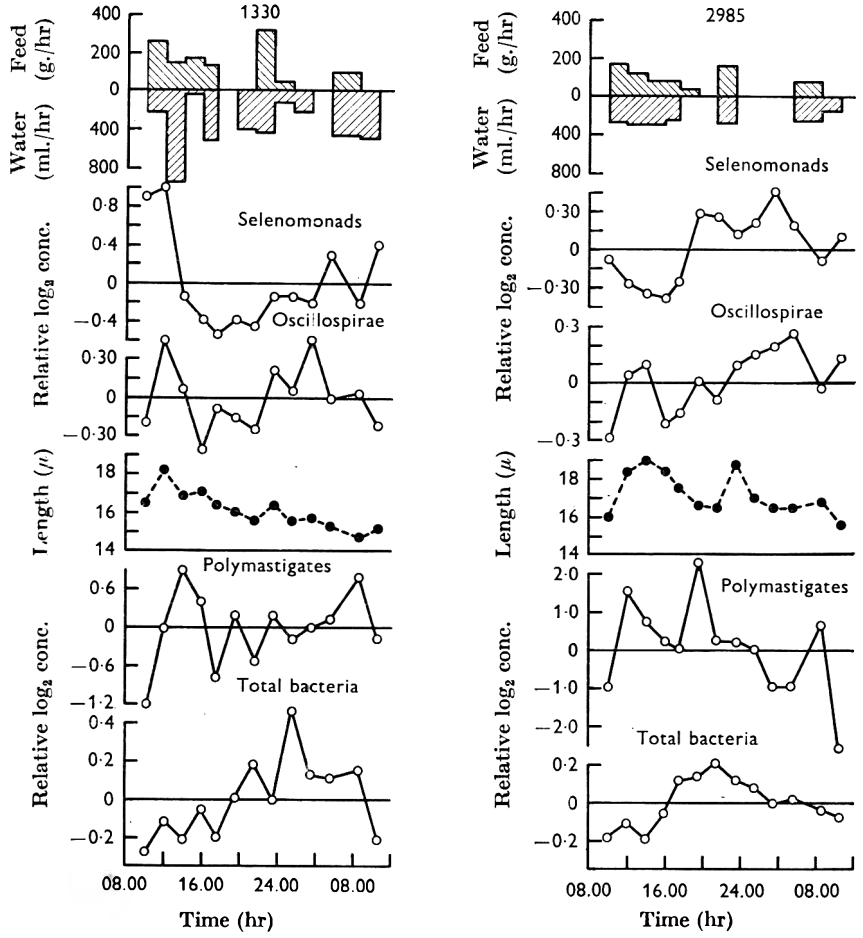

Fig. 2. Feeding and drinking behaviour and changes in rumen microbial population in sheep nos. 1330 and 2985, fed wheaten chaff to appetite. Basal concentrations in numbers/ml. were as follows (data for sheep nos. 1330 and 2985, respectively): selenomonads, $1{\cdot}1 \times 10^7$, $2{\cdot}4 \times 10^8$; oscillospirae, $1{\cdot}1 \times 10^6$, $3{\cdot}9 \times 10^7$; polymastigates, 7×10^3, 6×10^3; total bacteria, $2{\cdot}6 \times 10^{10}$, $3{\cdot}3 \times 10^{10}$.

RESULTS

Sheep fed dry roughage to appetite

Results are shown in Figs. 1 and 2. Feed and water were consumed in three periods: a long period near the middle of the day (sheep no. 1330, weighing 73 kg., ate 1360 g. feed, sheep no. 2985, weighing 49 kg., ate 820 g.); a short period around midnight (feed consumed 740 and 290 g., respectively); another short period early in the morning (feed consumed 310 and 205 g., respectively). In the first two periods the

sheep drank water equivalent to 2–3 times the weight of the feed eaten, but in the last period the ratio was 6–7 times.

The entodinia, diploplastrons and Eadie's ovals showed similar patterns of behaviour (Fig. 1), with lower concentrations but more actively dividing organisms

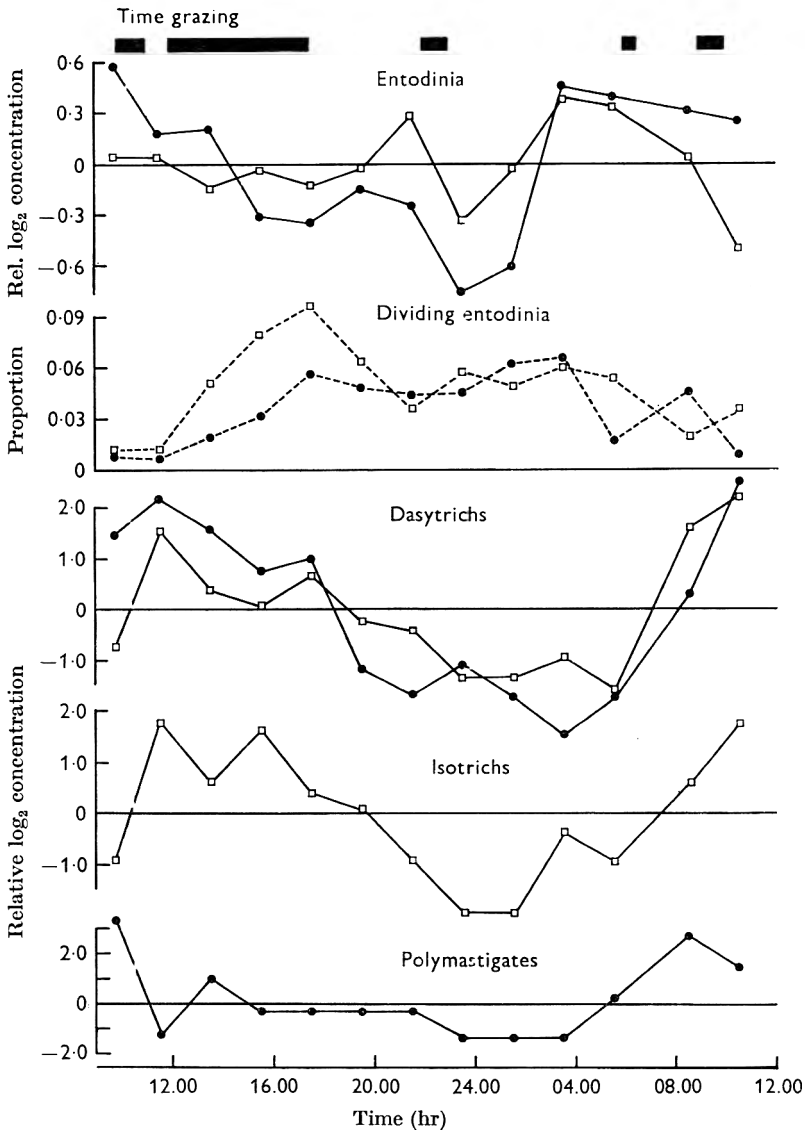

Fig. 3. Grazing behaviour and changes in rumen microbial population in sheep no. 1593 (□) and no. 2750 (●). Basal concentrations in numbers/ml. were as follows (data for sheep nos. 1593 and 2750, respectively): entodinia, $3{\cdot}9 \times 10^5$, $7{\cdot}3 \times 10^5$; dasytrichs, $1{\cdot}5 \times 10^4$, $3{\cdot}9 \times 10^4$; isctrichs, $3{\cdot}8 \times 10^3$, 0; polymastigates, 0, $2{\cdot}5 \times 10^3$.

when the animal was eating than when it was resting. The concentrations of selenomonads and total bacteria (Fig. 2) also tended to be greatest when the animal was resting. The peptostreptococci (Fig. 1) showed a considerable increase in concen-

tration towards the end of the first eating period, but no further increase after later meals.

The polymastigates (Fig. 2) showed considerable increases in concentration during the midday and early morning feeds, but none during the midnight feed. The holotrich ciliate protozoa (Fig. 1) showed deep minima early in the morning. The oscillospirae (Fig. 2) showed two peaks in concentration, the first associated with

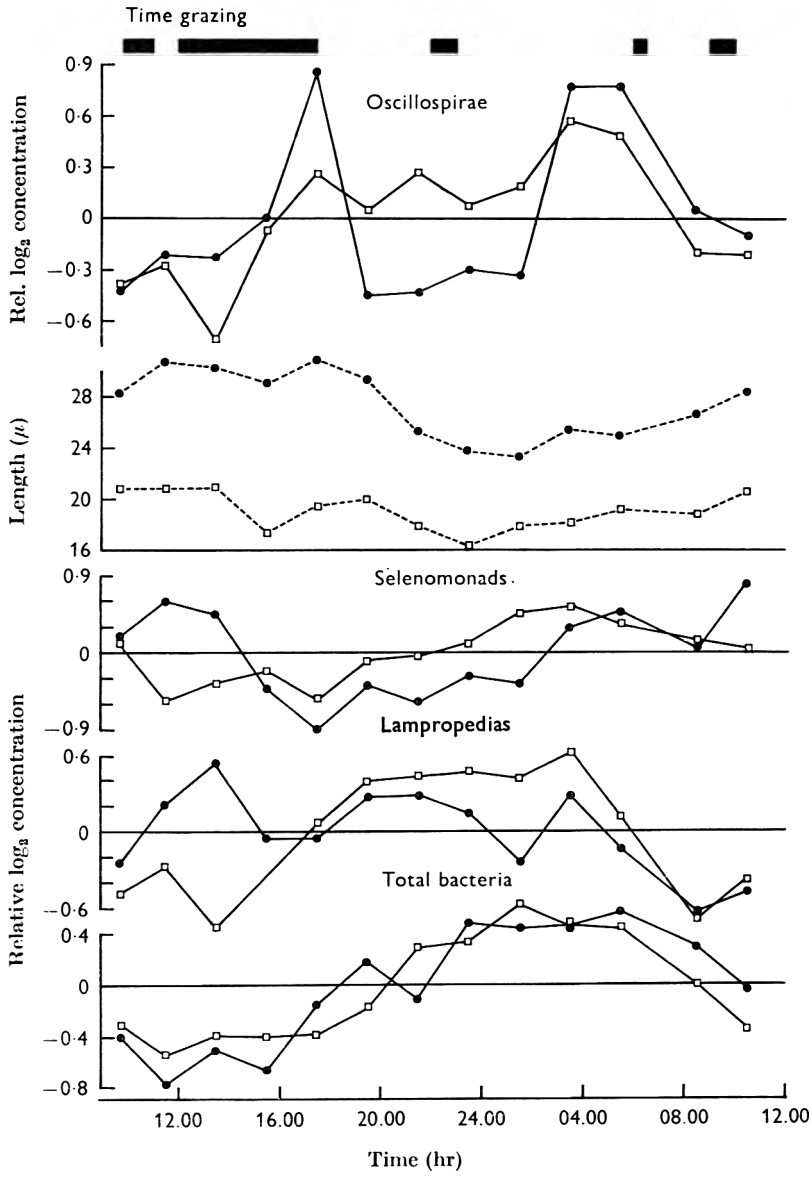

Fig. 4. Grazing behaviour and changes in rumen microbial population in sheep no. 1593 (□) and no. 2750 (●). Basal concentrations in numbers/ml. were as follows (data for sheep nos. 1593 and 2750, respectively): oscillospirae, $8{\cdot}8 \times 10^5$, $8{\cdot}9 \times 10^5$; selenomonads, $1{\cdot}6 \times 10^8$, $6{\cdot}6 \times 10^8$; lampropediae, $8{\cdot}0 \times 10^5$, $3{\cdot}5 \times 10^5$; total bacteria, $3{\cdot}7 \times 10^{10}$, $4{\cdot}6 \times 10^{10}$.

the midday feeding period, and with a peak in trichome length, the second early in the morning, associated with a generally decreasing trichome length.

Grazing sheep

Results are shown in Figs. 3 and 4. Assuming that the time spent grazing is a measure of the amount of feed consumed, these animals also consumed most of their feed during the middle of the day, with a short feeding period around midnight and another two short periods early in the morning. These sheep were not seen to drink.

The entodinia (Fig. 3) showed a decreasing or stationary concentration throughout the major grazing period and for a while thereafter, increasing rapidly in the early morning and then decreasing slowly again. The proportion of dividing entodinia increased during the major grazing period, particularly in sheep 1593, and then slowly decreased. Somewhat similar concentration changes were seen with the selenomonads and total bacteria (Fig. 4).

The concentration of lampropedias (Fig. 4) in sheep no. 1593 was low during the major grazing period, increasing rapidly to a high value which was maintained until early morning when the concentration decreased. A less regular behaviour was seen in sheep no. 2750. The polymastigates in both sheep were high in concentration early in the morning (Fig. 3) but low throughout the rest of the day. The holotrich ciliate protozoa (Fig. 3) reached maximal concentration shortly before noon, then slowly decreased in concentration to minima 12–18 hr later.

The concentration of oscillospirae showed two well-marked peaks for sheep no. 2750 (Fig. 4), the afternoon peak being associated with maximal length of trichome, the early morning peak with near-minimal length. In sheep no. 1593 the concentration increased during the early afternoon as in sheep no. 2750, but did not decrease before the further increase in the early morning; the mean length of the trichomes was higher in the afternoon than in the early morning, but the differences were not as marked as for sheep no. 2750.

DISCUSSION

Ruminants at pasture usually graze for 8–9 hr per day, and most of this time is spent in one major period of fairly continuous eating (Hughes & Reid, 1951; Arnold, 1962). In the present work, both the grazing and the penned sheep spent a fairly continuous period of 6–9 hr consuming 60–80 % of their day's total feed. In the previous studies, the sheep needed only about 1 hr to consume their whole ration, either in one continuous period (Warner, 1966*a*) or regularly distributed throughout the day (Warner, 1966*b*).

The rumens of the sheep in the present studies were more tightly packed with feed than in the previous work, and hence the digesta were presumably less well mixed. Bearing in mind this inefficient mixing, the patterns of microbial concentration change appeared generally similar to those found in sheep fed once daily (Warner, 1966*a*), rather than to the patterns found in frequently fed animals (Warner, 1966*b*). Hence it would appear that the conditions which control microbial growth in the rumens of animals feeding naturally to appetite, whether in pens or at pasture, resemble those in animals fed a restricted diet once daily, presumably owing to the basic similarity of eating behaviour.

Dilution rates were not measured in the present experiments. However, since

these animals ate more feed than the restricted ones, and since the amount of saliva secreted per unit of feed consumed is fairly constant (Bailey, 1961; Meyer, Bartley, Morril & Stewart, 1964; Stacy & Warner, 1966), it was expected that the dilution rates would be greater. Ulyatt (1964) found average dilution rates of 0·10–0·24 hr^{-1} for animals grazing normal pastures; these values are somewhat higher than the figure of 0·06 hr^{-1} found by Warner (1966*a*) for an animal on a restricted ration. Hogan (1964) also found greater flow rates through the rumens of grazing sheep and sheep fed to appetite than in animals on restricted rations. When considered over the full 24 hr, the dilution rate must equal the net growth rate of the micro-organisms, so that it was expected that the growth rates and hence the proportions of dividing organisms should be greater in the present work than previously. Table 1 shows that this expectation was realized. The magnitude of the difference suggests that the overall dilution rate was about double that found previously, i.e. was probably in the range 0·10–0·15 hr^{-1}, in both experiments. It seems likely that the dilution rate would not exceed 0·1 hr^{-1} except when the animal was actually eating.

The immediate effect of the act of eating on conditions in the rumen is a greatly increased dilution rate, rapidly followed by the leaching of solutes from the feed. At various, slower, rates, the metabolic products of the feed constituents then accumulate. The rumen micro-organisms respond to the incoming nutrients by growth, but usually after some delay. My previous work has shown that for some organisms many hours are needed for this response to develop fully, so that the characteristic pattern of concentration change is only shown when the animal spends long periods without eating.

Table 1. *Average proportions of dividing organisms*

	Feeding regimen			
	Restricted ration		Unrestricted ration	
Organism	Once daily*	3-hourly†	Chaff *ad lib.*	Grazing
Entodinia	0·018	0·018	0·035	0·045
Diploplastrons	0·050	0·033	0·075	0·09‡
Polyplastrons	0·059	—	0·16‡	0·10‡
Eadie's ovals	0·033	—	0·035	—
Holotrichs§	0·008	0·008	0·010	0·026

* Data taken from Warner (1966*a*); † Data taken from Warner (1966*b*); ‡ Data were not presented in Figs. 1–4 since concentrations were too low for accurate counts; the figure represents the proportion in the total of organisms counted; § These data were not reported previously but were taken from the same experiments.

As stated previously (Warner, 1966*a*) the concentration of organisms in the rumen can be affected by dilution, by division, by lysis and by sequestration and return. Lysis and sequestration do not appear to play any significant part in the behaviour of the ophryoscolecid protozoa, selenomonads, Eadie's ovals or lampropedias. The changes in the concentrations of the organisms appear to reflect only changes in dilution rate, e.g. in response to eating, and changes in growth rate, e.g. in response to the supply of nutrients. The curves for the entodinia in Fig. 3 show this particularly well, each feeding being accompanied by a decrease in concentration greater for the less actively dividing organisms, and by a slowly increasing rate of division.

In this same experiment, the effect of the midnight feeding is not so noticeable with the selenomonads and lampropedias (Fig. 4); possibly their growth rates were still increasing in response to the previous feed. Again, in the penned animals, the most noticeable increases in concentration of these organisms occurred between feeding periods (Figs. 1, 2).

The rate of decrease of concentration of the peptostreptococci in the penned sheep no. 2985 (Fig. 1) after its maximum at about 21.00 hr was greater than could be accounted for by the expected dilution rate ($r = -0{\cdot}15\ \mathrm{hr}^{-1}$, $D < 0{\cdot}1\ \mathrm{hr}^{-1}$ so that the net growth rate ρ was negative), suggesting as previously (Warner, 1966*a*) that lysis or engulfment was taking place. The rapid increase in concentration, beginning shortly after the major part of the day's ration had been consumed, was at a rate indicating a doubling time of about 2 hr, as before (Warner, 1966*a*).

The polymastigate flagellates behaved somewhat irregularly, but most of the considerable increases in concentration followed closely on the start of a period of eating (Figs. 2, 3). This is consistent with the previous tentative explanation of normal sequestration close to the rumen wall with return to the general rumen liquor in response to fresh nutrients (Warner, 1966*a*).

The holotrich ciliate protozoa also showed long periods when the rates of decrease of concentration were greater than could be accounted for by the dilution rate (from Figs. 1 and 3, $r = -0{\cdot}11$ to $-0{\cdot}21\ \mathrm{hr}^{-1}$). A minimum 14–22 hr after the beginning of the major feeding period was followed by a period of perhaps 8 hr during which the organisms divided at least 2–4 times. Dividing organisms were more irregularly distributed than in the previous work, but again the highest proportions of dividing organisms (6 dividing in 17 organisms seen) occurred at the period of minimum concentration.

Lastly, the oscillospirae again showed two peaks in concentration, the peak accompanying the maximum trichome length being associated with the major feeding period (Figs. 2, 4).

From the previous work (Warner, 1962, 1966*a*) and the present study, the following tentative conclusion emerges. Whenever the daily feeding behaviour of the animal includes a major period of time during which all or most of the ration is consumed, and long periods during which little or nothing is consumed, then the patterns of change of concentration of the rumen micro-organisms are characteristic of the species of micro-organism and little affected by the nature or amount of the diet. Penned animals fed a restricted diet once daily have patterns of rumen microbial concentration changes similar to those patterns found in animals eating to appetite in natural circumstances, so that studies under these strictly controlled conditions have considerable relevance to the relatively uncontrollable natural situation.

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Adansonian Classification of Mycobacteria

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SUMMARY

An Adansonian classification of mycobacteria has been done by using 94 characters. Fifty-nine strains of slowly growing mycobacteria were classified into five groups: (1) *Mycobacterium tuberculosis* and *M. bovis* (2) *M. kansasii*; (3) *M. avium*, 16 strains of non-photochromogens from human sources, 6 strains of non-photochromogenic mycobacteria from soil sources, and scotochromogens from human sources (*M. aquae*); (4) 2 strains of non-photochromogens from human sources; (5) 1 strain of non-photochromogen from human source.

The third group seemed to consist of three subgroups: (3, i) non-chromogens from soil sources; (3, ii) *Mycobacterium avium* and some non-photochromogens, which were inseparable from *M. avium*; (3, iii) some non-photochromogens from human sources resembling *M. avium* (but separable from it) and scotochromogens from human sources.

Slowly growing non-photochromogenic mycobacteria from soil sources (subgroup 3, i) were considered to form a new species, *M. terrae*. A description of this species is given.

Seventy-eight strains of rapidly growing mycobacteria were classified into seven groups: (6) 6 strains of miscellaneous species, *Mycobacterium marinum*, *M. balnei*, *M. platypoecilus*, *M. ranae* and *M. piscium*; (7) *M. thermoresistibile* (sp.nov.); (8) *M. phlei*; (9) *M. aurum* (sp.nov.); (10) *M. fortuitum* and group IV rapid growers; (11) *M. parafortuitum*; (12) *M. smegmatis*. These groups seemed to form independent species. *Mycobacterium thermoresistibile* is a new species capable of growing at 52°. *Mycobacterium aurum* is a new species consisting of rapidly growing scotochromogenic mycobacteria with urease, nicotinamidase and pyrazinamidase and some strains also with acetamidase and allantoinase.

INTRODUCTION

Adansonian classification of bacteria proposed by Sneath (1957*a*, *b*) was adapted successfully to mycobacteria by Bojalil & Cerbón (1961), Cerbón & Bojalil (1961) and Bojalil, Cerbón & Trujillo (1962). Although much information was obtained by their studies, the taxonomy of Runyon's group II scotochromogens and group III non-photochromogens (Runyon, 1955) was not clarified sufficiently. The purpose of the present paper is to report an Adansonian classification of slow-growing and rapid-growing mycobacteria by using an extended number of features, including a new differentiation system proposed by the present author (Tsukamura, 1965*d*). A new species of slow-growing mycobacteria and two new species of rapid-growing mycobacteria have been recognized.

METHODS

Organisms. The following strains were used: 4 strains of *Mycobacterium tuberculosis*, H37Rv, H37Ra, Aoyama-B, Frankfurt; 5 strains of *M. bovis*, Ravenel, D-4, Denken, Miwa, BCG; 3 strains of *M. kansasii*, Forbes-84*, Bostrum-D-35*, Nagai; 7 strains of *M. avium*, A71, 3717, 4110, 4121, 11755, Kirchberg, Flamingo; 19 strains of group 3 non-photochromogens isolated from human sources, N100616*, N121326*, Ueda (NJ-1), Tagami (NJ-2), Garnoh (NJ-3), Suhara (NJ-6), Hatsuno (NJ-7), Minamisawa (NJ-8), Iijima (NJ-9), Onari (NJ-10), Saito (NJ-16), Hasegawa, Ichihara, Tomida (SJ-7), Sakatani (NJ-17), Niikura, Iwai, Munaishi, Saito-T (NJ-18); 6 strains of non-photochromogenic mycobacteria from soil sources, 301, 315, 317 (NCTC 10424), 318, 361, 362; 15 strains of group 2 scotochromogens from human sources, SP-5*, SP-6*, Matsumoto (SJ-1), Ishii (SJ-2), Watanabe (SJ-3), Okubo (SJ-5), Nagashima (SJ-8), Aruga (SJ-9), Goto (SJ-13), Kubota, Nakagawa, Takahashi, Ezaki, Ito, Tominaga; *M. smegmatis*, SN1, SN2, SN3, SN4, SN5, SN6, SN7, SN8, SN9, SN10 (these ten strains were supplied by Dr R. Bönicke, Forschungsinstitut Borstel, Germany), Wa 63, Wa 290, Wa 236, Wa 237, Wa 402, R 1102, R 1103, CDC, SP-9, Trudeau (these ten strains from the collection of Dr G. P. Kubica, U.S.A., were supplied by Dr H. Saito, Hiroshima University), Denken, Kyushu, Jucho, Takeo (the last two strains were identified by Tsukamura, Toyama & Mizuno (1964)); *M. phlei*, SN101, SN102, SN103, SN104, SN105, SN106, SN107, SN108, SN109, SN110 (these ten strains were supplied by Dr R. Bönicke), 5, Wa 40, Wa 60, Wa 366, Wa 289, Trudeau, SP-11, CDC (these 8 strains were supplied by Dr H. Saito, Hiroshima University, who received them from Dr G. P. Kubica), Denken, Kyushu; *M. fortuitum*, 605*, 606*, 607*, 335, 334, 330, 313, 302, 306, 308 (the latter 7 strains were isolated and identified in this laboratory); Rapid growers (group IV) from human sources, Yamamoto, Nishiwaki, Mimura; *M. marinum*, Ross (Ross, A. J.); *M. balnei*, B916, B913 (Lausanne); *M. ranae*, L-17 (Lausanne); *M. platypoecilus*, Ross; *M. piscium*, Ross (these 6 strains were supplied by Dr K. Konno, Tohoku University); *M. parafortuitum* (Tsukamura, 1966*a*), 310 (NCTC 10410), 311 (NCTC 10411), 304, 305, 307, 314; *M. thermoresistibile* (sp.nov.), 316 (NCTC 10409), 1005, 1011, 1021, 1025; *M. aurum* (sp.nov.) (rapid-growing scotochromogenic mycobacteria to be presented as a new species in the present study), 358 (NCTC 10437), 312 (NCTC 10438), 303 (NCTC 10439), 309 (NCTC 10440).

Among the above, the non-photochromogens and scotochromogens from human sources, except for the strains with a symbol *, were isolated in Japan from 1948 to 1959 and were considered to be pathogenic for humans (Hibino, 1964). These were collections of Professor S. Hibino (Nagoya University) and were supplied by him in 1961. The strains with symbol * were from the collections of Dr E. H. Runyon and were supplied through Dr K. Takeya (Kyushu University) in 1960. The *Mycobacterium avium* strains were supplied by Dr K. Konno (Tohoku University) in 1964.

The strains had been maintained on Löwenstein-Jensen medium or Ogawa egg medium and were subcultured from single colonies.

The following tests were done in triplicate and, when different results were obtained in three replicates (these occurred rarely), the feature obtained twice was recorded. Data were expressed as either + or −.

(1) Gram reaction (*Mackie & McCartney's Handbook*, 1960).

(2) Acid-fastness was tested by the Ziehl–Neelsen stain (*Mackie & McCartney's Handbook*, 1960).

(3) Colony morphology was observed on the Sauton agar medium, in which sodium glutamate replaced asparagine, and the results were recorded as 'rough' or 'smooth' (colony morphology was more clearly characterized on the Sauton agar medium than on the Löwenstein–Jensen medium).

(4) Pigmentation of colonies was observed on 2- to 4-week cultures of slow-growing mycobacteria or on 7 day cultures of rapid-growing mycobacteria, growing on the Sauton agar medium (pigmentation on the Sauton agar was more stable than that on egg media).

(5) Reaction to light. Two- to 3-week cultures of slow-growing mycobacteria or 7 day cultures of rapid-growing mycobacteria, growing on the Löwenstein–Jensen medium were exposed to light after replacing a rubber stopper by a loose cotton-wool plug, and allowed to stand at room temperature (20–37°) for 2 days. The appearance of pigmentation was read as a positive reaction to light (photosynthesis of carotenoids).

(6) Growth rate was observed on both the Löwenstein-Jensen medium and the Sauton agar medium. Abundant growth on the egg medium within 3 days or on the Sauton agar within 7 days was recorded as rapid growth, and growth on the former after more than 1 week or on the latter after more than 2 weeks was recorded as slow growth.

(7) Catalase activity was tested by immersing one loopful of the organism into a 30 % aqueous solution of H_2O_2 and observing the occurrence of bubbling.

(8) Peroxidase activity was tested according to the method described by Tiranarayanan & Vischer (1957).

(9) Nitrate reduction. Fifty mg. (moist weight) of the organism were suspended in 5 ml. 0·067 M-phosphate buffer (pH 7·1) containing 0·1 % sodium nitrate, and incubated at 37° for 15–16 hr. Formation of nitrite was examined by adding two drops of 2 % (w/v) *p*-dimethyl-aminobenzaldehyde in 10 % HCl + 1·0 ml. 10 % HCl.

(10) Three-day arylsulphatase test was done according to the method of Wayne (1961).

(11) Two-week arylsulphatase test was done according to the method of Kubica & Vestal (1961) with a final concentration of 0·001 M-tripotassium phenolphthalein disulphate.

(12) Salicylate degradation was observed as a blackening of Sauton agar medium containing 0·1 % (w/v) sodium salicylate, after incubation at 37° for 7 days (Tsukamura, 1965*a*).

(13) Sodium *p*-aminosalicylate (PAS) degradation was observed according to a method described previously (Tsukamura, 1961; Tarshis, 1964; Tsukamura, 1965*a*). Blackening of the Ogawa egg medium or Löwenstein–Jensen medium containing 0·2 % (w/v) PAS after incubation at 37° for 7 days was defined as a positive degradation.

(14) *p*-Aminobenzoate degradation was recorded as a blackening of Sauton agar medium containing 0·2 % (w/v) sodium *p*-aminobenzoate after incubation of 4 weeks (Tsukamura, unpublished test).

(15) Hydroxylamine resistance was defined as an ability of slow-growing mycobacteria to grow on the Löwenstein–Jensen medium or the Ogawa egg medium

containing 0·025 % (w/v) hydroxylamine hydrochloride after incubation for 3 weeks or an ability of rapid-growing mycobacteria to grow on the media containing 0·05 % hydroxylamine after incubation for 1 week (Tsukamura, 1965*b*).

(16) The niacin test was done according to Konno (1963).

(17) Picric acid (0·1 %, w/v) tolerance was defined as ability to grow on the Sauton agar medium containing 0·1 % (w/v) picric acid after incubation for 3 weeks (Tsukamura, 1965*c*).

(18) Picric acid (0·2 %, w/v) tolerance. Ability to grow on Sauton agar containing 0·2 % picric acid after 3 weeks.

(19) Growth at 28°.

(20) Growth at 37°.

(21) Growth at 45°.

(22) Growth at 52° was observed on the Löwenstein–Jensen medium.

(23) Survival at 60° for 1 hr was observed on the Löwenstein–Jensen medium.

(24–33) Bönicke's amidase pattern was tested according to the description of Bönicke (1962). Incubation time was 16 hr. The following amides were used: (24) acetamide; (25) benzamide; (26) urea; (27) isonicotinamide; (28) nicotinamide; (29) pyrazinamide; (30) salicylamide; (31) allantoin; (32) succinamide; (33) malonamide.

(34–41) Utilization of organic acids as sole carbon sources was tested on the following medium: $(NH_4)SO_4$, 2·64 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; purified agar (Eiken Co., Tokyo, or Wako Chemical Co., Osaka), 20·0 g.; distilled water, 1000 ml. The medium was adjusted to pH 7·1 by adding 10 % (w/v) KOH. The medium was supplemented with organic acid Na salt to a final concentration 0·02 M, and re-adjusted to pH 7·1. The medium was then sterilized by autoclaving at 115° for 30 min. and made up as slopes (sterilization by autoclaving gave the same results as sterilization by heating at 100° for 15 min.). Series of media containing different organic acids and control medium without carbon source were inoculated with one loopful of stock cultures. Growth of slow-growing mycobacteria was observed after incubation for 4 weeks, and growth of rapid-growing mycobacteria after incubation for 2 weeks. For incubation, a loop, 3·5 mm. outside diameter and 2·0 mm. inside diameter, was used throughout. After touching the loop slightly on the growth of test organisms, the inoculum was streaked on the slope of fresh media. Care was taken not to bring any visible bacterial mass on the fresh media. This method of inoculation gave nearly similar results as a semi-quantitative method in which one loopful of 3-day cultures of rapidly growing mycobacteria or 10-day cultures of slow-growing mycobacteria growing in the Dubos liquid medium were used for inoculation. The following organic acids were used for tests: (34) acetate; (35) citrate; (36) succinate; (37) malate; (38) pyruvate; (39) benzoate; (40) malonate; (41) fumarate.

(42–54) Acid from carbohydrates. This was observed on the following medium: $(NH_4)_2SO_4$, 2·64 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; yeast extract, 0·2 g.; 0·2 % (w/v) bromthymol-blue in 0·02 M-NaOH, 20 ml.; purified agar, 20·0 g.; distilled water, 980 ml. The medium was adjusted to pH 7·0 and sterilized by autoclaving at 115° for 30 min. Carbohydrates were sterilized separately by heating in solution at 100° for 15 min. and added to the above medium aseptically to a final concentration of 0·5 % (w/v). When necessary the medium was re-adjusted

to pH 7·0 aseptically and poured into tubes. Slopes were inoculated with one loopful of the stock cultures and incubated. Control medium without carbohydrate was always set up. Acid formation was observed after 2 weeks incubation at 28° (rapidly growing mycobacteria) or after 4 weeks incubation at 28° (slowly growing mycobacteria). The following carbohydrates were used: (42) glucose; (43) mannose; (44) galactose; (45) arabinose; (46) xylose; (47) rhamnose; (48) trehalose; (49) maltose; (50) lactose; (51) raffinose; (52) inositol; (53) mannitol; (54) sorbitol.

(55–74) Utilization of carbohydrates (sugars, alcohols, glycols) as sole carbon sources. This was observed by using the same medium (except that yeast extract and bromthymol-blue were omitted) as that used in the tests for acid formation. Sugars were added to a final concentration of 0·5 % (w/v), and alcohols and glycols to a final concentration of 0·1 M. Growth was observed after incubation for 2 weeks (rapidly growing mycobacteria) or after incubation for 4 weeks (slow-growing mycobacteria). The following were used: (55) glycerol; (56) glucose; (57) fructose; (58) sucrose; (59) mannose; (60) galactose; (61) arabinose; (62) xylose; (63) rhamnose; (64) trehalose; (65) raffinose; (66) inositol; (67) mannitol; (68) sorbitol; (69) ethanol; (70) propanol; (71) propyleneglycol; (72) 1,3-butyleneglycol; (73) 1,4-butyleneglycol; (74) 2,3-butyleneglycol.

(75–86) Utilization for growth of nitrogen compounds as sole nitrogen sources (a selected series from Tsukamura, 1965*d*), where this method was shown to be useful. The tests were carried out in the following medium: glycerol, 30 ml.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; sodium citrate, 1·0 g.; purified agar, 20·0 g.; distilled water, 970 ml. Nitrogen compounds were added to the above medium to a final concentration of 0·02 M, and the medium was adjusted to pH 7·0. The medium was sterilized by autoclaving at 115° for 30 min. and sloped. Only nitrite medium was sterilized by heating at 100° for 5 min. or else nitrite solution was sterilized by Seitz-filtration and added to medium aseptically. The medium, together with control medium without nitrogen source, was inoculated with one loopful of the stock cultures and incubated. Growth was scored in comparison with growth on the control medium after incubation for 2 weeks (rapid-growing mycobacteria) or after incubation for 4 weeks (slow-growing mycobacteria). The following were used for the tests: (75) sodium L-glutamate; (76) L-serine; (77) L-methionine; (78) acetamide; (79) benzamide; (80) urea; (81) pyrazinamide; (82) isonicotinamide; (83) nicotinamide; (84) succinamide; (85) $NaNO_3$; (86) $NaNO_2$ (Tsukamura & Tsukamura, 1965).

(87–94) Utilization for growth of nitrogen compounds as sole nitrogen and carbon sources. This was shown recently to be very useful for classification of rapidly growing mycobacteria (Tsukamura, 1965*d*). The tests were carried out on the following medium: KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; purified agar, 20·0 g.; distilled water, 1000 ml. The medium was supplemented with a nitrogen compound to a final concentration of 0·02 M, and the medium was adjusted to pH 7·0 and sterilized by autoclaving at 115° for 30 min. After sloping, the medium was inoculated with one loopful of the stock cultures and incubated. Growth was read after incubation for 2 weeks (rapid-growing mycobacteria) or after incubation for 4 weeks (slow-growing mycobacteria). The results were insensitive to inoculum size and the readings were clear. The following compounds were used: (87) sodium L-glutamate; (88) L-serine; (89) glucosamine-HCl; (90) acetamide; (91) benzamide; (92) nicotinamide; (93) monoethanolamine (0·1 M); (94) trimethylene diamine (0·1 M).

Incubation was at 37° unless otherwise noted. As the source of inoculation for tests 3- to 4-week-old cultures of slow-growing mycobacteria or 1-week-old cultures of rapid-growing mycobacteria grown on Löwenstein–Jensen medium were used.

Similarity value. Similarity value (S-value) was expressed as a percentage $=\{$(the number of similar features)/(the number of characters tested)$\} \times 100$. A set of cards were prepared, in which the name of 94 characters (94 tests) each bearing symbols + and − were printed. Results obtained on each strain were recorded on a card by checking the symbol + or − with a circle. For comparison between two strains, two cards were overlapped and the number of the same features (or different features) was read by bringing a light behind the cards. If two strains gave the same features (positive or negative results in both strains), one could see only one circle at a given test (character). On the other hand, if two strains gave different features, one could see two circles at a given test. Thus, the number of similar features (or different characters) was counted easily by counting the number of single circles (or the number of pairs of circles).

When this work was done, the number of different (or similar) features was counted by eye. However, if the features are recorded in thick, non-transparent, hard cards or tapes by punching the circles, it would not be difficult to prepare photoelectric equipment suitable for counting the number of similar features (two similar features permit the passage of light).

RESULTS AND DISCUSSION

Adansonian classification of slowly growing mycobacteria. Grouping of slowly growing mycobacteria

The results are summarized in Fig. 1, which is a diagrammatic representation of the S-value table, prepared by shading squares according to the S-values between the strains of slow-growing mycobacteria. Figure 1 shows the results of 55 strains. Since strains H37Rv and Aoyama-B gave the same results as the H37Ra and Frankfurt strains, and strains Ravenel and D-4 gave the same results as the Denken and BCG strains, these latter four strains were omitted from Fig. 1. The S-values ranged from 72 to 100 %. These relatively high S-values seem to be due to many characters in which all the slow-growing mycobacteria gave negative results. These characters were included as they were useful for differentiation from rapid-growing mycobacteria. Slow-growing mycobacteria were classified into five groups:

Group 1 consisted of *Mycobacterium tuberculosis* and *M. bovis*.

Group 2 of *M. kansasii*.

Group 3 of *M. avium*, most non-photochromogens and scotochromogens.

Group 4 of the non-photochromogens N100616 and N121326.

Group 5 of the non-photochromogen Tomida. The last strain was slightly coloured, like *M. phlei*.

Group 3 could be divided into three subgroups: (3, i) non-photochromogenic mycobacteria from soil sources (6 strains); (3, ii) *Mycobacterium avium* (7 strains) and non-photochromogens from human sources inseparable from *M. avium* (8 strains); (3, iii) non-photochromogens from human sources resembling *M. avium* but separable from it (8 strains) and scotochromogens from human sources (15 strains). In Fig. 1 (3, a), (3, b + 3, c) and 3, d + 3, e) correspond to these three groups, respectively.

The subgroup 3, i (soil isolates) seemed to be a new species and is named *Mycobacterium terrae*. The subgroup 3, iii may be identified with *M. aquae*, since the scotochromogens from human sources had been named *M. aquae* by Bönicke (1962).

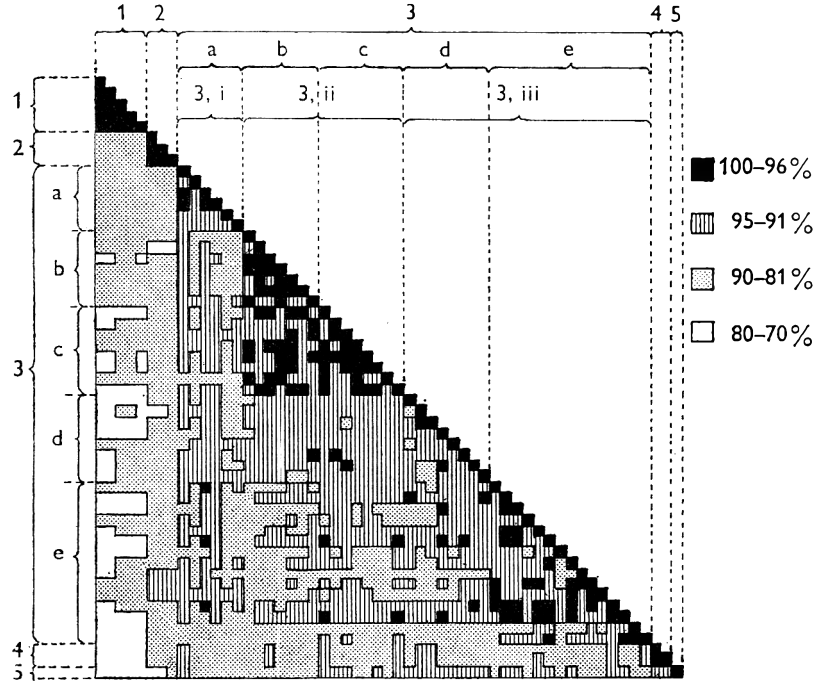

Fig. 1. Diagrammatic representation of the S-value table of slow growing mycobacteria, prepared by shading the squares according to the S-values between the strains. The order of strains given by the sorting procedure was as follows (from left to right or from top to bottom): Group 1: *Mycobacterium tuberculosis*, H37Rv, Aoyama-B, and *M. bovis*, Ravenel, D-4, Miwa; Group 2: *M. kansasii*, Forbes-84, Bostrum-D-35, Nagai; Group 3: (3,a) non-photochromogens from soil sources, (*M. terrae*) 301, 315, 317, 318, 361, 362; (3, b) *M. avium*, A71, 3717, 4110, 4121, 11755, Kirchberg, Flamingo, (3, c) non-photochromogens from human sources, Hatsuno, Minamisawa, Onari, Saito, Sakatani, Hasegawa, Ichihara, Niikura; (3, d) non-photochromogens from human sources, Iwai, Gamoh, Iijima, Saito-T, Munaishi, Ueda, Tagami, Suhara; (3, e) scotochromogens from human sources, (*M. aquae*) Ishii, Nagashima, Kubota, Nakagawa, Okubo, Aruga, Goto, Takahashi, Ezaki, Watanabe, Ito, Matsumoto, Tominaga, SP-5, SP-6; Group 4: non-photochromogens from human sources, N100616, N121326; Group 5: non-photochromogen from human source, Tomida.

Tests for the homogeneity of a group

Intermediate types would be omitted from a group by a diagrammatic representation of the S-values, but it is desirable to confirm 'homogeneity' of a group by a statistical method. If a group contained any strain which was markedly aberrant, that strain must have low S-values to other strains of the group. If the S-values are significantly low, the mean of the S-values for such a strain would be significantly lower than the mean of the S-values for all strains of the group. This comparison is done by the 't'-test.

Since groups 1, 2, 4 and 5 are apparently homogenous (S-values 97–100 %), the test for homogeneity was made on the subgroups of group 3. Examples of these tests are shown in Tables 1–3. These subgroups shown in the tables correspond to the groups 3, a, b, c, d, and e in Fig. 1 and do not always correspond to the taxonomic subgroups obtained by the Adansonian classification. An example on a taxonomic group will be shown later in the section on rapidly growing mycobacteria.

Table 1 shows the homogeneity test on the non-photochromogenic mycobacteria from soil, *Mycobacterium terrae*. If the 6 strains of this group were equally similar to each other for every strain, the mean S-values between that strain and the rest of the group should not differ significantly from the mean of all the S-values between the members of the group. However, when the mean for the whole group was compared with the mean for each strain by the 't'-test, the mean for strain 361 differed significantly from the mean for the group ($P = 0{\cdot}05$).

Mean for the group = 94·2 S.D. = 1·41 % ($n_1 = 15$). Mean for strain 361 = 92·0 S.D. = 2·00 % ($n_2 = 5$). d (difference) = 94·2–92·0 = 2·2

$$s^2 = \frac{(n_1-1)(s_1)^2+(n_2-1)(s_2)^2}{n_1+n_2-2} = \frac{(15-1)(1{\cdot}41)^2+(5-1)(2{\cdot}00)^2}{15+5-2}$$

$$= 2{\cdot}44$$

$$s = 1{\cdot}56,$$

where s_1 and s_2 are the standard deviations for the group and for the strain.

$$d \pm t_{n_1+n_2-2,\ 0{\cdot}05}\, s\sqrt{\left(\frac{1}{n_1}+\frac{1}{n_2}\right)} = 2{\cdot}2 \pm 2{\cdot}10 \times 1{\cdot}56 \times \sqrt{\left(\frac{1}{15}+\frac{1}{5}\right)}$$

$$= 2{\cdot}2 \pm 1{\cdot}30 \text{ which is } > 0$$

The difference d is therefore significant at a level of $P = 0{\cdot}05$ (Bennett & Franklin, 1956).

Table 1. *Test for homogeneity of strains of Mycobacterium terrae (subgroup 3, a)*

Strain	Mean S-value for strain (n = 5)*	Mean S-value for strain (n = 4)†
301	94·0 ± 2·40	95·0 ± 1·83
315	94·0 ± 0·87	94·0 ± 1·00
317	95·0 ± 3·04	96·0 ± 2·22
318	95·0 ± 2·82	97·5 ± 2·77
361	92·0 ± 2·00‡	
362	95·0 ± 0·71	94·5 ± 0·52
	Mean S-value for group (n = 15)§	Mean S-value for group (n = 10)
	94·2 ± 1·41	95·0 ± 1·71

* Mean of the S-values to other five strains.

† Mean of the S-values to other four strains after strain 361 was omitted.

‡ A significant difference between the mean S-value for this strain and the mean S-value for the group ($P = 0{\cdot}05$).

§ The number of S-values in the group, (5 values) × (6 strains) = 30. This was divided by 2, as one half of values are a reflected figure. The number of 'n' in the group was calculated similarily also in other cases.

The other strains showed no significant difference from the mean for the group (Table 1). Thus, it was considered that strain 361 should be omitted from the group. After omitting strain 361, mean S-values for the remaining strains were calculated afresh and the same test was carried out. This time, there was no significant difference between the mean for the group and each mean for strains (Table 1). The five strains, 301, 315, 317, 318 and 362 were considered to form a 'homogeneous' group.

Table 2. *Test for homogeneity of strains of Mycobacterium avium (subgroup 3, b)*

Strain	Mean S-value for strain ($n = 6$)*	Strain	Mean S-value for strain ($n = 6$)*
A 71	96·0 ± 1·17	11755	96·0 ± 1·34
3717	96·2 ± 1·34	Kirchberg	96·6 ± 1·48
4110	96·2 ± 1·42	Flamingo	95·0 ± 0·78
4121	97·1 ± 0·78		

Mean S-value for group ($n = 21$): 96·2 ± 1·21

* Mean of S-values to other six strains.
No significant difference between the mean for each strain and the mean for group.

Table 3. *Test for homogeneity of strains of the non-photochromogens inseparable from Mycobacterium avium (subgroup 3, c)*

Strain	Mean S-value for strain ($n = 7$)*	Strain	Mean S-value for strain ($n = 7$)*
Hatsuno	96·5 ± 1·91	Sakatani	97·0 ± 1·29
Minamisawa	95·0 ± 1·08	Hasegawa	96·0 ± 1·58
Onari	95·0 ± 1·64	Ichihara	94·6 ± 1·15
Saito	96·5 ± 1·29	Niikura	96·4 ± 2·00

Mean S-value for group ($n = 28$): 96·0 ± 1·64

No significant difference between the mean for each strain and the mean for group.
* Mean of S-values to other seven strains.

Table 4. *Test for homogeneity of strains of the non-photochromogens separable from Mycobacterium avium (subgroup 3, d)*

Strain	Mean S-value for strain ($n = 7$)*	Strain	Mean S-value for strain ($n = 7$)*
Gamoh	91·9 ± 4·14	Ueda	92·9 ± 9·58
Iijima	92·9 ± 3·24	Tagami	92·3 ± 2·24
Saito-T	92·9 ± 1·63	Suhara	92·2 ± 2·38
Munaishi	92·6 ± 2·45	Iwai	92·2 ± 2·45

Mean S-value for group ($n = 28$): 92·5 ± 2·59

No significant difference between the mean for each strain and the mean for group.
* Mean of S-values to other seven strains.

Similarly, 7 strains of *Mycobacterium avium* seemed to be homogeneous (Table 2). These may be considered as standard *M. avium* strains. The group of eight strains of non-photochromogens inseparable from *M. avium* was also considered to be homogeneous (Table 3). Non-photochromogens separable from *M. avium* were also homogeneous (Table 4). However, the mean value of the group was 92·5 % which was lower than the values of the other subgroups. This might indicate that this consisted of intermediate types. When the scotochromogens were subjected to the tests, strains SP-5 and Goto were first distinguished by their low S-values, and, secondly, strains SP-6 and Ezaki were omitted. The remaining eleven strains were shown to be homogeneous (Table 5).

Table 5. *Test for homogeneity of strains of Mycobacterium aquae (subgroup 3, c)*

Strain	Mean S-value for strain (n = 14)*	Mean S-value for strain (n = 12)†	Mean S-value for strain (n = 10)‡
SP-5	90·0 ± 3·30§	·	·
SP-6	92·8 ± 2·64	93·2 ± 1·88§	·
Ishii	93·5 ± 2·97	94·3 ± 2·36	94·9 ± 1·38
Nagashima	94·0 ± 2·57	94·6 ± 2·13	94·9 ± 2·03
Kubota	93·5 ± 2·67	94·0 ± 2·35	94·4 ± 2·16
Nakagawa	94·0 ± 2·08	94·5 ± 1·57	94·3 ± 1·60
Okubo	95·5 ± 1·94	96·0 ± 1·62	96·2 ± 1·63
Aruga	95·6 ± 2·52	96·0 ± 2·30	96·6 ± 2·11
Goto	91·0 ± 3·09§	·	·
Takahashi	94·8 ± 2·29	96·0 ± 1·65	95·5 ± 1·38
Ezaki	92·5 ± 2·39	93·0 ± 1·63§	·
Watanabe	95·0 ± 2·42	96·0 ± 1·73	95·6 ± 1·76
Ito	93·5 ± 2·85	94·0 ± 2·09	94·7 ± 1·97
Matsumoto	95·5 ± 2·40	96·0 ± 1·76	96·5 ± 1·53
Tominaga	95·6 ± 2·64	96·0 ± 2·59	96·1 ± 2·85
Mean S-value for group	(n = 105) 93·8 ± 2·61	(n = 78) 95·0 ± 2·00	(n = 55) 95·4 ± 1·99

* Mean of S-values to other 14 strains.
† Mean of S-values to other 12 strains, after strains SP-5 and Goto were omitted.
‡ Mean of S-values to other 10 strains, after strains SP-6 and Ezaki were omitted.
§ Significant difference between the mean for strain and the mean for group (P = 0·05).

To find the taxonomic distance from standard *Mycobacterium avium* strains, mean S-values were calculated for non-photochromogens and scotochromogens compared to the standard *M. avium* strains. These values are shown in Table 6. The eight strains of subgroup 3, c found inseparable from standard *M. avium* strains showed no significant difference from the mean S-value of *M. avium*. On the other hand, all of the other strains showed significant difference. Thus, the results of the statistical method agreed with the results of the diagrammatic representation.

Sneath's Adansonian classification (Sneath, 1957*a*, *b*) made it possible to indicate the correlation among bacterial strains. In this method, grouping was made by a diagrammatic representation of the S-values. A method has been presented in this paper for testing the homogeneity of a group formed by the Adansonian method. If there is no significant difference at a level of P = 0·05, the group may be considered a homogeneous one. If there is a significant difference, strains with significant difference are omitted from the group, and the same test is made

Table 6. *Comparison of non-photochromogens and scotochromogens with Mycobacterium avium*

Strain	Mean S-value to *M. avium* ($n = 7$)*	Strain	Mean S-value to *M. avium* ($n = 7$)*
N 100616	87·5 ± 2·4	SP-5	84·0 ± 1·4
N 121326	87·5 ± 2·4	SP-6	87·0 ± 1·7
Gamoh	94·2 ± 1·6	Ishii	89·6 ± 1·6
Iijima	93·5 ± 2·0	Nagashima	91·5 ± 1·5
Saito-T	91·5 ± 0·8	Kubota	88·0 ± 2·1
Munaishi	93·5 ± 0·8	Nakagawa	88·6 ± 2·3
Tomida	88·0 ± 1·8	Okubo	90·5 ± 1·7
Ueda	93·5 ± 1·4	Aruga	91·6 ± 1·9
Tagami	92·0 ± 1·5	Goto	89·5 ± 2·1
Suhara	91·5 ± 1·7	Takahashi	90·8 ± 2·1
Hatsuno	95·0 ± 1·6†	Ezaki	85·6 ± 1·6
Minamisawa	95·0 ± 1·7†	Watanabe	88·7 ± 1·9
Onari	95·0 ± 2·0†	Ito	88·5 ± 2·2
Saito	95·5 ± 1·4†	Matsumoto	90·6 ± 1·8
Sakatani	95·5 ± 1·1†	Tominaga	91·5 ± 1·8
Hasegawa	95·0 ± 1·2†	301	91·7 ± 1·7
Ichihara	95·5 ± 1·6	315	88·5 ± 2·3
Niikura	95·0 ± 1·6†	317	91·5 ± 1·1
Iwai	91·7 ± 1·3	318	90·0 ± 1·1
M. phlei Kyu	59·8 ± 1·7	361	86·9 ± 1·8
M. smegma K	37·2 ± 1·9	362	87·7 ± 2·2

* Mean of S-values to 7 standard *M. avium* strains.

† No significant difference between the mean for a test strain and the mean S-value for the *M. avium* strains (96·2 ± 1·72; $P = 0{\cdot}05$ by the '*t*'-test: see Table 2).

successively on the residual group. The homogeneous group is a unit of classification though it is not always identical with a species. The homogeneity test would be useful for two other purposes.

Species description. Unusual strains might often be grouped erroneously. To decrease the chance of mistakes, it is better to omit unusual strains from species descriptions. Description of the species should preferably be done on the homogeneous group and type strains should be selected from it. If one wishes to make descriptions from a small number of strains, exclusion of unusual strains is needed in order to avoid overestimation of rare characters.

Application to identification. Identification according to Adansonian classification principles would follow the following steps: (i) S-values between the test strain and type strains *a*, *b*, *c*, of various species *A*, *B*, *C*, are measured and the type strain *f* showing the highest S-value is found. The test strain most probably belongs to this species, *F*; (ii) S-values of the test strain to the other type strains are compared with those between this type strain *f* and the other type strains. The former values should not differ significantly from the latter; (iii) A homogeneity test is done, the test strain being supposed to belong to the proposed species *F*. The test strain is incorporated into a homogeneous group of standard strains of the proposed species. If the group thus formed proves to be homogeneous by the 't'-test, the test strain would belong to species *F*.

Description of species (slow-growing mycobacteria)

Biological and biochemical characters of the slow-growing mycobacterial species are shown in Table 7. In this table are shown the characters of the species which were shown to be homogeneous.

Table 7. *Characters of slowly growing mycobacteria*

Typical strain and group ...	*M. tuberculosis* H37 Rv Gr. 1	*M. bovis* D-4 Gr. 1	*M. kansasii* Forbes-84 Gr. 2	*M. avium* 4121 Gr. 3, ii	*M. aquae* Matsumoto Gr. 3, iii	*M. terrae* 317 Gr. 3, i	Non-photochromogens N100616	Non-photochromogens Tomida
Characters†								
(3) Colony morphology	R	R	R	S	S*	S	S	S
(4) Pigmentation	−	−	+	−	+	−	−	±‡
(5) Light reaction	−	−	−	−	−	−	−	−
(6) Growth rate	Slow	Slow	Slow	Slow	Slow	Slow	Slow	Slow
(7) Catalase	+	+	−	±	±	±	+	+
(8) Peroxidase	−	−	−	−	−	−	−	−
(9) Nitrate reduction	−	−	−	−	−	−	±	+
(11) Two-week arylsulphatase	−	−	−	−	−	−	−	±
(15) NH_2OH resistance	+	+	+	+	+	+	±	±
(16) Niacin	−	−	−	−	−	−	−	−
(17) Picric acid resistance (0·1%)	+	+	+	+	+	+	±	+
(19) Growth at 28°	+	+	+	±	±	±	±	+
(20) Growth at 37°	+	+	+	+	+	+	+	+
(21) Growth at 45°	−	−	−	+	±	−	−	−
(26) Urease	−	−	−	−	−	−	−	−
(28) Nicotinamidase	−	−	−	+*	±	−	±	−
(29) Pyrazinamidase	−	−	−	+*	−	−	+	−
(34) Acetate as C	−	−	−	+*	±*	+	+	+
(36) Succinate as C	−	−	−	−	−	−	±	−
(37) Malate as C	−	−	−	−	−	−	±	−
(38) Pyruvate as C	−	−	−	−*	−*	−	±	±
(55) Glycerol as C	−	−	±	±	−	−	+	+
(56) Glucose as C	−	−	±	−*	−*	−	+	+
(58) Sucrose as C	−	−	+*	−	−	−	−	−
(69) Ethanol as C	−	−	−	−*	±*	−	−	+
(70) Propanol as C	−	−	−	+	−*	−	−	+
(75) Glutamate as N	+	+	+	±	±	+	+	+
(76) Serine as N	−	−	−*	−	±	+*	+	+
(77) Methionine as N	−	−	−	−	−	−	−	+
(80) Urea as N	−	−	+	+	+	+	+	+
(81) Pyrazinamide as N	−	−	−	±	±	±*	+	+
(82) Isonicotinamide as N	−	−	−	−	−	−	+	−
(83) Nicotinamide as N	−	−	−	+*	±*	+*	+	+
(84) Succinamide as N	−	−	±	±	±	±	+	+
(85) Nitrate as N	−	−	−	+	±	+	+	+
(86) Nitrite as N	−	−	−	−	−	−	+	+
(87) Glutamate as N and C	−	−	−	−	−	+*	−	+

* One or more strains gave different results

† The number shown here is equal to the number in the section 'Methods'. All strains were Gram-positive and acid-fast. Characters in which all strains gave negative results are not shown.

‡ Strain Tomida is non-photochromogenic in young cultures but orange-coloured in old cultures.

A group of non-photochromogenic mycobacteria isolated from soil are presented as a new species, *Mycobacterium terrae*, of which no previous description has been found. Differentiation of this species from other species can be done from Table 7.

Description of *Mycobacterium terrae* (sp.nov.):

Holotype: strain 317 (NCTC 10424).

Colonial morphology: white, wet, smooth on Löwenstein–Jensen medium and on Sauton agar. Non-photochromogenic.

Growth rate: visible growth at 7 days on Löwenstein–Jensen medium and at 7–14 days on Sauton agar. Growth after 2 weeks as single colonies on Löwenstein–Jensen medium.

Catalase positive. Peroxidase negative. Nitrate not reduced. No salicylate degradation. No PAS degradation. Grows on hydroxylamine medium.

Three-day arylsulphatase activity negative (a few strains were weakly positive); 2-week-arylsulphatase activity positive.

Tolerance to 0·1 % picric acid positive; tolerance to 0·2 % picric acid negative.

Growth temperature: growth at 28° and 37°; no growth at 45°.

Amidase activity for Bönicke's series of ten amides all negative.

Acetate and pyruvate utilized as sole carbon source; citrate, succinate, malate, benzoate, malonate, fumarate not utilized. No acid from glucose, mannose, galactose, arabinose, xylose, rhamnose, trehalose, maltose, lactose, raffinose, inositol, mannitol and sorbitol. None of the above sugars and alcohols utilized as sole carbon source. Fructose, sucrose, ethanol, propanol, propylene glycol, 1,3-butylene-glycol, 1,4-butyleneglycol, 2,3-butyleneglycol not utilized as sole carbon source.

Only L-glutamate utilized as sole simultaneous nitrogen and carbon source; L-serine, glucosamine, acetamide, benzamide, monoethanolamine and trimethylene-diamine not utilized as such source.

L-Glutamate, L-serine, urea, pyrazinamide, nicotinamide, succinamide and nitrate utilized as sole nitrogen source; L-methionine, acetamide, benzamide, isonicotinamide and nitrite not utilized as sole nitrogen source.

Differentiation of this species from *Mycobacterium avium* is done as follows: (1) *M. avium* is sensitive to 0·1 % picric acid, but *M. terrae* is tolerant to it; (2) *M. avium* grows at 45°, but *M. terrae* does not; (3) *M. avium* usually possesses nicotinamidase and pyrazinamidase, but *M. terrae* lacks them; (4) *M. avium* usually gives a negative 2-week arylsulphatase, but *M. terrae* gives an intensive reaction; (5) *M. avium* does not utilize L-glutamate as sole simultaneous nitrogen and carbon source, but *M. terrae* usually does.

Differentiation of this species from *Mycobacterium aquae* is done as follows: (1) *M. aquae* has a marked orange colour, but *M. terrae* is colourless; (2) *M. aquae* is sensitive to 0·1 % picric acid, but *M. terrae* is tolerant to it; (3) some strains of *M. aquae* give a positive urease, but *M. terrae* does not; (4) *M. aquae* does not utilize L-glutamate as sole nitrogen and carbon source, but *M. terrae* usually utilizes it.

Bönicke (1962) proposed a species name, *Mycobacterium aquae*, for the Runyon's group II scotochromogens, as these were distinguished from other species by the amidase pattern. In the present study, it was noted that some of the non-photochromogens (Runyon's group III) could not be differentiated from *M. aquae* by the Adansonian classification. Characters of *M. aquae* were studied more extensively in the present study. The results are shown in Table 7.

Non-photochromogens N 100616 and N 121326 and non-photochromogen Tomida were distinguished from other mycobacteria by the Adansonian classification. These may form new species, but at present, no conclusions can be drawn as only one or two isolates were studied.

It has been reported by a number of investigators that the non-photochromogens resembled *Mycobacterium avium*. Bojalil & Cerbón (1960) and Meissner (1960) stated that these two organisms were similar in their physiological and morphological characters. Palmer, Edwards, Hopwood & Edwards (1959), Takeya, Zinnaka, Yamaura & Toda (1960), and Smith & Johnstone (1964) stated that they showed a cross-tuberculin reaction. Murohashi, Tokunaga, Mizuguchi & Maruyama (1963)

stated that they could not be separated by phage typing. Furthermore, Bönicke (1962) reported that they had the same amidase pattern, and Bojalil *et al.* (1962) stated that they were not separable by their biochemical characters even though the similarity was based mainly on negative data. On the other hand, there are a few papers in which differences between these two organisms are described. Differences were reported in arylsulphatase activity (Kubica & Beam, 1961), acid phosphatase activity (Urabe, Saito, Tasaka & Matsubayashi, 1965) and virulence to chickens (Durr, Smith & Altman, 1959). Meissner (1960) also observed a difference in virulence to fowls, but she preferred, considering this was the only difference, to include the non-photochromogens in *M. avium*.

The results of the present study showed that 8 of 19 strains of non-photochromogens were inseparable from *Mycobacterium avium* and may be included in that species. It was noticed that another 8 strains also had an S-value of more than 91 % to the standard *M. avium* strains, and formed part of a large group, Group 3, in which *M. avium* was included.

Adansonian classification of rapid-growing mycobacteria

Grouping of rapidly growing mycobacteria

By arranging strains with more negative features to left and those with more positive features to right, an S-value table was prepared, by which 78 strains could be divided clearly into seven groups (Fig. 2): (6) Six strains of various named species; (7) *Mycobacterium thermoresistibile* (sp.nov.); (8) *M. phlei*; (9) rapidly growing scotochromogens (*M. aurum* (sp.nov.)); (10) *M. fortuitum* and rapid growers (Runyons group IV); (11) *M. parafortuitum*; (12) *M. smegmatis*.

Mycobacterium parafortuitum, a new species described by Tsukamura (1966), occupied an independent position.

Mycobacterium thermoresistibile sp.nov. (holotype: strain 316, NCTC 10409) was distinguished from other species by the Adansonian classification and was considered to form a new species. It can grow at 52°, although *M. phlei* is the only mycobacterium known hitherto that is capable of growing at this temperature. Thirty-nine isolates were obtained from soil and formed an homogeneous group. Further details of this species will be described in another paper so only one strain is shown in Fig. 2 for comparison. It was noticed that most soil isolates growing at 52° obtained around Nagoya belonged to this type. Strains of this type grew more slowly than *M. phlei*, and their characters resembled those of slowly growing mycobacteria (compare Tables 7 and 10).

Rapid-growing scotochromogenic mycobacteria from soil sources were recognized as a new species (Fig. 2). This was named *Mycobacterium aurum*, sp.nov. (holotype: strain 358, NCTC 10437). Fourteen isolates of this species were obtained from the soil. The author had considered that this type of scotochromogenic mycobacterium might be a variety of *M. parafortuitum* (Tsukamura, 1966), but the results of the present study show that the species are differentiated by the following characters: (1) *M. aurum* is markedly orange-coloured, but *M. parafortuitum* is non-pigmented or slightly orange or brownish coloured; (2) *M. aurum* grows at 37° but does not grow at 45°, whereas *M. parafortuitum* grows at 45°; (3) *M. aurum* does not utilize malonate as sole carbon source, but *M. parafortuitum* does;

(4) *M. aurum* utilizes monoethanolamine as sole simultaneous nitrogen and carbon source, but *M. parafortuitum* does not; (5) *M. aurum* usually does not utilize pentoses, but *M. parafortuitum* utilizes them. *Mycobacterium aurum* is differentiated by the amidase pattern from *M. acapulcensis*, a rapidly growing scotochromogenic species (Cerbón & Trujillo, 1963). A fuller description of this species will be made elsewhere.

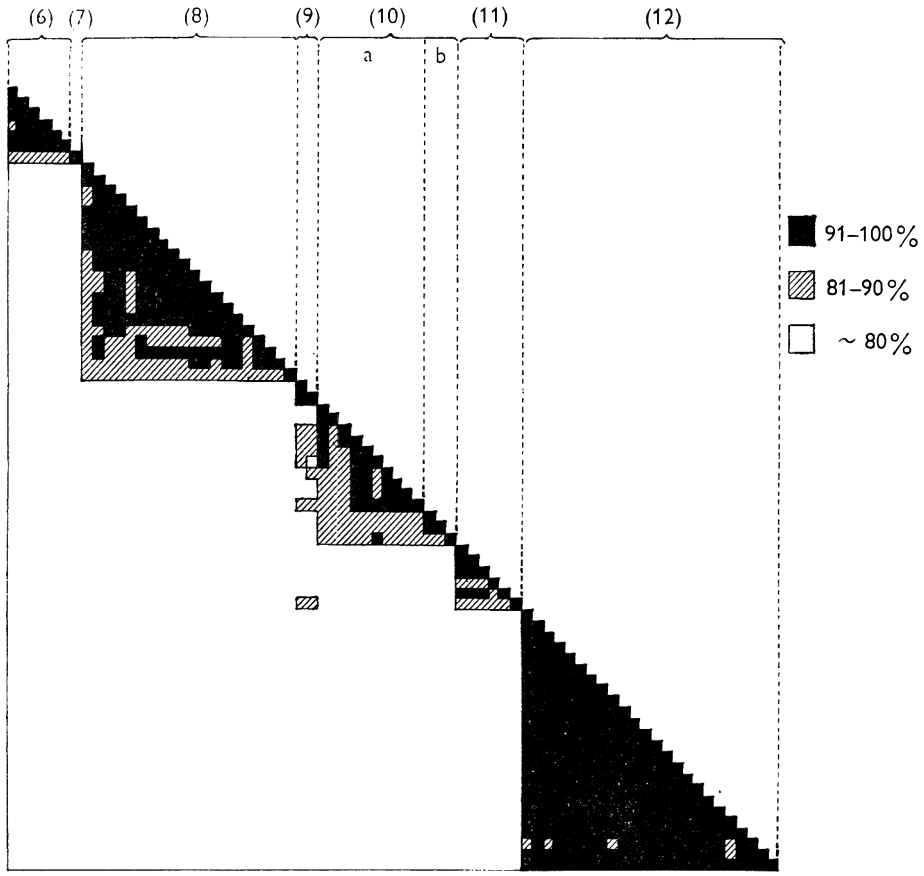

Fig. 2. Diagrammatic representation of the S-value table of rapid-growing mycobacteria. From left to right: (6) *Mycobacterium platypoecilus* Ross, *M. piscium* Ross, *M. ranae* L-17, *M. marinum* Ross, *M. balnei* B916, B913; (7) *M. thermoresistibile* 316 (NCTC 10409); (8) *M. phlei* SN101, SN102, SN103, SN104, SN105, SN106, SN107, SN108, SN109, SN110, Kyushu, 5, Wa40, Wa60, Wa366, Wa289, Trudeau, SP-11, CDC, Denken; (9) *M. aurum* 303 (NCTC 10439), 309 (NCTC 10440); (10, a) *M. fortuitum* 606, 607, 605, 335, 334, 330, 313, 302, 306, 308; (10, b) Rapid growers Yamamoto, Nishiwaki, Mimura; (11) *M. parafortuitum* 310 (NCTC 10410), 311 (NCTC 10411), 304, 307, 305, 314; (12) *M. smegmatis* SN1, SN2, SN3, SN4, SN5, SN6, SN7, SN8, SN9, SN10, Wa 63, Wa 290, Wa 236, Wa 237, Wa 402, R1102, R1103, CDC, SP-9, Trudeau, Denken, Kyushu, Jucho, Takeo.

Group 6, comprised *Mycobacterium platypoecilus*, *M. marinum*, *M. balnei*, *M. ranae* and *M. piscium*. These resembled each other closely, irrespective of the presence or absence of photochromogeneity. The identity of *M. marinum* and *M. balnei* was reported by McMillen & Kushner (1959) and Bojalil *et al.* (1962)

Strains of this group grew much more slowly than other rapidly growing mycobacteria and, as shown later, their natural position seemed to be in the group of slowly growing mycobacteria.

Homogeneity tests

Mycobacterium smegmatis: Of 24 strains tested, 9 were omitted successively because of low mean S-values and the remaining fifteen, SN 2, SN 5, SN 6, SN 7, SN 8, SN 10, Wa 63, Wa 290, Wa 237, R 1102, R 1103, CDC, SP-9, Denken, Takeo, formed a homogeneous group. The mean S-value for this group was $96{\cdot}5 \pm 1{\cdot}25$ % ($n = 105$). *M. phlei*: of 20 strains tested, 13 strains, SN 102, SN 103, SN 104, SN 105, SN 106, SN 107, SN 108, SN 109, SN 110, Kyushu, 5, Wa 60, Wa 366, formed a homogeneous group showing a mean value of $94{\cdot}0 \pm 2{\cdot}15$ % ($n = 78$).

Mycobacterium fortuitum. The 13 strains of *M. fortuitum*, included three rapid growers from human sources. These three were omitted by the first test. The similarity value between strains Yamamoto and Nishiwaki was 95 %, and they may form a subgroup (10, b) within the species. Among strains identified as *M. fortuitum*, 8 strains, 606, 335, 334, 330, 313, 302, 306, 308, formed a homogeneous group (Table 8).

Table 8. *Homogeneity test for strains identified as Mycobacterium fortuitum*

	Mean S-value for each strain			
Strain	($n = 12$) First test	($n = 9$) Second test	($n = 8$) Third test	($n = 7$) Fourth test
606	$90{\cdot}2 \pm 2{\cdot}34$	$91{\cdot}2 \pm 1{\cdot}80$	$91{\cdot}0 \pm 1{\cdot}77$	$90{\cdot}8 \pm 1{\cdot}92$
335	$90{\cdot}5 \pm 3{\cdot}23$	$92{\cdot}0 \pm 1{\cdot}34$	$91{\cdot}2 \pm 1{\cdot}85$	$92{\cdot}3 \pm 1{\cdot}41$
334	$91{\cdot}0 \pm 2{\cdot}84$	$92{\cdot}0 \pm 1{\cdot}95$	$92{\cdot}5 \pm 1{\cdot}97$	$93{\cdot}0 \pm 1{\cdot}36$
313	$90{\cdot}2 \pm 4{\cdot}05$	$91{\cdot}8 \pm 2{\cdot}00$	$92{\cdot}0 \pm 2{\cdot}14$	$92{\cdot}2 \pm 2{\cdot}16$
302	$88{\cdot}5 \pm 5{\cdot}41$	$91{\cdot}2 \pm 2{\cdot}80$	$91{\cdot}6 \pm 2{\cdot}56$	$92{\cdot}0 \pm 2{\cdot}48$
306	$88{\cdot}0 \pm 5{\cdot}24$	$90{\cdot}0 \pm 3{\cdot}82$	$91{\cdot}0 \pm 3{\cdot}25$	$92{\cdot}0 \pm 2{\cdot}34$
308	$89{\cdot}4 \pm 4{\cdot}69$	$91{\cdot}3 \pm 2{\cdot}82$	$92{\cdot}3 \pm 2{\cdot}30$	$92{\cdot}7 \pm 2{\cdot}12$
330	$90{\cdot}5 \pm 2{\cdot}94$	$91{\cdot}0 \pm 2{\cdot}43$	$91{\cdot}7 \pm 2{\cdot}24$	$92{\cdot}0 \pm 2{\cdot}28$
607	$88{\cdot}5 \pm 2{\cdot}57$	$88{\cdot}2 \pm 6{\cdot}25$*	.	.
605	$88{\cdot}5 \pm 3{\cdot}20$	$89{\cdot}5 \pm 2{\cdot}00$	$89{\cdot}5 \pm 2{\cdot}27$*	.
Yamamoto	$85{\cdot}9 \pm 4{\cdot}33$*	.	.	.
Nishiwaki	$85{\cdot}0 \pm 4{\cdot}67$*	.	.	.
Mimura	$86{\cdot}0 \pm 2{\cdot}21$*	.	.	.
Mean S-value for group	$89{\cdot}0 \pm 3{\cdot}98$ ($n = 78$)	$90{\cdot}9 \pm 2{\cdot}46$ ($n = 45$)	$91{\cdot}5 \pm 2{\cdot}30$ ($n = 36$)	$92{\cdot}0 \pm 2{\cdot}05$ ($n = 28$)

* Significant difference from the mean S-value for group at $P = 0{\cdot}05$.

Mycobacterium parafortuitum. The S-value table of this group is shown in Table 9. Six strains formed a homogeneous group by the usual test. However, the mean for the group was low, $88{\cdot}5 \pm 4{\cdot}54$ % ($n = 15$), suggesting that some unusual strains were included in the group. Strains 310, 311, 304 and 305 form a more compact group with a mean of $93{\cdot}8 \pm 2{\cdot}19$ % ($n = 6$). Mean S-values for the strains 307 and 314 were $84{\cdot}5 \pm 2{\cdot}39$ % and $84{\cdot}5 \pm 1{\cdot}32$% respectively, and they differed significantly from the mean of the group of 4 strains. Thus, the hypothesis that the strains 307 and 314 entered the group was rejected. As typical strains of *M. parafortuitum*, the following 4 strains have therefore been listed: 310 (NCTC 10410), 311 (NCTC 10411), 304 and 305.

Table 9. *S-value table for strains identified as Mycobacterium parafortuitum*

	S-value (%)					
Strain	310	311	304	305	307	314
310	100	.	.	.	.	.
311	98	100	.	.	.	.
304	93	93	100	.	.	.
305	92	93	93	100	.	.
307	86	87	83	82	100	.
314	84	86	83	84	85	100

Six miscellaneous strains, *Mycobacterium marinum*, *M. balnei*, *M. piscium*, *M. ranae* and *M. platypoecilus*, which were in the past classified as different species, formed a homogeneous group, the mean S-value for the group being $92{\cdot}5 \pm 2{\cdot}58$ % ($n = 15$). The correct name of this group seems to be *M. marinum* Aronson.

Description of species of rapidly growing mycobacteria

Characters of the species are shown in Table 10. As has been stated elsewhere (Tsukamura, 1965*d*), the pattern of utilization of nitrogen compounds as sole (simultaneous) nitrogen and carbon source seemed to be very useful for differentiation of rapid-growing mycobacteria. It was noticed, however, that the ability to utilize an amide as sole nitrogen and carbon source for growth is not always correlated with the amidase activity corresponding to the amide. For example, *Mycobacterium phlei* does not possess detectable acetamidase, but is able to utilize acetamide as sole nitrogen and carbon source. On the other hand, *M. parafortuitum* has acetamidase, but does not utilize acetamide as sole nitrogen and carbon source. However both organisms utilize acetate and ammonia as sole carbon and nitrogen sources. The explanation for the discrepancies is not yet known.

General view of the relationship among mycobacterial species

An S-value table was prepared by sorting one to three representative strains of both slow- and fast-growing species (Fig. 3). The genus *Mycobacterium* seemed to be separated into two large groups; one, A, consisting of *M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. marinum* (*M. balnei*, *M. ranae*, *M. piscium* and *M. platypoecilus*), *M. avium*, *M. aquae*, *M. terrae*, *M. thermoresistibile* and strains N100616 and Tomida; another, B, consisting of *M. phlei*, *M. fortuitum*, *M. aurum*, *M. parafortuitum* and *M. smegmatis*.

As seen in the figure, *Mycobacterium thermoresistibile* and strains N100616 and Tomida seem to be intermediate types between the two large groups. *M. marinum* should be placed in the former group.

Strains of group A grow slowly, are not tolerant to 0·1 % picric acid, do not utilize L-glutamate as sole nitrogen and carbon source, and do not utilize fumarate, malate and succinate as sole carbon source. On the other hand, strains of group B show the opposite features.

The intermediate form *Mycobacterium thermoresistibile* is tolerant to 0·1 % picric acid and utilize L-glutamate as sole nitrogen and carbon source, and even fumarate as sole carbon source.

Table 10. *Characters of rapidly growing mycobacteria*

Characters in which all test strains gave a negative reaction are omitted from table.

Species	*M. marinum*	*M. thermo-resistibile*	*M. phlei*	*M. fortuitum*	*M. aurum*	*M. para-fortuitum*	*M. smegmatis*
Typical strain and group ...	B 916 (6)	316 (7)	SN 106 (8)	334 (10, a)	358 (9)	310 (11)	SN 2 (12)
Character†							
Colony morphology	Rough	Smooth	Rough*	Smooth	Smooth	Smooth	Smooth*
Pigmentation	+*	−	±	−	+	±	−
Photochromogeneity	−*	−	−	−	−	−	−
Growth rate	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid
Catalase	+	+	+	+	+	+	+
$NaNO_3$ reduction	+	+	+	+	−	+	+
Third-day-arylsulphatase	−	−	−	+	−	−	−
Second-week-arylsulphatase	+*	−	+	+	+	+	+
Salicylate degradation	−	−	−	+	−	−	−
PAS degradation	−	−	−	+	−	−	−
NH_2CH resistance	−	−	−	+	−	−	−
Picric acid tolerance (0·1%)	−	+	+	−	−	+	+
Picric acid tolerance (0·2%)	−	+	−	−	−	+	+
Growth at 28°	+	−*	+	+	−	+	+
Growth at 37°	+*	+	+	+	+	+	+
Growth at 45°	−	+	+	−	−	+	+
Growth at 52°	−	+	+	−	−	−	−
Acetamidase	−	−	−	+	−*	+	+*
Benzamidase	−	−	−	−	−	−	+
Urease	+*	+	+	+	+	+	+
Isonicotinamidase	−	−	−	−	−	−	+
Nicotinamidase	−	+*	+	+*	+	+	+
Pyrazinamidase	−	+*	+	+*	+	+	+
Allantoinase	+*	−	−	+	−*	+	−
Succinamidase	−	−	−	−	−	−	+
Utilization of organic acids as sole carbon source:							
Acetate	−*	+	+	+*	+	+	+
Citrate	−	−	−*	+*	−*	+	+
Succinate	−	±	+	+	+	+	+
Malate	−	±	+	+	+	+	+
Pyruvate	−	+	+	+	+	+*	+
Benzoate	−	−	−	−	−	−	+*
Malonate	−	−	+	−	−	+	+
Fumarate	−	+	+	+	+	+	+
Acid from:							
Glucose	−	−	−*	+	+	+	+
Mannose	−	−	−*	+	+	+	+
Galactose	−	−	−	−	−	−	+
Arabinose	−	−	−*	−	−	+	+
Xylose	−	−	−	−	−	+	+
Rhamnose	−	−	−	−	−	−	+
Trehalose	−	−	−	−*	+	+	+
Inositol	−	−	−	−	−*	+	−
Mannitol	−	−	−*	−*	−*	+	−
Sorbitol	−	−	−*	−	−	−	−
Utilization of carbohydrates as sole carbon source:							
Glycerol	+	+	+	+	+	+	+
Glucose	−	−*	+	+	+	+	+
Fructose	−	−	+	+	+	+	+
Sucrose	−	−	−*	−*	+	+*	+
Mannose	−	−	+	+	+	+	+
Galactose	−	−	+	−*	−	+	+
Arabinose	−	−	+*	−*	−	−	+
Xylose	−	−	−*	−*	−	+	−
Rhamnose	−	−	−	−*	−	+	+
Trehalose	−	−	+	+	−	+	+
Raffinose	−	−	−	−	+*	−*	−
Inositol	−	−	−	−*	+	+	+
Mannitol	−	−	+	+	+	+	+
Sorbitol	−	−	+	+	−	+	+
Ethanol	−	−	+	+	+	+	+
Propanol	−	−	+	+	+	+	+
Propylene glycol	−	−	−*	−*	+	+	+
1,3-Butylene glycol	−	−	+	+	+	+	+
2,3-Butylene glycol	−	−	+	+	+	−*	+
Utilization of nitrogen compounds as sole nitrogen source:							
L-Glutamate	+	+	+	+	+	+	+
L-Serine	−*	+	−*	+	+	+*	+
L-Methionine	−	−	−*	+	−	−	+
Acetamide	−	−	+	+	+*	+*	+
Benzamide	−	−	−	−	+*	−	+
Urea	+*	+	+	+	+	+*	+
Pyrazinamide	+*	+	+	+	+	+	+
Isonicotinamide	−	−	−	+	−*	−*	+
Nicotinamide	−*	−*	+	−	+	+*	+
Succinamide	+	+	−	+	+	+*	+
Nitrate	+	+	+	+	+	+	+
Nitrite	−	−	+	+	−	−	+
Utilization of nitrogen compounds as sole nitrogen and carbon source:							
L-Glutamate	−*	+	+	+	+	+	+
L-Serine	−	−	−	−*	−*	−	+
Glucosamine	−	−	−*	+	+	+	+
Acetamide	−	−	+	+	−*	−	+
Benzamide	−	−	−	−	−	−	+
Nicotinamide	−	−	−	−	−	−	+*
Monoethanolamine	−	−	−	+	+	−	+
Trimethylene diamine	−	−	+	+	+	+	+

* A few or several strains of the homogeneous group showed different reactions.

† Utilization means utilization for growth.

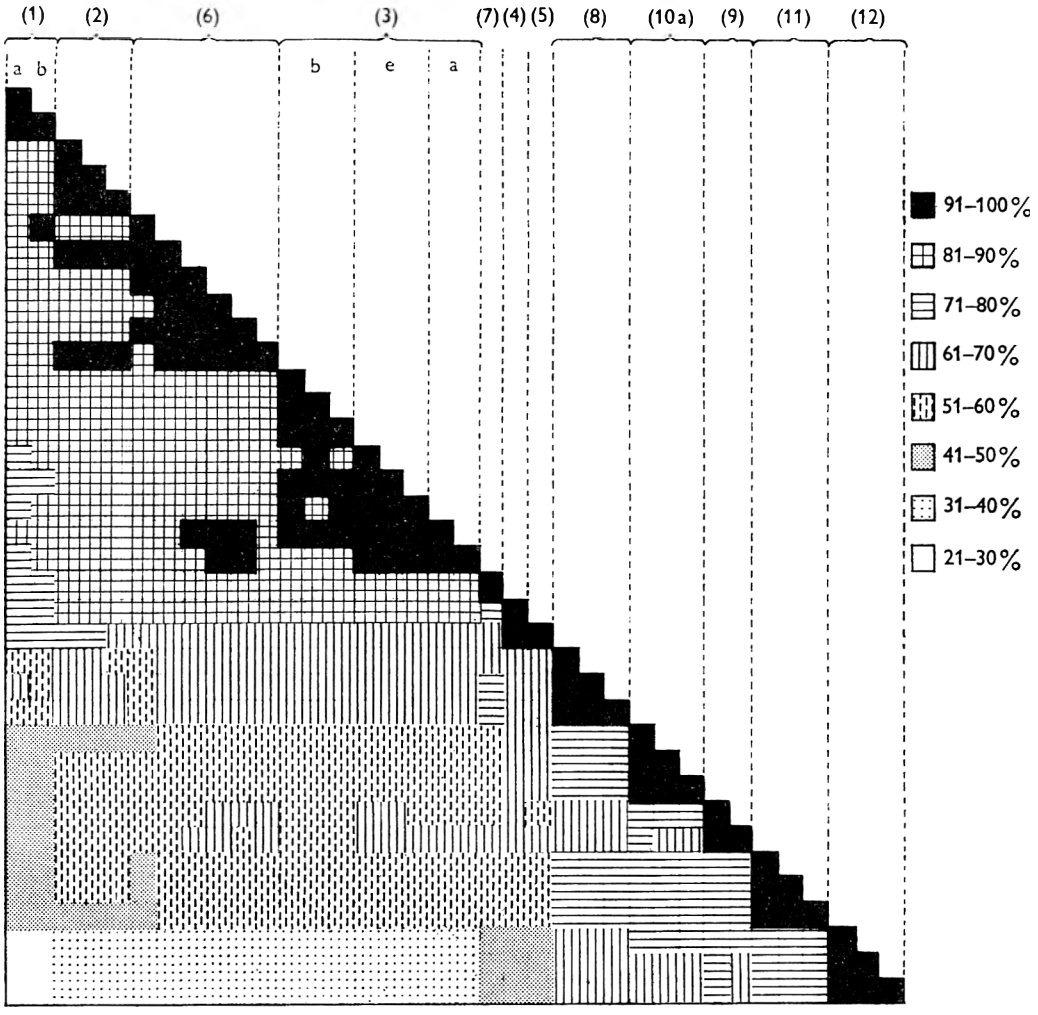

Fig. 3. Diagrammatic representation of the S-value table of both fast- and slow-growing mycobacteria. The groups are numbered as in Figs. 1 and 2.

From left to right: (1a) *Mycobacterium tuberculosis* H37Rv, (1b) *M. bovis* D-4; (2) *M. kansasii* Forbes-84, Bostrum-D-35, Nagai; (6) *M. platypoecilus* Ross, *M. balnei* B916, B193, *M. marinum* Ross, *M. ranae* L-17, *M. piscium* Ross; (3, b) *M. avium* 3717, 4121, Kirchberg; (3e) *M. aquae* Okubo, Aruga, Matsumoto; (3,a) *M. terrae* 317 (NCTC 10424), 318; (7) *M. thermoresistibile* 316 (NCTC 10409); (4) non-photochromogens N100616 (NCTC 10425); (5) non-photochromogen Tomida (NCTC 10428); (8) *M. phlei* SN103, SN106, SN108; (10,a) *M. fortuitum* 335, 334, 330; (9) *M. aurum* 303 (NCTC 10439), 309 (NCTC 10440); (11) *M. parafortuitum* 310 (NCTC 10410), 311 (NCTC 10411), 304; (12) *M. smegmatis* SN2, SN5, SN6.

In this figure, *M. avium*, *M. aquae* and *M. terrae* form one group (group 3). Although these three species form one large group, they are divided into subgroups if the diagrammatic representation is done more finely (at 5% levels; see Fig. 1).

An ideal taxonomy would be based on the similarity of the DNA structure or the gene structure. From this point of view, it is desirable that the characters to be used for the classification show 'one gene-one character' correspondence. If this is the case, all characters would be equivalent. There is no reason for supposing some characters (for example, immunological features) to be more valuable than others. As has been stated by Sneath (1957*a*) only when some characters are associated with several or many other characters, would they be regarded as more important ones.

It should always be remembered that we observe only a small sample of numerous characters. It is most important that the characters to be used for the classification are selected at random. Even if we produced a number of strains (that differed from a known species in several characters) by successive selections, such strains should not be regarded as having changed to another species. In such a case, selection would have been done deliberately to obtain certain characters. These selected characters should not be used as characters for the classification. Even if we observed several differences with respect to the selected characters, it is probable that the organisms have many similar features in characters not tested. Only when the organisms prove to differ significantly from a known species in respect of characters selected at random, should they be regarded as another species. Classification is a matter of statistics and probability.

The following strains are deposited in the National Collection of Type Cultures as type strains: (1) *Mycobacterium terrae* 317 (NCTC 10424); (2) *M. thermoresistibile* 316 (NCTC 10409); (3) *M. aurum* 358 (NCTC 10437), 312 (NCTC 10438), 303 (NCTC 10439) and 309 (NCTC 10440); (4) *M. parafortuitum* 310 (NCTC 10410) and 311 (NCTC 10411).

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Bacterial Survival in Suspension in Polyethylene Glycol Solutions

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SUMMARY

The % survivals of *Escherichia coli* (B, Jepp, commune), *Serratia marcescens* (UK 8) and *Aerobacter aerogenes* (Hinshelwood), in the presence of polyethylene glycol were studied as a function of molecular weight and % polyethylene glycol. Polyethylene glycol is shown to cause a loss of viability; experiments are described which suggest loci for its action.

INTRODUCTION

During an investigation of the protective action of solutes on the survival of bacteria as aerosols it was observed that polyethylene glycol (PEG) was toxic (Cox, 1965). The present paper reports *in vitro* experiments which were designed to elucidate this toxic action on *Escherichia coli* (B, Jepp, commune), *Serratia marcescens* (UK 8) and *Aerobacter aerogenes* (Hinshelwood).

METHODS

Organisms. Cultures of *Escherichia coli* (B, Jepp, commune), *Serratia marcescens* (UK 8) and *Aerobacter aerogenes* (Hinshelwood), were grown in a 2 % tryptone medium (pH 7·6) for 16 hr at 37° by a shake flask technique. Each day suspensions of organisms were prepared by centrifugation from the culture fluid and resuspension in phosphate buffer (pH 7·6), unless stated otherwise.

Procedure. The bacterial suspensions were serially diluted in phosphate buffer (pH 7·6) to give the chosen concentration, usually 10^5 colony-forming units/ml.; 0·5 ml. of this suspension was added to 4·5 ml. of buffer + polyethylene glycol (PEG) (supplied by L. Light and Co. Ltd., Colnbrook) mixtures of differing PEG compositions, % (w/v), and were allowed to equilibrate. After a standard time, 1 ml. samples were removed and added to 9 ml. buffer. The degree of survival in each sample was determined by surface drop plates (Miles & Misra, 1938) on 1 % tryptone agar or 1 % peptone agar in Petri dishes. After overnight incubation at 37° the colonies were counted on six plates/sample.

So that more information could be obtained, this procedure was modified to allow a study of the ability of *Escherichia coli* B to reproduce phage T7, when these *E. coli* organisms were treated with PEG. The technique with phage in aerosol studies was reported previously (Cox & Baldwin, 1964, 1966). The bacteria, after centrifugation, were resuspended to give a concentration of 3×10^9/ml. in tryptic meat broth at 37°

containing 0·004 M-potassium cyanide added to prevent phage development. After 2 min, to allow the cyanide to take effect, phage T7 was added and the mixture maintained at 37° for a further period of 10 min. to allow adsorption to occur. After centrifugation, which left unadsorbed phage in the supernatant fluid, the deposited bacteria were resuspended at room temperature in broth containing 0·004 M-cyanide, and the centrifugation and resuspension steps were repeated. Dilution in phosphate buffer gave 10^5 phage T7 infected *E. coli* B organisms/ml. suspension. The procedure then followed that detailed above for non-infected bacteria except that the ability to reproduce phage was determined by plaque formation in 0·5 % soft agar in tryptic meat broth layered on 1 % tryptone agar in Petri dishes. As a check on the handling procedure the experiments with infected bacteria were conducted in the presence of *Bacillus subtilis* var. *niger* spores, at a concentration similar to that of the *E. coli* B. In this manner the % plaque formation (and the % colony formation for a control of non-infected *E. coli* B) could be related to the initial bacterial concentrations allowing for dilution, or to the number of *B. subtilis* var. *niger* colonies, for the sample under consideration.

The estimates of colony formation of *Escherichia coli* B, and for plaque formation by phage T7, initially were conducted with tryptone plates containing crystal violet which inhibited the growth of *Bacillus subtilis*. This procedure, however, also partially inhibited the growth of *E. coli* B so crystal violet was omitted. The two bacteria were readily differentiated on the control plates, since *E. coli* B formed white colonies and *B. subtilis* var. *niger* formed orange colonies; the presence of *B. subtilis* var. *niger* did not interfere with phage plaque formation.

RESULTS

Time of contact between polyethylene glycol (PEG) and bacteria

Results with each of the five organisms in the presence of PEG of molecular weights of 200 and 15,000 at 50 % PEG and 40 % PEG in phosphate buffer (pH 7·6) respectively, were obtained. The curves approximated to a two-stage loss of viability in that an initial rapid decay (0–1 min.) occurred, followed by a much slower decay (1–60 min.). A convenient standard equilibration time of 30 min. was adopted, unless stated otherwise.

Age of bacterial culture

The influence of age of the bacterial culture was investigated in detail for only the Jepp strain of *Escherichia coli*. It was found that there was no significant difference in the behaviour of cultures of various ages from 5 hr to 8 days, timed from the end of the 16-hr growth period. This enabled cultures to be prepared weekly. Less detailed studies suggested that the same was true for the other four organisms.

Temperature for equilibration

The % survival of a suspension of *Escherichia coli* Jepp in PEG (1540) and PEG (9000) was determined at 4°, 10°, 22°, 30° and 37° as a function of % PEG made up in phosphate buffer (pH 7·6). The effects were least at 4° and greatest at 22°. As a consequence the experimentally convenient temperature of the room (21°) was used for later experiments.

Bacterial survival as a function of molecular weight and % PEG

The % survival (mean of two determinations) of each of the five organisms was studied as a function of % PEG of molecular weights 200, 300, 400, 600, 1540, 9000, 15,000 made up in phosphate buffer (pH 7·6) Figures 1–4 illustrate the survival observed for PEG 200, 600, 1540, 15,000, respectively, The data were plotted as a function of % PEG, rather than moles/l., because the molecular weight dependence

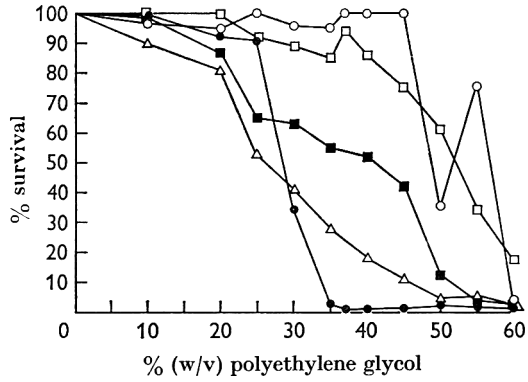

Fig. 1

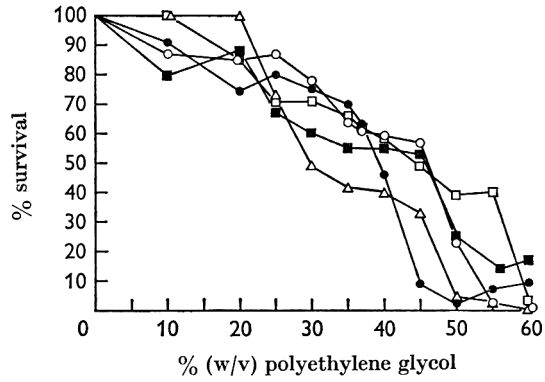

Fig. 2

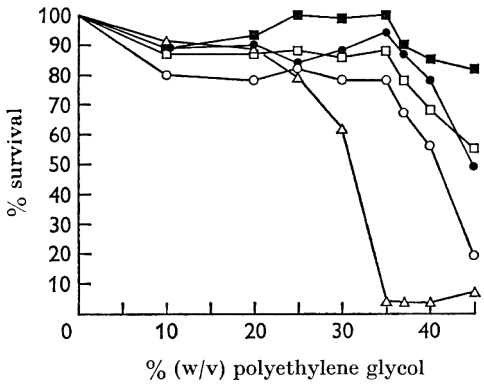

Fig. 3

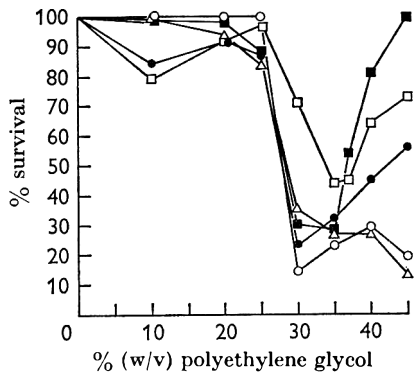

Fig. 4

Figs. 1–4. The survival of bacteria as a function of polyethylene glycol (of different molecular weights) + phosphate buffer mixtures at 21°. Fig. 1. Molecular weight 200. Fig. 2. Molecular weight 600. Fig. 3. Molecular weight 1540. Fig. 4. Molecular weight 15,000. ○, *Escherichia coli* B; Δ, *E. coli* Jepp; □, *E. coli* commune; ■, *Serratia marcescens*; ●, *Aerobacter aerogenes*.

was less apparent and virtually disappeared with *Escherichia coli* Jepp. Results obtained with PEG (300) were similar to those obtained with PEG (200) ,while with PEG (400) the survivals were intermediate between those using PEG 200 and 600. For the polymer fractions of 9000 and 15,000, the pattern for the five organisms was similar in that the survival curves all showed a break at about 25% PEG, followed by a marked change of slope at about 30% PEG. For lower molecular weight fractions the patterns were less consistent.

Effects of bacterial concentration

Escherichia coli (Jepp, B) was studied for the effect of the bacterial concentration. The results for concentrations of 1×10^9 and 1×10^4 bacteria/ml. were similar and showed no marked effect of concentration over this range.

Clumping behaviour

Suspensions at a bacterial concentration of 1×10^9 bacteria/ml. were studied to determine whether the clumping behaviour of the bacteria was modified in the presence of PEG (15,000). With *Escherichia coli* B a stable dispersion formed at 30 % PEG, corresponding to the minimum in the survival curve for PEG (15,000) (Fig. 4); at PEG concentrations higher and lower than 30 %, flocculation or aggregation (LaMer & Healy, 1963) occurred, i.e. the state of clumping and % survival appeared to be related. In PEG (200) a similar relationship was observed between clumping behaviour and % survival.

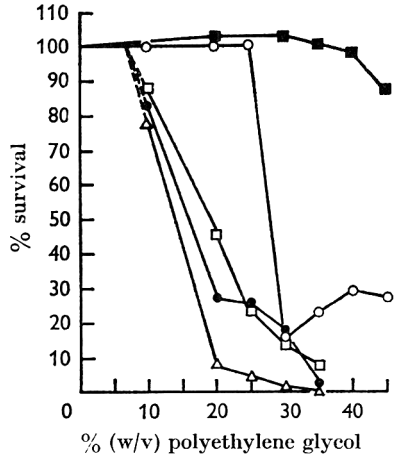

Fig. 5

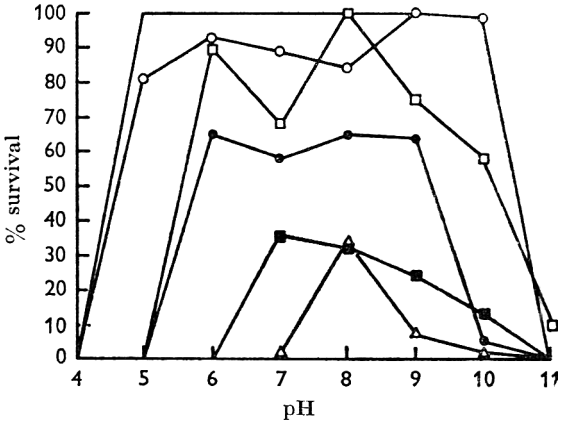

Fig. 6

Fig. 5. The survival of *Escherichia coli* B in polyethylene glycol (molecular weight 15,000) at 21°. ○, Polyethylene glycol in phosphate buffer; organisms collected in phosphate buffer. ■, Polyethylene glycol in tryptic meat broth; organisms collected in tryptic meat broth. Δ, Polyethylene glycol in distilled water; organisms collected in distilled water. ●, Polyethylene glycol in distilled water; organisms collected in phosphate buffer. □, Polyethylene glycol in distilled water; organisms collected in tryptic meat broth.

Fig. 6. Survival of *Escherichia coli* B in polyethylene glycol + phosphate buffer mixtures as a function of polyethylene glycol (15,000) concentration and pH value. —, 0 % PEG; ○, 10 % PEG; □, 20 % PEG; ●, 25 % PEG; ■, 30 % PEG; Δ, 40 % PEG.

Competitive action of other solutes with PEG

Protection of *Escherichia coli* Jepp by sucrose in the presence of PEG (1540) at 30 % PEG, but not at 60 % PEG, was reported previously (Cox, 1965). As an extension of these observations, but with *E. coli* B, solutions of PEG (15,000) in distilled water, phosphate buffer and tryptic meat broth, were studied (Fig. 5). The concentration at which PEG became toxic was dependent upon the presence of other

solutes. The substantial protection afforded by tryptic meat broth was also markedly apparent for 0·8 % tryptone (or greater), provided that the tryptone was present during contact of the bacteria with PEG (15,000). Contact of bacteria with tryptic meat broth after PEG treatment did not completely prevent the PEG stress. The degree of survival was dependent upon the diluting fluid used for the assay of survival (Fig. 5). The protective action of tryptone, even at 2 %, was not marked for PEG (200).

Influence of pH value

One of the differences between distilled water, phosphate buffer and tryptic meat broth was the pH value of these liquids. Figure 6 gives % survival of *Escherichia coli* B as a function of pH value over the range pH 4–11 and of % PEG (15,000); it shows that the toxic action of PEG (15,000) was least in the region of pH 8. The distilled water used for the data of Fig. 5 showed a value of pH 5; the curve at pH 5 in the presence of buffer closely follows the curve for distilled water. The addition to distilled water of a small quantity of buffer salt (0·001 M) to give pH 8 resulted in behaviour close to that at pH 8 in Fig. 6, i.e. the apparently greater toxicity of PEG (15,000) in distilled water was due to the pH value rather than to the low ionic strength.

Table 1. *Survival in polyethylene glycol of Escherichia coli* B *and Escherichia coli* B + *phage T7 complex*

	No. of determinations	Arithmetic mean	Standard deviation
% survival of control	7	26·4	3·4
% lysis*	5	94·2	8·5
% lysis†	4	56·7	3·8

* On non-dyed agar plates. † On agar plates + crystal violet.

The Escherichia coli B + *phage T7 complex*

Escherichia coli B infected with phage T7 in an undeveloped state were prepared by the method described. The results of subjecting these bacteria and the non-infected controls to 30 % PEG (15,000) for 10 min. are given in Table 1. The data included results for culture ages of from 16 hr to 7 days, and for phage:bacteria ratios of 1·2, 1·5, 3, 7 and 30:1. It is concluded that the action of PEG (15,000) upon *E. coli* B, did not affect those reactions involved in phage reproduction; however, the combined action of PEG (15,000) and crystal violet partially inhibited phage growth and lysis. In the terms used previously (Cox & Baldwin, 1964, 1966) the action of PEG (15,000) upon *E. coli* B is to prevent *E. coli* B reproduction.

The result of subjecting the *Escherichia coli* B infected with phage T7 and the non-infected controls to PEG (200) at 60 % for 10 min. was that lysis and survival values of the order of 12 % were obtained. This suggests that PEG (200) influenced the *E. coli* B phage productive capacity (Cox & Baldwin, 1964, 1966). i.e. the action of high and low molecular weight PEG would seem to be at different loci of action.

DISCUSSION

The use of phage showed that the reproductive capacity of *Escherichia coli* B was impaired by polyethylene glycol (PEG; 15,000). Since the latter penetrates the cell wall very slowly, if at all, and does not penetrate the cytoplasm, both the cell wall and cell division seem to be implicated as at least one locus of action of PEG (15,000). PEG of molecular weight 200, being able to pass through the cell wall but not the cytoplasmic membrane, may be able to operate at a second locus of action concerned with *E. coli* B phage productive capacity, but which cannot be reached and preserved by tryptone. The clumping behaviour of bacteria in PEG (15,000 and 200) also suggests that changes in the cell surface occur, since clumping behaviour depends upon the interactions of cell-wall surfaces. It is perhaps a coincidence that the stable suspension forms at the % PEG, which gives a minimum in the survival curve (Fig. 1, 4), although the action of tryptic meat broth is both to prevent the action of PEG (15,000) and also to prevent dispersion.

Since the data exhibit a more regular pattern when related to a % rather than a molar concentration scale, the concentration of the repeat unit of the PEG may be the important factor. Percentage PEG values are approximately converted to moles repeat unit/l. by multiplying the % value by a factor of 0·22. Of the five organisms, *Escherichia coli* Jepp showed the least dependence upon molecular weight, although any such relationship may be fortuitous, since it was shown that PEG of low and high molecular weight had different actions. Other substances (from the Dow Chemical Co. Michigan) such as polypropylene glycol (400); Ambiflo H7, H52, H438 (300, 1000, 3500 respectively) a dialkoxyether of polyoxyalkylene glycol; Hyprin GP30 (266) a hydroxypropyl glycerol; polyglycol 15-200 (2600) $CH_2OR_nH.CHOR_nH.CH_2OR_nH$, where R is C_2H_4O—C_3H_6O, behaved somewhat like PEG. Thus the phenomenon does not appear to be specific with regard to the nature of the agent.

The action of PEG may involve its adsorption; it was suggested (Cox, 1965) that the wetting properties of the bacterial surface might be effected. However, since phage T7 reproduction was not impaired by PEG (15,000) a change of wetting properties is not a likely explanation. Polymer phase separations in which a mixture of polymers forms a single homogeneous phase or separates into phases depending upon concentration, etc., may be pertinent. Such systems have been studied by Dervichian (1954), by Hyde, Langbridge & Lawrence (1954), by Albertsson (1960) and by Polson *et al.* (1964). Polson *et al.* found that with mixtures of protein + polyvinyl alcohol type, precipitation and denaturation of protein occurred, whereas mixtures of protein + dextran or + PEG resulted in phase separation without denaturation. The protein precipitation was dependent upon protein concentration, temperature, pH value and PEG concentration; this behaviour is similar to that described in the present paper.

The protective action of sucrose and perhaps protein is consistent with phase separation since multiplicity of hydroxyl groups produces marked increases in solubility (Hyde *et al.* 1954). The effect of pH value is consistent also with phase separation of an ionic polymer, since the solubility of such a polymer is related to its charge and this will change with the pH value. An understanding of the influence of temperature in terms of phase separation would require knowledge of the phase

diagram, since temperature changes can cause dramatic effects (Albertsson, 1960), while the marked recovery in viability for PEG of high molecular weight (Fig. 4) is not contrary to phase separation (Hyde *et al.* 1954). It therefore seems possible that the action of PEG might be explained through the phase separation of groups which normally would be in solution and attached to the cell membranes. Such an action would then have to cause a detrimental change in the structure of the cell membranes.

The author thanks Mr I. H. Silver for his interest in this work and thanks Mr M. C. Aireton for technical assistance.

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The Survival of *Escherichia coli* in Nitrogen Atmospheres under Changing Conditions of Relative Humidity

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SUMMARY

The consequence of relative humidity (RH) changes before collection of bacterial aerosols are described; it is shown how *Escherichia coli* (B, commune), but not *E. coli* Jepp, gave good survival over the range 100–20 % RH provided that the following conditions applied: (1) a pure nitrogen atmosphere; (ii) a protective agent in the spray fluid; (iii) sucrose in the collecting fluid; (iv) an RH change to 100 % or to a nominal 30 % (depending upon the experimental conditions) before collection of the aerosol. Differences in survival of *E. coli* commune sprayed from suspension in solutions of raffinose into an atmosphere of nitrogen which were previously attributed to the effect of raffinose concentration in the spray fluid were found to be caused by the use of variant organisms.

INTRODUCTION

Previous papers (Cox, 1966; Cox & Baldwin, 1966) showed that the survival of *Escherichia coli* as an aerosol was greatly enhanced at low relative humidity (RH) when nitrogen replaced air. In the high RH range, regions of marked aerosol instability occurred for the bacteria sprayed from suspension in distilled water, while the addition of a protective agent altered both the size and the RH value of the minima in plots of survival versus RH. The possibility was considered that a shift in RH from high (unstable) to low (stable) values before collection might indicate whether the death of *E. coli* occurred in the aerosol at high humidity or as a result of the collection technique. The present paper gives the results obtained and shows the extent to which decrease of survival values may be attributed to the method of sampling. In previous work (Cox, 1966) the effect of the protective agent appeared markedly to depend on its concentration for *E. coli* commune, whereas for *E. coli* (B, Jepp) the effect was much less marked. The possibility that the apparent effect of concentration of protective agent might have been associated with a change of stock culture was also examined, even though different stock cultures gave suspensions having very similar survival characteristics under the conditions of test when sprayed from distilled water.

METHODS

The techniques used were as previously reported (Cox, 1966), with the addition that chambers of 2 l. capacity as in Fig. 1 were used to bring about the changes in the RH shifts. Partial filling of the chambers and wetting of the muslins, with

distilled water or with saturated lithium chloride ($LiCl_2$) solution, enabled RH values of approximately 100 and 30 %, respectively, to be achieved. The value of 30 % RH is in doubt since this was the value achieved when 12 l. wet nitrogen/min. flowed through the chamber. In equilibrium conditions an RH value of 12 % should be achieved; since the aerosol cloud remained static in the chambers for 30 sec. before collection the true value of RH will have been between these two extreme values. This RH range corresponded to a plateau region of good survival for *Escherichia coli* in nitrogen (Cox, 1966). The chambers were maintained under an atmosphere of nitrogen (> 99·9 %) at all times.

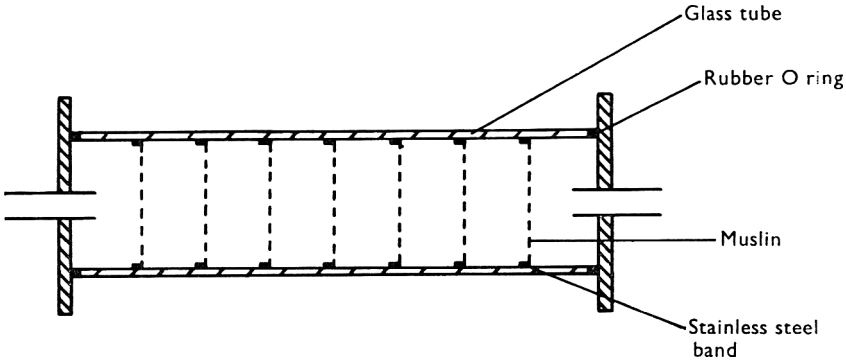


Fig. 1. Chamber of 2 l. capacity used for making relative humidity changes.

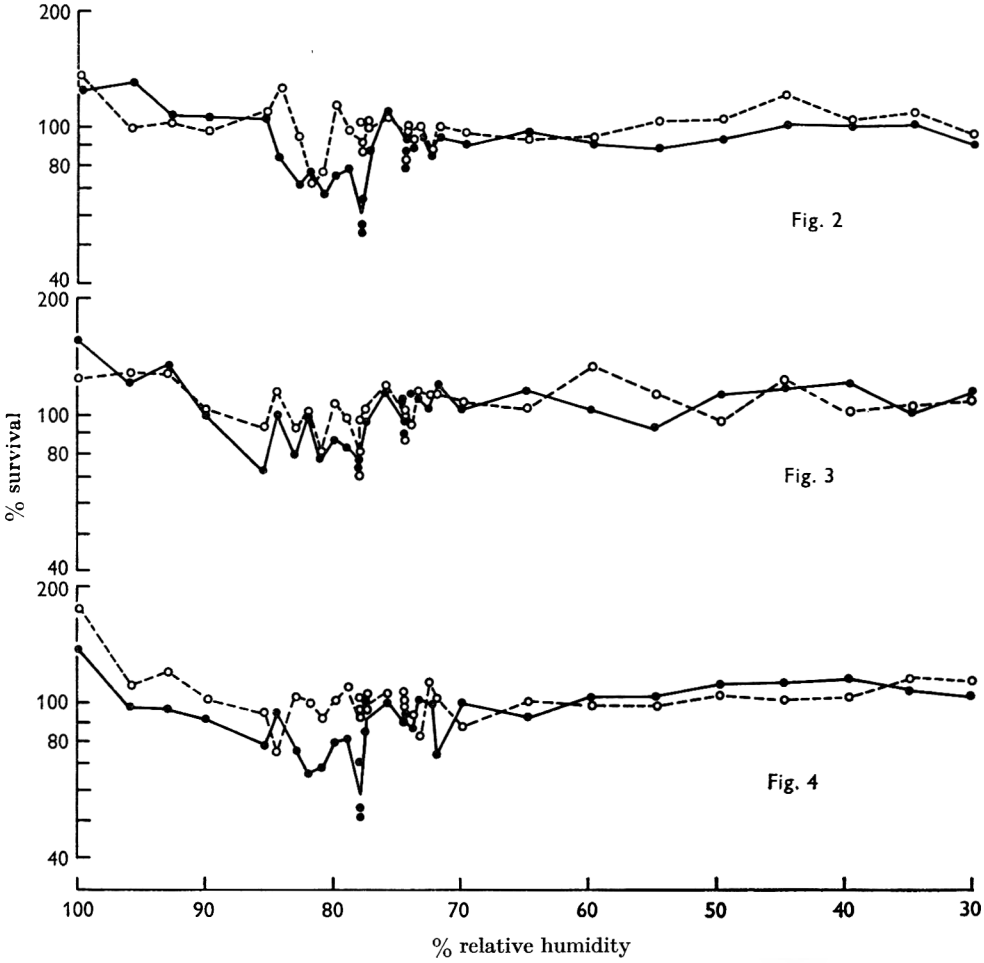
RESULTS

Figure 2 shows the survival of *Escherichia coli* commune 2 when sprayed into nitrogen from 0·13 M-raffinose in distilled water (added to the bacteria immediately before spraying), at an aerosol age of 25 min. for collection by raised impinger (May & Harper, 1957) into phosphate buffer and into M-sucrose + phosphate buffer. Similar to previous results (Cox, 1966) collection in phosphate buffer was marked by instability within the range 72–86 % RH, whereas collection in M-sucrose + phosphate buffer revealed only one clear minimum at RH 81–82 %. However, with this variant of *E. coli* commune the extent of the instability was less than that previously observed.

Figures 3 and 4 show the effect of changing the RH of the nitrogen atmosphere either to 100 % or to 30 % before collecting the aerosol. In general phosphate buffer and M-sucrose + phosphate buffer gave similar results outside the unstable zone of 72–86 % RH. In the unstable zone phosphate buffer remained inferior to M-sucrose + phosphate buffer when the shift was to 30 % RH, but the difference was smaller when the shift was to RH 100 %. Suspensions prepared from a third stock culture of *Escherichia coli* commune gave similar results under static and changing RH conditions.

Table 1 includes results for three strains of *Escherichia coli* when sprayed into nitrogen from distilled water and from 0·13 M-raffinose in distilled water (added to the bacteria immediately before spraying) at an aerosol age of 25 min. The test RH values were those corresponding to minima in the plots of survival versus RH, or were at 30 % RH where the stability was much greater. The data agree with that

previously published (Cox, 1966) and show that when sprayed from distilled water RH changes before collection did not markedly effect the survival of *E. coli* (B, Jepp) stored at RH 85 %. However, for *E. coli* commune 2 at RH 81 % a change to 100 %, but not to 30 %, produced a large increase in the survival in both collecting fluids. When the organisms were sprayed from raffinose the pattern of results was



Figs. 2–4. Survival of *Escherichia coli* commune 2 in nitrogen, sprayed from 0·13 M-raffinose at an aerosol age of 25 min. ●, Collection in phosphate buffer; ○, collection in M-sucrose + phosphate buffer. Fig. 2. At the storage relative humidity. Fig. 3. At the storage relative humidity followed by a change in relative humidity to 100 % before collection. Fig. 4. At the storage relative humidity followed by a change in relative humidity to 30 % before collection.

more complex. A change from RH 83 to 100 %, but not to 30 %, eliminated the difference between collection in the two fluids for *E. coli* B and gave 100 % survival. For *E. coli* Jepp neither a change to RH 100 % nor to 30 % gave enhanced survival for either collecting fluid. *E. coli* commune 2 gave 100% survival when the RH changed to 100 %, but the change to 30 % resulted in survival being dependent upon collecting fluid.

For aerosols stored at a controlled RH value of 30 % no really marked effect of changes of RH occurred for *Escherichia coli* B and commune 2. However, for *E. coli* Jepp, as might be expected from its instability at high RH (Cox, 1966) a change to RH 100 % caused a rapid decrease of survival. The results for *E. coli* Jepp also showed the previously reported (Cox, 1966) poorer survival in M-sucrose + phosphate buffer than in phosphate buffer alone which occurred at low RH, but not at high RH; these results contrast with those obtained with *E. coli* B and commune at low RH.

Table 1. *The influence on stored aerosols of Escherichia coli in nitrogen at 26·5° of relative humidity changes before collection*

			Percentage survival					
			At storage RH		At 100 % RH		At 30 % RH	
Strain of *E. coli*	Spray fluid	Percentage RH for storage	PB*	PBS*	PB	PBS	PB	PBS
B	D†	85	0·5	0	0	2	0	0
		30	80	77	65	35	85	61
Jepp	D	85	0	0·5	0·9	0·4	0	0·2
		30	50	7	9	0·9	42	3
commune 2	D	81	3·3	6	18	21	1	4
		30	84	82	83	70	82	90
B	R‡	83	39	102	100	100	38	100
		30	110	113	124	127	104	105
Jepp	R	82	2·5	15	1	2	5	15
		30	97	22	64	28	94	25
commune 2	R	82	77	73	99	100	66	108
		30	94	100	115	114	104	117

* PB = phosphate buffer collecting fluid; PBS = M-sucrose + phosphate buffer collecting fluid.
† D = distilled water. ‡ R = 0·13 M-raffinose in distilled water added immediately prior to spraying.

DISCUSSION

The difference in behaviour of *Escherichia coli* commune when sprayed from 0·13 and 0·3 M-raffinose, located outside the cell wall, was previously attributed (Cox, 1966) to the difference in raffinose concentration. From the results of the experiments given in the present paper it now seems to have been caused by the use of stock cultures which contained different variants of *E. coli* commune. These variants were not distinguishable under the conditions of test in aerosol behaviour when sprayed as a suspension in distilled water, but were distinguishable in the presence of raffinose. These findings are perhaps an indication of the subtlety of the action of raffinose as a protective agent. As far as can be judged, each stock culture gave rise to suspensions of bacteria showing consistent behaviour, and it has been possible to achieve similar results for suspensions arising from different stock cultures. The three stock cultures all gave suspensions of bacteria which, when sprayed from raffinose, showed the same trends, namely, good survival at high and low RH and poorer survival in the RH 72–86 % region, with minima at 74 and 78 % (and at 81 % RH for one culture, others not tested at this RH). Hence it was the degree of response rather than the response itself which was dependent upon the stock culture

used. This difference in stock culture so far has not been found for *E. coli* B and Jepp. Dr G. J. Harper (personal communication) has found that *E. coli* commune gives variants.

Previous work (Cox, 1966) showed that part of the survival decrease of *Escherichia coli* occurred during or following collection by raised impinger (May & Harper, 1957). Transfer from the unstable relative humidity zone to a stable zone before collection should indicate the proportions in which death occurred in the aerosol and in the impinger. The stable state most easily obtained for *E. coli* (B, Jepp, commune) was in the region of RH 30 %. A second region near RH 100 % was reasonably stable for bacteria protected with raffinose, but not when sprayed from distilled water alone. The results (Table 1) show that a shift to RH 100 % usually increased the survival of *E. coli* B, commune but not of *E. coli* Jepp. A shift to RH 30 % had no significant effect for *E. coli* B and Jepp with either spray fluid; however, for *E. coli* commune sprayed from raffinose, but not distilled water, the shift in RH was beneficial.

Other slight anomalies remain. Since little difference had been found between phosphate buffer and M-sucrose + phosphate buffer as collecting fluids at low RH for *Escherichia coli* B and commune, then if an aerosol stored at high RH were changed to low RH before collection, it might be expected that the collection difference between phosphate buffer and M-sucrose + phosphate buffer observed at high RH might disappear, but in fact it did not. *Escherichia coli* Jepp at low RH had shown poorer survival for collection in M-sucrose + phosphate buffer than in phosphate buffer, and the converse was true at high RH (Cox, 1966). Hence, if an aerosol were stored at low RH, then transferred to RH 100 % before collection it might be expected that M-sucrose + phosphate buffer would be a better collecting fluid than phosphate buffer alone. However, it was not. The collection difference would therefore not seem to be caused simply by water activity differences. Dramatic effects, both of a beneficial and of a detrimental nature, were reported by Cox (1965) and by Silver (1965) for shifts in RH to 100 % before collection.

Provided that raffinose located outside the cell wall is used to protect *Escherichia coli* B and commune and that the necessary collecting fluid is used together with an RH change to either 100 % or 30 %, depending upon the conditions, then death at or following collection can be eliminated and good survival can be achieved over the range RH 20–100 %. For *E. coli* Jepp these procedures are not sufficient to overcome the instability in the region of RH 82 %.

The author thanks Mr I. H. Silver for his interest and for much helpful discussion, and thanks Mr M. C. Aireton for technical assistance.

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Automatic Control of Dissolved Oxygen Concentration in Stirred Microbial Cultures

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SUMMARY

Equipment was developed for the automatic control of dissolved oxygen concentration in microbial cultures, by using the Mackereth electrode as a sensing probe. Factors affecting the choice of components are discussed and the performance of the equipment is described during typical batch and continuous culture runs. The equipment provided precise and reliable control over a wide range of dissolved oxygen concentrations, and retained a high degree of stability over long periods of continuous use.

INTRODUCTION

Ever since the inception of aerobic techniques for cultivating micro-organisms there has been a need for quantitatively assessing the concentration of dissolved oxygen in the culture medium. Until relatively recently, the main concern was to ensure that growth was not impaired by an inadequate supply of oxygen. Over the last decade, however, there has been an increasing interest in the response of micro-organisms to conditions of limited oxygen availability. Thus the necessity arose not only for the direct measurement of dissolved oxygen in microbial cultures, but also for the automatic control of this factor.

The implementation of automatic control of dissolved oxygen in culture media has been considerably delayed by the lack of a suitable measuring probe. Such a probe must remain stable over long periods of time without requiring recalibration, must have a linear response to increasing oxygen concentration and must be capable of sterilization. The requirement for stability becomes even more rigorous when the electrode is to be used in continuous processes, which may be required to run for periods of a month or more. It is in long-term stability that most electrodes described to date have proved deficient. An electrode suitable for use with automatic control equipment, however, has recently been described by Mackereth (1964), and a smaller version suitable for laboratory use is now commercially available (Electronic Instruments Ltd., Richmond, Surrey).

Herbert, Phipps & Tempest (1965) briefly described an 'oxystat' for controlling the dissolved oxygen concentration in continuous microbial cultures, by using the Mackereth electrode. The authors stated that reasonably good control was achieved over a wide range of dissolved oxygen concentrations; however, few details of the control equipment and none of the performance were given. Siegell & Gaden (1962) also described equipment for the automatic control of dissolved oxygen concentrations in fermentations; however, again, the performance of the equipment was not treated in detail and results were given only for short batch cultures (about

8 hr) at relatively high dissolved oxygen concentrations (equivalent partial pressure, 0·1 atmosphere). Lengyel & Nyiri (1965) described an automatic aeration system based on measurement of the redox potential of the culture. No experimental results were presented, however, about the calibration of the electrode, or the accuracy and stability of the system. Furthermore, the nature of the relationship between the redox potential and the dissolved oxygen concentration is not clear. The present paper describes the design and performance of equipment for the accurate control of dissolved oxygen concentration in batch cultures and in continuous cultures of micro-organisms.

METHODS

Units. The oxygen content of a gas or liquid can be expressed either as oxygen concentration or as oxygen partial pressure. The partial pressure of oxygen in a liquid is equal to the partial pressure of oxygen in a gas in equilibrium with that liquid, and is related directly to the oxygen concentration as follows:

$$P = fC,$$

where P is the partial pressure or tension of oxygen in the liquid, C is the oxygen concentration in the liquid and f is an activity coefficient. It is important to note that the value of f depends on the nature of the liquid and thus may vary with different media or even with different cultural conditions. Membrane electrodes such as the Mackereth electrode measure the partial pressure of oxygen rather than the concentration (Siegell & Gaden, 1962). The term 'dissolved oxygen tension' will be used throughout this work to denote the partial pressure of oxygen in the liquid phase and will be expressed as mm. Hg. The differences between oxygen concentration and oxygen tension have been further discussed by Kinsey & Bottomley (1963).

Characteristics of the Mackereth oxygen electrode

The Mackereth electrode is a lead–silver galvanic cell sheathed in a protecting membrane. The cell generates a current which is directly proportional to the rate of diffusion of oxygen through the membrane, and hence to the dissolved oxygen tension in the surrounding medium. The membrane is necessary to prevent poisoning of the electrode; however, it is responsible for a time lag between a change of dissolved oxygen tension in the culture and the response of the electrode. Mackereth (1964) estimated the lag at 17·7° to be about 15 sec. for the electrode to register 90 % of a change, and about 90 sec. for a 100 % response. Such a lag in the electrode might introduce some difficulty in automatic control.

Besides long-term stability, perhaps the greatest advantage of the Mackereth electrode is the high sensitivity (electrode current/dissolved oxygen tension). Sheathed with the standard polythene membrane (thickness 0·0025 in.), the electrode produces approximately 2·5 μA/mm. Hg, O_2, in water at 31°; with a fluoroethylenepropylene (FEP) membrane (thickness 0·001 in.) the output is increased to about 5·0 μA/mm. Hg, O_2 (MacLennan & Pirt, 1965). Such currents can be easily measured directly without need for pre-amplification, by measuring the potential drop across a resistor on a self-balancing potentiometric recorder.

Mackereth (1964) found that the response time of the electrode decreased with decreasing resistance in the external circuit. Thus it was desirable to keep this resistance as low as possible, commensurate with the production of an accurately measurable potential drop across it. A full-scale deflexion of 0–1 mV is about the lowest measuring range readily available in industrial grade self-balancing potentiometric recorders. For the polythene sheathed electrode (output about 400 μA at air saturation and 30°) a resistance of about 2·5 ohms would produce such a potential drop, and minimize lag in response.

An extremely important factor influencing the performance of the electrode is the necessity for sufficient turbulence in the culture to ensure that the rate of diffusion of oxygen through the membrane is always the limiting factor. This is essential, since the output from the electrode is directly proportional to this rate of diffusion (Mackereth, 1964). In vigorously stirred fermentor vessels, however, this should not present any problem.

Another desirable characteristic of the electrode is the low residual current at zero oxygen tension (1 % of air saturation current or better).

Process characteristics

The first factor to be considered in relation to the process was the method by which the dissolved oxygen tension in the culture was to be increased or decreased. The rate of solution of oxygen from a gas into culture medium can be described by the following equation (Arnold & Steel, 1958):

$$N_A = K_g.a(P_g - P_e),$$

where: N_A is the rate of oxygen transfer; K_g is the overall oxygen transfer coefficient expressed in terms of a partial pressure driving force; a is the total interfacial area, generally included with the overall mass transfer coefficient as $K_g.a$; P_g is the partial pressure of oxygen in the gas; P_e is the dissolved oxygen tension in the culture medium.

When it is necessary for the controller to change the rate of oxygen transfer N_A, in order to maintain the dissolved oxygen tension in the culture P_e at a predetermined value, then from the above equation the change must be brought about by altering either the mass transfer factor $K_g.a$, or the partial pressure of oxygen in the gas P_g. In a stirred fermentor $K_g.a$ can be varied within limits by changing the stirring rate, and P_g can be varied by changing the oxygen content of the inflowing gas. Although both methods are feasible for controlling dissolved oxygen tension, each method has certain advantages. Control by stirring, as used by Herbert *et al.* (1965), requires only one gas, usually air, whereas the partial pressure method may also require an inert carrier gas such as nitrogen. The stirring method, however, could not be applied to larger-scale equipment because of the extreme difficulty in quickly changing the speeds of large and highly powered motors. Another disadvantage of the stirring method is that the mixing efficiency might vary enormously. As the immediate use for the automatic control equipment was to study the growth of bacteria on hydrocarbon substrates, mixing was a vital factor and hence control by variation of oxygen partial pressure became essential. Such a control could be achieved by automatically controlling the adjustment of a valve which governed the flow of an oxygen-containing gas into a constant flow of an inert carrier gas.

Before the automatic control equipment could be selected, however, there were two basic characteristics of the process which had to be taken into account: load changes and process lag.

A change in the dissolved oxygen tension due to altered conditions in the process constitutes a *load change*. Both the magnitude and the rate of load change must be considered. Such changes may result from changes in the rate of oxygen uptake by the culture. In batch cultures, for example, the oxygen uptake rate increases enormously through the growth period, although these changes do not occur very rapidly. An example of relatively large and rapid load changes is the fluctuation in dissolved oxygen tension observed during the 'steady state' continuous culture of *Klebsiella aerogenes* at 'critical' dissolved oxygen tensions (Harrison & Pirt, 1965). Load changes could also result from a variation in the flow rate or composition of the aerating gas, or from periodic addition of antifoam to the culture. In the former case the changes would probably be relatively small and slow, but the addition of antifoam might cause large and rapid changes in dissolved oxygen tension in the culture.

The *process lag* is the time required for the dissolved oxygen tension to reach a new value when load changes occur. This lag is considerably influenced by the oxygen capacitance of the process, which is defined as the quantity of oxygen necessary to raise the dissolved oxygen tension in the process by 1 mm. Hg, O_2. Although a low process capacitance is desirable in decreasing the process lag, too low a 'demand side' capacitance (that of the medium) can be a disadvantage in that the system is extremely sensitive and cannot absorb even small load changes without reflecting considerable change in the dissolved oxygen tension. This effect becomes even more troublesome when the 'supply side' capacitance (that of the gas volume above the medium in a vortex-aerated fermentor) is appreciable, because after corrective action by the controller has ceased, oxygen will continue to be transferred to the culture until the oxygen tensions in the gas phase and in the medium are the same. If the supply side capacitance is high, considerable overshoot will result. In the vortex-aerated fermentor used in the present work the supply side capacitance was appreciable, and since the demand side capacitance was low, some difficulty was expected with overshoot.

Selection of controller

Summarizing the relevant characteristics of the system, the electrode might have a considerable lag, and under some conditions the process might exhibit large and rapid load changes. In addition, the process lag might be significant and the oxygen capacitance of the system might give rise to considerable overshoot. For such a system the simplest mode of control, two position, would not be suitable, since it would give rise to excessive cycling. A more refined method involving proportional action would thus be necessary. The proportional controller moves the control valve proportionally to the deviation from the set-point. It moves the valve to a fixed position for each value of dissolved oxygen tension in the proportional band. However, when a load change occurs, a different valve position to that initially fixed will be required to maintain the dissolved oxygen tension at the set-point. As the proportional controller cannot change the fixed relationship between the valve position and the dissolved oxygen tension it will begin to control about a new value,

thus producing 'offset'. The introduction of automatic 'reset' action effectively eliminates offset by shifting the proportional band about the set-point.

Although proportional + reset control may be satisfactory, the lags in both the electrode and the process might cause difficulty, particularly during fast load changes. Thus, in addition, 'rate action' was considered to be desirable. Rate action applies a correction to the movement of the control valve according to the rate of change of the controlled variable. It greatly speeds return to the set-point by instigating a large initial over-correction, then begins to remove this effect, leaving only the proportional + reset action to determine the final position of the valve. A treatise covering all relevant aspects of automatic process control is given by Porter & Considine (1950), but the technical bulletin 'Fundamentals of Industrial Instrumentation' (Minneapolis-Honeywell Regulator Co., Philadelphia, Pa., U.S.A.) gives an adequate description for most purposes.

Selection of control valve

The most convenient choice for a valve to control the gas flow rate would be an electrically operated model working directly from the output of the controller. However, since such a valve was not available, it was necessary to use a pneumatic system consisting of an electro-pneumatic transducer and a pneumatic control valve. To achieve good automatic control it is essential that the control valve is the correct size. A wide range of suitable miniature pneumatic control valves with interchangeable trims are available from Research Controls Inc., Tulsa, Oklahoma, U.S.A. A single valve trim can be used for a wide range of requirements by suitably adjusting the composition of the oxygen-containing gas or the flow of inert gas into the fermentor. However, it is important to note that the higher the flow rate of gas through the fermentor the faster is the response to corrective action and the better the control. The trim used in the present work was chosen to allow a maximum gas flow of about 150 ml./min. with a 5 lb./in.^{2} pressure drop across the valve.

Equipment assembly

The oxygen control equipment was fitted to a 2-litre 'Porton type' fermentor similar to that described by Elsworth, Meakin, Pirt & Capell (1956). An unbaffled vortex aeration system was used, with a stirrer speed of 1440 rev./min. The inflowing gas mixture was introduced into the volume above the culture and the effluent gas was removed via the overflow weir. The temperature was automatically controlled to within $\pm 0{\cdot}25°$. The oxygen electrode was sterilized by ethylene oxide (Callow & Pirt, 1956).

A schematic diagram of the control equipment is shown in Fig. 1; each component is specified in Table 1. Basically the equipment is a closed loop control system. The Mackereth electrode produces a current which is directly proportional to the dissolved oxygen tension in the culture, and which is measured by determining the potential drop across a resistance on a self-balancing potentiometric recorder. Depending on the deviation from the set-point, and on the rate of change of deviation, the 3-action control unit gives out a corrective signal varying between 1 and 5 mA (DC). The signal is fed into an electro-pneumatic transducer which converts it to a pneumatic signal varying proportionally between 3 and 15 lb./in.^{2} This signal, by acting on the diaphragm of the pneumatic control valve, varies the adjustment

of the valve, which in turn varies the flow of the oxygen-containing gas proportionally to the initial corrective signal from the controller. The gas is then directed into a stream of inert gas flowing at a constant rate into the fermentor. The variation in flow of the oxygen-containing gas causes a corresponding variation in the oxygen partial pressure of the gas mixture above the culture, which is responsible for any necessary corrective action to the dissolved oxygen tension in the culture. The inert gas used was nitrogen, and the oxygen-containing gas was air.

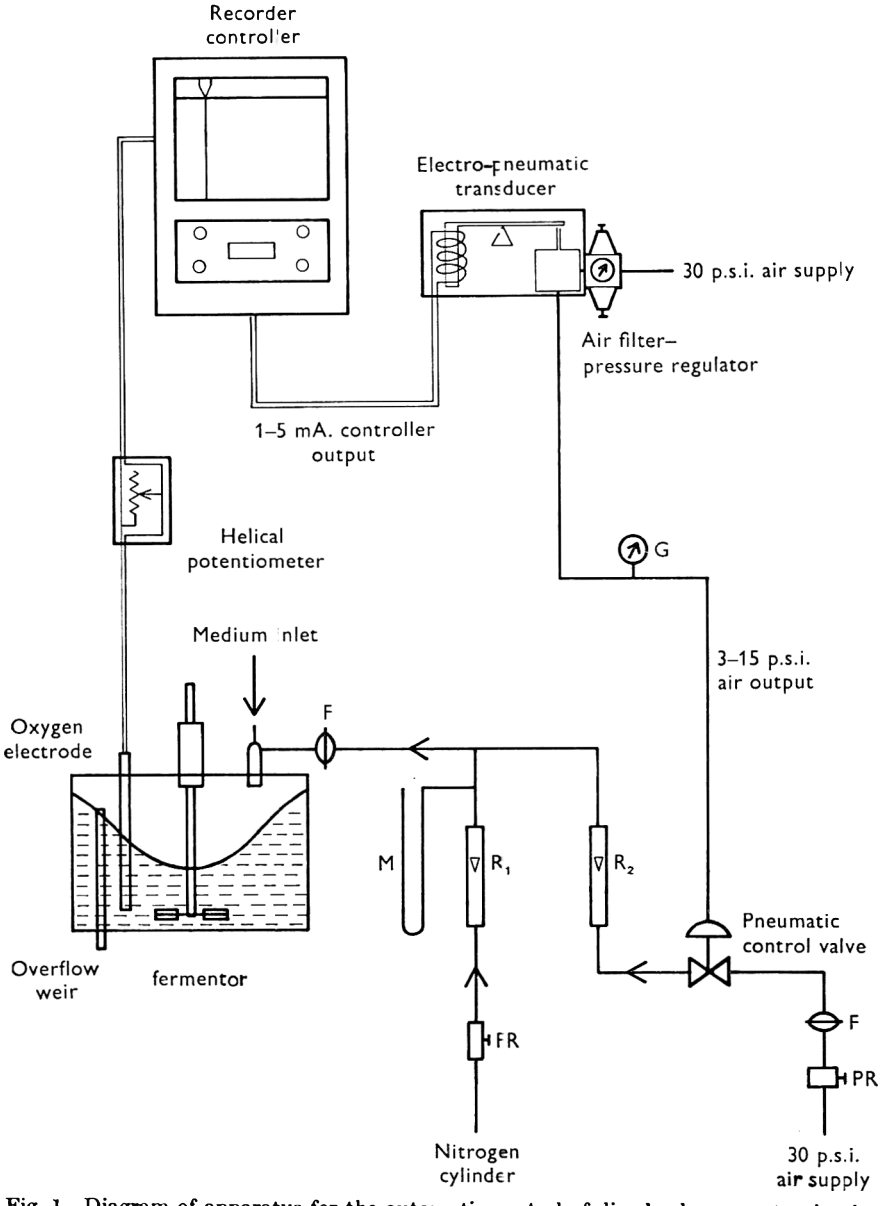

Fig. 1. Diagram of apparatus for the automatic control of dissolved oxygen tension in stirred microbial cultures. F, air filters; M, manometer; R_1, R_2, Rotameters; G, pressure gauge; FR, flow regulator; PR, pressure regulator. See Table 1 for specifications of components.

Table 1. *Specifications of equipment for the automatic control of dissolved oxygen tension in stirred microbial cultures*

Electrode. 'Mackereth' laboratory oxygen electrode manufactured and supplied by Electronic Instruments Ltd., Richmond, Surrey. The electrode was modified for insertion through the fermentor head-plate by extending the length with a perspex sleeve. Price £31.

Helical potentiometer. 0–10 ohm, 10 turn 'Reliance' miniature helical potentiometer, type HEL 07–10, with 'Kilo' analogue dial and brake, type 462, manufactured and supplied by Reliance Controls Ltd., Walthamstow, London, E. 17. Price £5.

Recorder controller. 0–1 mV range 'Speedomax H' self-balancing potentiometric recorder with integrally mounted Series 60 3-action control unit, manufactured and supplied in the U.K. by Leeds and Northrup Ltd., Tysely, Birmingham. Recorder details: type, strip chart; chart speed, 1 in./hr; balance, 1·2 sec. nominal. Controller details: action, proportional + reset + rate; type, current adjusting; output, 1–5 mA at 15 V (DC). Price £330.

Electro-pneumatic transducer. 'Fisher' electro-pneumatic transducer, type 543, with integrally mounted air filter–pressure regulator type 67 FR, manufactured and supplied by Fisher Governor Co. Ltd., Rochester, Kent. Transducer details: resistance, 2500 ohms; current input, 1–5 mA (DC); pneumatic output, 3–15 lb./in.^{2} Price £45.

Pressure gauge (G, Fig. 1). 0–20 lb./in.^{2} Price £2.

Pneumatic control valve. $\frac{1}{4}$ in. air-to-open 'Minim' air operated miniature control valve; body, type 304 stainless steel with P4 trim (C_v max. (U.S.) 0·0006), manufactured by Research Controls Inc., Tulsa, Oklahoma, U.S.A., and supplied by G. A. Platon Ltd., Croydon, Surrey. Price £67; additional trim about £16.

Pressure regulator for culture air supply (PR, Fig. 1). 'Manostat' pressure regulator type 10, $\frac{1}{4}$ in. BSP, output range 2–25 lb./in.^{2}, with high sensitivity adjustment, manufactured in the U.K. by John Watson and Smith Ltd., Leeds, Yorkshire, and supplied by G. A. Platon Ltd., Croydon, Surrey. Price £7.

Flow regulator for culture N_2 supply (FR, Fig. 1). 'Flostat minor' automatic flow regulator, MK.II AL, manufactured and supplied by G. A. Platon Ltd., Croydon, Surrey. Price £8.

Gas flow indicators (R_1, R_2, Fig. 1). Rotameters, ranges R_1, 10–100 ml./min., R_2, 5–150 ml./min.; manufactured and supplied by the Rotameter Manufacturing Co. Ltd., Croydon, Surrey. Price £16.

Total cost £511.

Calibration

The Mackereth electrode was calibrated in sterile medium before inoculation. The system was allowed to equilibrate with an air flow rate of about 200 ml./min. until a steady recorder reading was obtained. With a freshly prepared electrode this usually took about 24 hr. The measuring range was then set by adjusting the helical potentiometer so that the recorder indicated a particular value between 0·95 and 0·98 mV. The brake on the potentiometer dial was then locked. The linearity of the electrode was checked by substituting at least two other gases of known composition before determining zero dissolved oxygen tension with oxygen-free nitrogen. After correction for barometric pressure, back pressure, and saturated water vapour pressure, the partial pressure (mm. Hg) of oxygen in the gas phase for each gas mixture was plotted against the corresponding recorder readings (mV). Since the partial pressure of oxygen in the gas phase was in equilibrium with the oxygen dissolved in the medium, the dissolved oxygen tension in the medium could thus be read directly from the graph. The current produced by the electrode at any time was calculated by dividing the relevant potential drop (mV) by the resistance, the latter being read directly from the dial of the helical potentiometer.

The measuring system was always re-calibrated after an experiment, to check for

drift. Before re-calibration the culture was discarded, the fermentor thoroughly flushed with sterile medium, and the equipment left overnight to equilibrate.

The calibration procedure described is for a measuring range of 0–100% air saturation (or approximately 0–155 mm. Hg dissolved oxygen tension). By substituting gases of appropriate composition the recorder can be calibrated over any desired range. The following gases have been used for calibration (obtained from British Oxygen Co. Ltd., North Wembley, Middlesex): 10, 5, 2, and 1% (v/v) oxygen in nitrogen; oxygen-free nitrogen.

Tuning of the controller

Once the equipment had been installed, all that was necessary to set it in operation was to select the set-point, fix the nitrogen flow rate, and 'tune' the controller by adjusting three dials which determined the width of the proportional band and the degree to which reset and rate actions were required. The controller was tuned according to the directions in the comprehensive explanatory booklet supplied by the manufacturers. After becoming familiar with the equipment no difficulty was experienced in the tuning process. When the conditions in the process were to be drastically changed from those under which the controller was tuned, a further adjustment was often necessary to ensure good control. Table 2 shows typical proportional band, rate and reset adjustments used under various experimental conditions.

RESULTS

The equipment has been used in a series of experiments involving both batch and continuous culture of a pseudomonad where the dissolved oxygen tension has been controlled at various values down to 0·26 mm. Hg. During this work the system has shown a high degree of stability over periods up to 5 weeks, and proved capable of controlling the dissolved oxygen tension to within very fine tolerances. The experiments are summarized in Table 2.

Fig. 2*a* and *b* show sections of typical charts for dissolved oxygen tension controlled at 30 mm. Hg and 1 mm. Hg, respectively, during the continuous culture of the pseudomonad grown on the hydrocarbon decane. Fig. 2*a* is the control trace obtained when the culture was grown under nitrogen-limited conditions, at a concentration of organisms equiv. 2·22 mg. dry wt/ml. Fig. 2*b* shows the trace when the dissolved oxygen tension was growth-limiting, the corresponding concentration of organisms being equiv. 1·44 mg. dry wt/ml. It can be seen that the control traces were virtually straight lines even when the dissolved oxygen tension was the growth-limiting factor.

Fig. 3*a* and *b* show the response of the controller to a disturbance artificially induced by switching off the stirrer in the fermentor. The electrode registered a decrease in dissolved oxygen tension followed by an increase when the stirrer was switched on. In the figure the arrow denotes the point at which the true control action began. It can be seen that in returning the dissolved oxygen tension to the set-point no overshoot or hunting occurred.

Fig. 4 shows a chart for dissolved oxygen tension controlled at 30 mm. Hg during the batch culture of the same pseudomonad with glucose as the carbon source. Over the 9-hr period shown in the figure, the concentration of bacteria and the oxygen

Table 2. *Summary of experiments with the automatic equipment for controlling dissolved oxygen tension*

Cultural details refer to growth of a pseudomonad on defined medium at 30°. Foam was controlled throughout all experiments by the automatic addition at 2-hr intervals of about 0·1 ml. of polyglycol P-2000 (Dow Chemical Co., U.K., Ltd., London, W. 1). In all cases the inert gas was N_2 at a flow rate of about 90 ml./min. The oxygen-containing gas was air.

Controlled dissolved O_2 tension (mm. Hg)	Growth-limiting factor	Steady-state bacterial concn. (equiv. mg. dry wt./ml.)	Air flow (ml./min.)	Efficiency of oxygen utilization (% of O_2 input)	Controller adjustment: Proportional band (% recorder scale)	Controller adjustment: Reset (repeats/min.)	Controller adjustment: Rate time (min.)	Fig. no. of typical chart
Continuous culture, dilution rate 0·1 hr^{-1}; C source decane								
30·0	N source	2·22	117	57	25	0·06	0·04	2*a*, 3*a*
14·0	N source	2·06	90	70	25	0·06	0·04	—
5·7	N source	2·18	71	80	19	0·06	0·025	—
3·0	N source	2·12	53	84	19	0·06	0·025	3*b*
1·0	Dissolved O_2 tension	1·44	19	86	19	0·06	0·025	2*b*
Batch culture, 9-hr duration; C source glucose								
30·0	None	Increase from 0·64 to 3·46	Increase from 55 to 160	—	30	0·3	0·0	4

uptake increased about fivefold. It can be seen that the controller satisfactorily maintained the dissolved oxygen tension at the set-point despite the large load changes encountered. It is important to note that for use with a batch culture, the controller had to be tuned to cope with the very large load changes which occur towards the end of exponential growth, i.e. a high reset value was required. However, in the initial stages of the batch culture where load changes were relatively smaller and slower, the high reset rendered the system ultrasensitive, thus giving rise to cycling. This effect can be seen in Fig. 4. The deviations in the initial stages would be of little consequence for most practical purposes.

With the exception of the batch culture (Fig. 4), all work reported in this paper was done with the electrode sheathed with a 0·001 in. thick membrane made from fluoroethylenepropylene polymer (FEP; Du Pont Co., U.K., Ltd). The FEP membrane was used primarily because of its resistance to hydrocarbons (MacLennan & Pirt, 1965). However, the same control precision was obtained when using the standard polythene membrane (0·0025 in. thickness) supplied with the electrode, in systems not involving hydrocarbons. The batch culture trace shown in Fig. 4 was obtained with the standard polythene membrane.

One defect in all membrane electrodes is their sensitivity to temperature fluctuation. Fig. 5 shows a typical recording of the dissolved oxygen tension in sterile medium at air saturation (155 mm. Hg, O_2) when using an FEP membrane. The cycle in the trace is entirely due to temperature fluctuations resulting from automatic temperature control ($\pm 0{\cdot}25°$). The temperature sensitivity for the FEP membrane was thus about 2% of the reading/degree, and not 5% as estimated previously

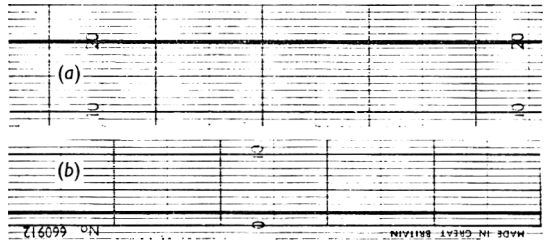

Fig. 2. Sections of typical charts showing controlled dissolved oxygen tension during a continuous culture of a pseudomonad on a defined medium (C source, decane). (*a*) Controlled at 30 mm. Hg (growth limited by N source); (*b*) controlled at 1 mm. Hg (growth limited by dissolved oxygen tension). The heavy vertical lines represent intervals of 1 hr. Each small horizontal division corresponds to 1·6 mm. Hg dissolved oxygen tension.

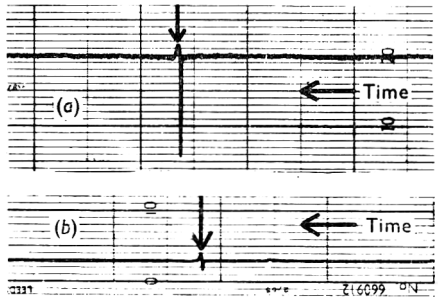

Fig. 3. Charts showing the response of the controller to an artificially induced interference in the dissolved oxygen tension caused by switching off the stirrer during a continuous culture of a pseudomonad on a defined medium (C source, decane). The vertical arrows indicate the point at which true control action began. (*a*) Controlled at 30 mm. Hg dissolved O_2 tension; (*b*) controlled at 3 mm. Hg. The heavy vertical lines represent intervals of 1 hr. Each small horizontal division corresponds to 1·6 mm. Hg dissolved oxygen tension.

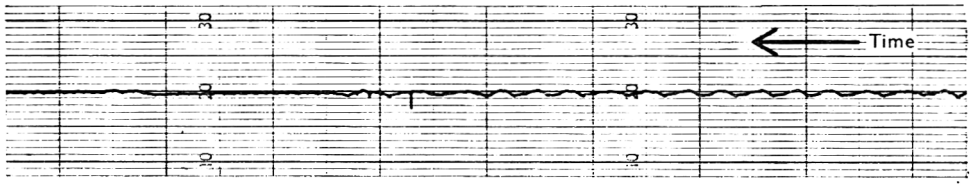

Fig. 4. Chart showing dissolved oxygen tension controlled at 30 mm. Hg during a batch culture of a pseudomonad on a defined medium (C source, glucose). Over the 9-hr period shown the concentration of bacteria and the oxygen uptake increased about five-fold. The Mackereth electrode was sheathed with the standard polythene membrane (0·0025 in. thickness). The heavy vertical lines represent intervals of 1 hr. Each small division corresponds to 1·6 mm. Hg dissolved oxygen tension.

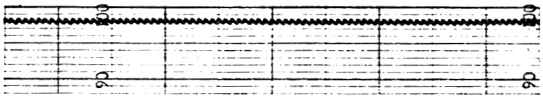

Fig. 5. Chart showing the effect of temperature fluctuation on the output from the Mackereth electrode (FEP membrane, 0·001 in. thick) measured in sterile medium at 30° and at a dissolved oxygen tension of 155 mm. Hg. The cycle in the trace is due to fluctuations of ±0·25° in the automatic temperature control. The temperature coefficient of the electrode reading is about 2 %/degree. The heavy vertical lines represent intervals of 1 hr. Each small horizontal division corresponds to 1·6 mm. Hg dissolved oxygen tension.

(MacLennan & Pirt, 1965). The lower the dissolved oxygen tension the less appreciable the temperature effect became. In Figs. 2*a* and 3*a* (30 mm. Hg, O_2) the effect is barely visible, and in Figs. 2*b* (1 mm. Hg, O_2) and 3*b* (3 mm. Hg, O_2) it cannot be seen at all.

Contrary to expectation the periodic addition of antifoam (2-hr intervals) as used throughout this work produced no change in the dissolved oxygen tension in the culture. It is significant that the rate of antifoam addition was such as to prevent the formation of foam rather than to control the foam level.

An important feature of this automatic control system, particularly from the industrial point of view, was the high efficiency of oxygen utilization. From Table 2 it can be seen that the efficiency ranged from 57 % at a controlled dissolved oxygen tension of 30 mm. Hg to 86 % at 1 mm. Hg.

The stability of the electrode can be assessed from Fig. 6, which shows calibration curves for the electrode both before and after a 5-week continuous culture run in which the sole carbon source was decane. It can be seen that the output from the

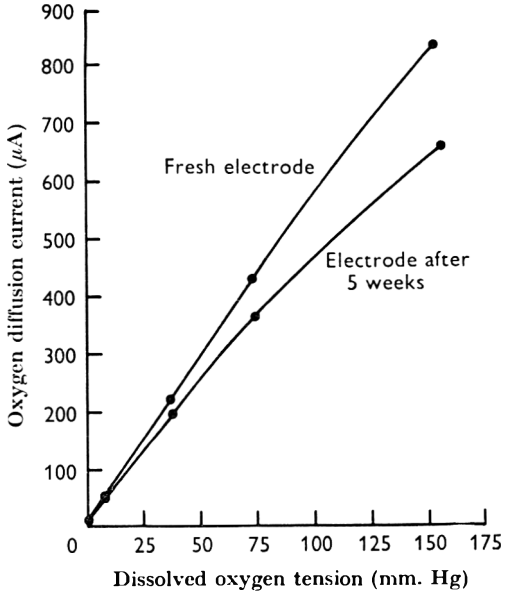

Fig. 6. Calibration curves for a Mackereth electrode (FEP membrane, 0·001 in. thick) before and after a 5-week continuous culture of a pseudomonad on a defined medium (C source, decane). The dissolved oxygen tension was controlled at various values between 30 and 1 mm. Hg.

fresh electrode tended to show a slight change from linearity at high dissolved oxygen tensions (above about 100 mm. Hg). At the end of the run the output from the electrode had decreased and showed a more marked change from linearity at high dissolved oxygen tensions. However, in the latter curve the deviation from linearity was negligible below about 75 mm. Hg, O_2. It is in this region that the most interesting effects of dissolved oxygen tension on the behaviour of microbial cultures are to be expected. In this region the electrode sensitivity decreased from 5·82 to 5·02 μA/mm. Hg, O_2, over the 5-week period. The sensitivity could thus be expressed as the mean of these two values, 5·42 μA/mm. Hg, O_2, with a maximum deviation of 7·5%. Alternatively, the average decrease in sensitivity was 0·4% of the initial value/day. The residual current remained at the same value, 9 μA, throughout the period. All of the work described so far was done with an early model of the Mackereth electrode (model A 15A). More recently, however, a better performance has been obtained with a later model of the electrode (model A 15000, 0·001 in. thick FEP membrane), during the continuous culture of the pseudomonad in which the sole carbon source was glucose. In this work the recorder was calibrated over a range 0–39 mm. Hg dissolved oxygen tension, and the equipment was used to control the dissolved oxygen tension at various values between 0·26 and 30 mm. Hg. Over the 5-week period the mean sensitivity was 7·67 μA/mm. Hg, O_2, with a maximum deviation of 3·6% (average decrease in sensitivity, 0·2% of the initial value/day). The residual current remained constant at 2·9 μA. Thus within the range 0–75 mm. Hg, O_2, both models of the electrode showed a sufficiently high degree of stability to ensure accurate and reliable automatic control of dissolved oxygen tension over periods of at least 5 weeks.

DISCUSSION

The equipment described in this paper provides, for the first time, a precise, reliable and versatile system for the automatic control of dissolved oxygen tension in microbial cultures. The equipment was selected with plant operation as well as laboratory use in mind. All the components used are commercially available and are of first quality, precision industrial grade. Such equipment is expensive, but by far the most economical in the long run because of its stability, reliability and trouble-free operation, factors often lacking in cheaper instruments. The equipment is not limited to the use of the Mackereth electrode, but can be used with any oxygen electrode whose output can be converted to millivolts. The extremely low range of the recorder is a great asset in this respect.

The straight line control traces obtained in this work could possibly have been the result of such an extremely sluggish and insensitive electrode response that the traces did not reflect actual changes occurring in the dissolved oxygen tension. This, however, is considered most unlikely on the grounds of the rapid response to artificially induced disturbances shown in Fig. 3*a* and *b*, and also because small changes in controlled dissolved oxygen tension (for example, from 1 to 0·26 mm. Hg) have produced significant and reproducible changes in culture mass.

It is important to note that the decrease in sensitivity of the FEP-sheathed electrodes was the largest observed for any Mackereth electrode used in these laboratories. The best performance was obtained with an electrode with a polythene membrane (0·0025 in. thick), a negligible decrease being observed over a

period of 8 weeks continuous use followed by a 10% decrease in the following week (D. E. F. Harrison, personal communication). The higher current output from the FEP-sheathed electrodes would probably be responsible for the increased deviation from linearity at high dissolved oxygen tensions and the change in long-term stability observed. In the present work the thin FEP membrane was used specifically to decrease the response time of the electrode (Mackereth, 1964), since it was initially thought that the slow response of the electrode would be the critical factor in obtaining precise automatic control. Subsequent work, however, showed that good control could be achieved when using the thicker polythene membrane (see Fig. 4). Hence a thicker FEP membrane could be used to decrease the current output and possibly improve the long-term stability.

One criticism of the partial pressure method of controlling dissolved oxygen tension in continuous cultures was that it would be difficult to determine the oxygen uptake of the culture because of variation in oxygen input. In our experience, the air input has been found to remain remarkably constant once a steady state has been attained, and no difficulty has been encountered in determining oxygen uptake rates, irrespective of whether the culture was grown at excess, critical or limiting dissolved oxygen tensions. This might be due to the stabilizing effect of *controlled* dissolved oxygen tension, which would tend to damp rather than amplify potential oscillations in the culture metabolism.

At the present time the extent to which rate action is necessary in controlling dissolved oxygen tension in fermentation systems has not been fully investigated. It has been included in the equipment to provide maximum versatility in coping with any situation which may arise during microbial cultivation, and has been found necessary in all circumstances so far examined.

Although no experimental evidence of control below 0·26 mm. Hg, O_2, is available, there is no reason why it should not be feasible, since the Mackereth electrode produces a linear response down to zero dissolved oxygen tension, and the control equipment has ample potential to cope with sensitive and unstable systems. It is important to note that the equipment described is capable of controlling the dissolved oxygen tension well below the levels which can be tolerated by such 'strict anaerobes' as *Clostridium tetani* (2 mm. Hg, O_2; Gordon, Holman & McLeod, 1953).

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Biochemical Studies of Lethal Processes in Aerosols of *Escherichia coli*

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SUMMARY

The effects of holding time and relative humidity on the survival of populations recovered from aerosols of *Escherichia coli* strain B was examined. β-Galactosidase was chosen as a convenient model of a bacterial protein in this organism. Populations recovered from aerosols of *E. coli* strain B were shown: (i) to have suffered no detectable damage to β-galactosidase; (ii) to have suffered a temporary and severe loss of ability to synthesize β-galactosidase. The significance of these results in identifying lethal mechanisms in bacterial aerosols is discussed. For these studies a method was developed for the determination of total bacterial numbers recovered from aerosols by labelling the organisms directly with [^{14}C]. *Bacillus globigii* was shown to be unsuitable as a tracer for the determination of total numbers in biological aerosols.

INTRODUCTION

The propagation of many plant and animal diseases depends on the transfer of the causative organisms in an aerosol. Although many pathogenic and non-pathogenic organisms are known to be less stable in aerosols than in aqueous suspensions, comparatively little is known of processes in aerosols which lead to death or loss of infectivity or in what way these processes resemble similar phenomena resulting from stresses such as starvation, drying, exposure to radiation or ageing. *Escherichia coli* was chosen as a convenient simulant of the pathogenic bacteria, but since this organism is not normally pathogenic only changes in viability could be studied. Changes in the viability of this model organism can be taken only as an indication of possible changes of infectivity of a pathogen since some pathogenic bacteria recovered from aerosols are viable *in vitro* yet not infective *in vivo* (e.g. evidence summarized by Goodlow & Leonard, 1961).

Stresses which may act on bacteria responsible for the spread of natural infections include radiation, relative humidity, temperature and composition of the atmosphere, and physical forces involved in the generation and recovery of organisms from the aerosol. The viability of recovered aerosolized organisms may also be markedly influenced by the presence of extraneous material such as spent culture fluid or tissue exudates in fluids used in the generation and recovery of the aerosols. The present communication is primarily concerned with biochemical processes in an aerosol generated from a washed suspension of *Escherichia coli* strain B, maintained at controlled temperature and humidity in the dark in a suitable apparatus. Changes in the viability and certain biochemical properties of the bacterial populations recovered from aerosols were determined and related to changes in the relative

humidity and age of aerosols in an attempt to locate the actual site of damage in the organism.

METHODS

Organisms. Escherichia coli strain B was grown from freeze-dried cultures on tryptic meat agar at 37° for 18 hr. A typical colony was selected, grown in meat broth (37°, 18 hr) and stored at 4°. This culture was used as a stock inoculum for 4 months, then discarded. *Bacillus globigii* (*B. subtilis* var *niger*) spores were obtained from several sources; a laboratory strain kindly provided by Mr F. C. Belton was used for most of the work.

Media. The tryptone medium (H. E. Wade, private communication) contained Oxoid tryptone, 2%; sodium chloride, 0·3%; dipotassium hydrogen phosphate, 0·005 M; ferric citrate 0·001 M; calcium chloride, 0·001 M; magnesium sulphate, 0·004 M; adjusted to pH 7·2 with sulphuric acid. The agar medium for general use contained: 'Oxoid' tryptone, 1·0%; agar, 1·5%; glucose, 1·0%; sodium chloride, 0·5%. An agar medium used only for the growth of pure cultures of *Bacillus globigii* contained: Lab Lemco, 0·2%; peptone, 0·5%; sodium chloride, 0·5%; glucose, 0·5%; agar, 3%.

Phosphate buffer alginate. Potassium dihydrogen phosphate, 0·45%; ammonium sulphate, 0·05%; ammonium chloride 0·05%; sodium alginate ('Manucol SS/LH' by Alginate Industries Ltd., London W.C. 2) 0·25%; sodium hydroxide to pH 7·2, approximately 0·1%. This medium was used in the collection of bacteria and for all serial dilutions. Alginate was incorporated to minimize losses of organisms by physical processes (Henderson, 1952). Bacteria could be maintained in this fluid at room temperature for over 30 min. without any change in colony count.

Radiochemicals. [U-^{14}C]-D-Glucose (3·9 mc/m-mole) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Measurement of radioactivity. The apparatus, methods and materials used for the determination of radioactivity by a 'coincidence counting' technique employing a liquid scintillator were as described by Anderson & Smith (1965). Aqueous samples were generally mixed with an equal volume of formamide and stored in solid carbon dioxide until assayed.

Growth of organisms. For [^{14}C]-tracer studies the tryptone medium (10 ml.) containing [U-^{14}C]-D-glucose (about 60 μg./ml.; 1·25 μC/ml.) was inoculated (to 5×10^7 organisms/ml.) with a suspension of *Escherichia coli* then shaken (100 ml. flasks, 5 cm. throw, 100 oscillations/min.) at 37° for 16 hr. The bacteria were stored for 5 hr at 4° before use and contained about 30% of the original added tracer. Non-labelled bacteria were grown in a similar manner but in the absence of [U-^{14}C]-D-glucose.

Where appropriate, β-galactosidase formation was induced in this organism by overnight growth in the liquid tryptone medium supplemented with *iso*-propyl-thio-β-D-galactopyranoside (0·10 m-mole/ml.; Rickenberg, Cohen, Buttin & Monod, 1956). This substrate induced higher galactosidase values than did its thio-methyl analogue or lactose itself.

When dead [^{14}C]-labelled *Escherichia coli* was required as a tracer, a washed suspension of the organisms (10^{10} bacteria/ml.) was shaken with [U-^{14}C]-D-glucose (1·5 μC/ml.; 70 μg./ml.) in Locke's solution at 37° for 30–60 min. Following this

treatment bacteria killed with formaldehyde (4 %, w/v; 30 min.) contained 30–60 % of the original tracer after two washings with water.

Spores of *Bacillus globigii* produced after growth (4 days, 37°) on peptone agar medium were washed off with water, heated (60°; 2 hr), stored (4°, 7 days), reheated (60°, 2 hr), then washed again and resuspended in water (5×10^{10} spores/ml.). The spore suspension was stored at 4° and renewed after 4 months; it was reheated (60°, 30 min.) immediately before use to kill any vegetative organisms.

Determination of survival values. Viability was defined as the ability of an organism to produce a visible colony in 18 hr at 37° on the tryptone agar medium. Non-sprayed organisms were given a nominal viability of 100 %. Colony counts and radioactivity determinations were so arranged that the determined values of the viability estimates generally had a 95 % confidence belt of ± 10 %.

Mixed suspensions of *Escherichia coli* and *Bacillus globigii* were assayed by differential colony counts on tryptone agar plates.

Total numbers of organisms recovered from aerosols were generally calculated from the observed radiotracer content of the recovered suspensions and from the radiotracer and colony count of non-aerosolized organisms. [^{14}C]-Labelled *Escherichia coli* was used to determine spore counts in studies with *Bacillus globigii*. *B. globigii* itself was used as a tracer of total bacterial numbers for comparative purposes only, in the few cases where the use of radiotracer was not possible.

Apparatus for the study of bacterial aerosols. Aerosols generated in a modified Collison atomizer were diluted with a stream of secondary air in a stainless steel mixing tube to give an atmosphere of the desired relative humidity (Henderson, 1952). The relative humidity of the aerosol could be adjusted over a range of from 35 to 100 % by alteration of the water content of the secondary air. The characteristics of the Collison spray are such that a suspension containing 2 to 4×10^9 organisms/ml. yields an essentially monodisperse aerosol. Such a system was chosen to eliminate possible protective, or damaging, interactions between organisms in multicellular particles. The apparatus was adjusted to give flow rates of from 25 to 60 l./min. and aerosol concentrations up to 2×10^7 organisms/l.

Aerosols emerging from the mixing tube were passed into a rotating stainless steel drum (75 l.) for storage. The construction and operation of the apparatus was similar to that described by Goldberg, Watkins, Boerke & Chatigny (1958). The relative humidity of the atmosphere was determined by means of suitable conversion tables (Hodgman, 1948) from the observed differences between readings of 'wet and dry bulb' thermometers placed in the air stream. The temperature of the room containing the appartus was generally between 19° and 22° and did not vary by more than 0·2° during experiments; the relative humidity of air leaving the apparatus seldom varied by more than ± 2 %.

Aerosol samples were generally collected for 45 sec. into phosphate buffer alginate (7–10 ml.) by using a sonic impinger with a throughput of 11 l./min. (raised Porton impinger; May & Harper, 1957).

Preparation of suspensions for the generation of bacterial aerosols

The method used in the preparation of suspensions of organisms for aerosolization depended on whether spores (1), [^{14}C]-labelled killed bacteria (2), or [^{14}C]-labelled living bacteria (3), were to be used as indicator of total bacterial numbers.

1. *Bacillus globigii* spores were heated, mixed with the labelled or unlabelled culture of *Escherichia coli* and the organisms separated from the culture fluids by centrifugation. The organisms were then washed once with water by centrifugation and resuspended in water (about 2×10^9 of each organism/ml.).

2. A mixture of equal numbers of organisms of a culture of *Escherichia coli* with [^{14}C]-labelled formalized bacteria as tracer were treated exactly as the spore tracer for preparation of spray suspensions.

3. Suspensions of living [^{14}C]-labelled organisms could, however, be aerosolized directly after washing with water as above. This tracer method was used wherever possible to indicate total numbers of recovered populations.

Appropriate enzyme, colony counts and radioactivity determinations were made on suspensions before and after spraying to ensure that no change had taken place in populations during generation of the aerosol.

Determination of the galactosidase content of pre-induced bacteria

Samples of [^{14}C]-labelled induced organisms were collected and portions (2 ml. containing 1 to 2×10^7 organisms in phosphate buffer alginate) were treated with a solution of substrate (3 ml. of 2·5 mM-*o*-nitrophenol-β-D-galactopyranoside in 0·05 M-sodium phosphate buffer, pH 7·2); benzene (0·1 ml.; Lederberg, 1950) and incubated at 37° for 30 min. Sodium carbonate solution (1·0 M; 3 ml.) was then added and the mixture filtered (Whatman No. 1 paper) and the liberated *o*-nitrophenol determined colorimetrically at 420 mμ.

Samples of suspensions taken directly from the spray pot at the beginning and end of the experiment were diluted (1/500 in phosphate buffer alginate) and assayed in a similar manner. The organism hydrolysed about 5 μmole of *o*-nitrophenol-β-D-galactoside/10^{10} bacteria/min.

Determination of the ability of stressed organisms to produce galactosidase

Samples of bacterial suspensions recovered from aerosols (4·5 ml. containing about 5×10^7 organisms in phosphate buffer alginate) were added to an inducer (0·5 ml. of a 5 mM-solution of methyl-thio-β-D-galactopyranoside; Lederberg, 1950) in tryptone medium, and gently rotated in a tube at 37° for 30 min. The presence of this small quantity of growth medium enhanced galactosidase production. Diluted suspensions (1/500 or 1/1000 in phosphate buffer alginate) of samples removed from the spray pot at the beginning and end of the experiment were treated similarly. The induced bacteria were then treated with substrate (0·5 ml. of a 15 mM-solution of *o*-nitrophenol-β-D-galactopyranoside in 0·05 M-sodium phosphate buffer, pH 7·2) and benzene (0·1 ml.). After incubation at 37° for 30 min. the *o*-nitrophenol content was determined as above.

RESULTS

Development of a radiotracer technique for estimating total numbers of organisms in recovered aerosols

Spores of *Bacillus globigii* have been used as a tracer for determining total numbers in bacterial aerosols despite doubts about the stability of this organism (Harper, Hood & Morton, 1958; Cox, 1966). In initial studies with this tracer

apparent viabilities of up to 160% of those of unsprayed organisms were obtained when populations of *Escherichia coli* were recovered from recently generated aerosols. These high apparent viabilities might have been caused by division or disaggregation of *E. coli* or death of *B. globigii*. No change in numbers was detected in suspensions of *B. globigii* or *E. coli* remaining in the spray pot at the end of experiments in which these organisms would have re-circulated 50 times through the jets of the spray. The use of [^{14}C]-labelled *E. coli* was therefore introduced to investigate the suspected death of *B. globigii* in the aerosol and to provide a tracer for biochemical work where the presence of another organism would invalidate results.

Table 1. *Evidence for the biological similarity of aerosols of* [^{14}C]*-labelled and non-labelled Escherichia coli*

	Relative humidity = 53%		Relative humidity = 88%	
Age of aerosol cloud	Viability (%) of [^{14}C]-labelled *E. coli* (*Bacillus globigii* tracer)	Viability (%) of non-labelled *E. coli* (*Bacillus globigii* tracer)	Viability (%) of *E. coli* labelled internally with [^{14}C]	Viability (%) of non-labelled *E. coli* with external [^{14}C]-tracer
unsprayed control	(100)	(100)	(100)	(100)
1·2 sec.	78	77	73	75
2 min.	43	45	52	57
10 min.	32	38	9	9
30 min.	16	19	3	5

Before using [^{14}C]-labelled *Escherichia coli* as a tracer for total bacterial numbers the possibility of damage by radioactivity had to be eliminated, since the organisms were grown and stored in the presence of radioisotope and might be particularly sensitive to radiation in the aerosol. Comparison of the viability of recovered aerosols of [^{14}C]-labelled and non-labelled *E. coli*, using *Bacillus globigii* as a tracer, indicated that the radioisotope was not harmful (Table 1). This comparison was carried out under identical conditions with the same batch of spore tracer to minimize the effects of death of the spore. Similar results were obtained when [^{14}C]-labelled *E. coli* was compared with the non-labelled bacteria using dead [^{14}C]-labelled organisms as an external tracer (Table 1). An external tracer was used to test the possibility that, if radiation damage only occurred in the aerosol, the spatial separation of radioisotope from the living organism and consequent diminished radiation flux might result in a measurable difference in viability. The external [^{14}C]-tracer also confirmed the results obtained with the spore tracer. Further proof of the biological similarity of [^{12}C]- and [^{14}C]-organisms was provided by comparison of the effect of heat stress (Table 2). Finally, the viable count of cultures of [^{12}C]- and [^{14}C]-organisms were shown to be similar, and to remain so during storage at 4° for up to 1 week.

Application of the radiotracer technique to mixed suspensions of [^{14}C]-labelled *Escherichia coli* (as a tracer for the spore tracer) and *Bacillus globigii* spores showed that there was, initially, a rapid death of the spore tracer. Viability determinations

on spores recovered from aerosols 1·2 sec. after generation, at 37 points, at relative humidities ranging from 40 to 97% suggest that the initial decrease in viability tended to increase with increasing relative humidity (correlation coefficient = −0·34; P = approximately 4%). The regression of viability % (V) on relative humidity % (H) gave the equation $V = 87{\cdot}9 - 0{\cdot}267\,H$ with 95% fiducial limits of ±28% (approximately over the relevant range). From the wide range of the fiducial limits of this equation the reproducibility was obviously unsatisfactory. The lethal effect on a proportion of the population appeared to be almost instantaneous, with no evidence of further change at up to 30 min. (Table 3). The use of this spore was therefore discontinued. The viability of spore suspensions remained unchanged on agitation in an impinger for up to 3 min. Death of the spore during impingement found under different experimental conditions by Levine & Cabelli, (1963) can therefore be discounted.

Table 2. *Evidence for the biological similarity of [^{14}C]-labelled and non-labelled organisms of Escherichia coli during exposure to heat stress*

10^7 *E. coli*/ml. in phosphate buffer alginate; nominal viability of non-stressed organisms = 100%

Period of stress (min.)	Temperature	Viability (%) of non-labelled organisms	Viability (%) of [^{14}C]-labelled organisms
15	49°	108	101
	50°	79	77
	55°	7	5
30	49°	51	57
	50°	11	11
	55°	0	0
45	49°	28	31
	50°	8	6
	55°	0	0

Table 3. *Evidence for the absence of any significant decrease in the viability of recovered aerosols of Bacillus globigii after the initial rapid death*

Age of aerosol cloud	Viability (%) of *Bacillus globigii* at a relative humidity of 54%	Viability (%) of *Bacillus globigii* at a relative humidity of 94%
unsprayed suspension	(100)	(100)
1·2 sec.	72	77
5 min.	75	82
10 min.	71	86
20 min.	75	74
30 min.	84	69

Measurement of the effect of relative humidity and holding time on aerosols of Escherichia coli

Figure 1 shows the effect of a range of twenty-seven relative humidity values on the viability of aerosolized populations of *Escherichia coli*. To simplify the graphical

representation, data have only been presented for bacterial clouds recovered at 1·2 sec., 5 min. and 30 min., although samples had also been taken at 10 and 20 min. These figures suggest that there were zones of relative humidity where the viability of the bacterial populations decreased rapidly. Figure 2 represents graphically 3 of the 27 log viability-time curves which illustrate the typical initial rapid decay of the organism, followed by a slower decay which was reasonably linear in the range 5–30 min. The dot diagram (Fig. 3) of these twenty-seven regression coefficients against relative humidity suggests that after the initial rapid decay the death rate is minimum at about 80 % and rises rapidly at higher humidities.

Measurement of the stability of cell protein following generation and recovery of aerosols of Escherchia coli

Bacterial death in organisms recovered from aerosols might be due to damage to cell proteins. To test this β-galactosidase was chosen as a model protein, since a

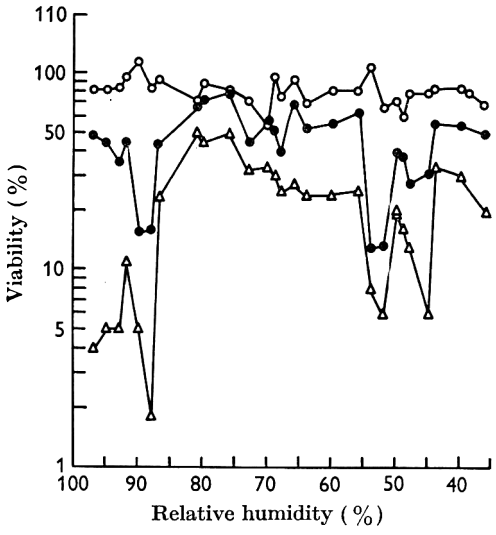

Fig. 1

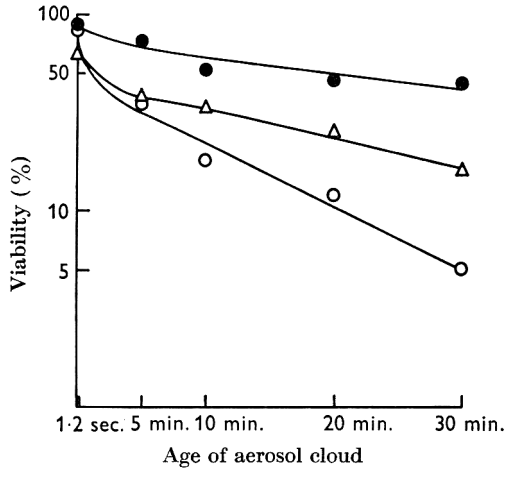

Fig. 2

Fig. 1. Viability of populations of *Escherichia coli*, strain B, recovered from aerosols 1·2 sec (—○—○—), 5 min. (—●—●—) and 30 min. (—△—△—) after generation.

Fig. 2. Viability of populations of *Escherichia coli*, strain B, held at relative humidities of 80 % (—●—●—), 49 % (—△—△—) and 93 % (—○—○—) at intervals up to 30 min.

convenient sensitive assay method was available, and its enzymic activity could be used as a criterion of structural integrity. β-Galactosidase formation was induced in *Escherichia coli* by growth in a medium containing *iso*-propyl-thio-β-D-galacto-pyranoside. The general properties and death rate on aerosolization of the induced *E. coli* were similar to those of similar bacteria grown without an inducer. The specific activity of the β-galactosidase, in populations recovered from aerosols, was determined and expressed as a percentage of that in unsprayed organisms. Table 4 summarizes results obtained with bacterial clouds sampled at the longest time after generation (30 min.) when damage to β-galactosidase would be expected to be greatest. Aerosolization had little effect on the specific β-galactosidase activity of

Table 4. *Changes in the β-galactosidase value and viability of pre-induced organisms of Escherichia coli recovered from aerosol clouds held at various relative humidities for 30 min.*

Relative humidity:	42	51	58	68	68	77	84	90	94	99
β-Galactosidase level*:	104	108	130	120	102	91	106	101	97	126
Viability*:	19	13	42	56	44	12	12	9	1	6

* The nominal specific activity (enzyme/total no. bacteria) of the β-galactosidase of non-aerosolized organisms = 100 %. The nominal viability of these organisms = 100 %.

E. coli despite large changes in viability; this was true at various relative humidities and different holding times. Combined results of all 10 experiments for samples removed at aerosol ages of 1·2 sec., 5 min., 10 min., 20 min. and 30 min. strongly indicate a small positive regression (significance level being 0·001 %) in the plot of

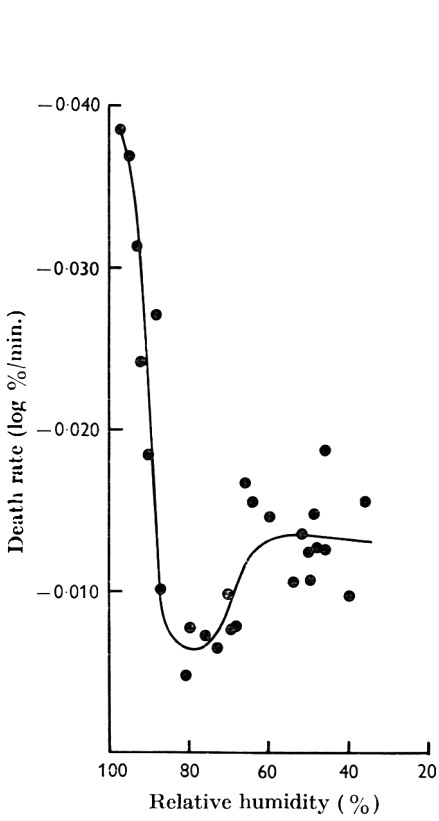

Fig. 3

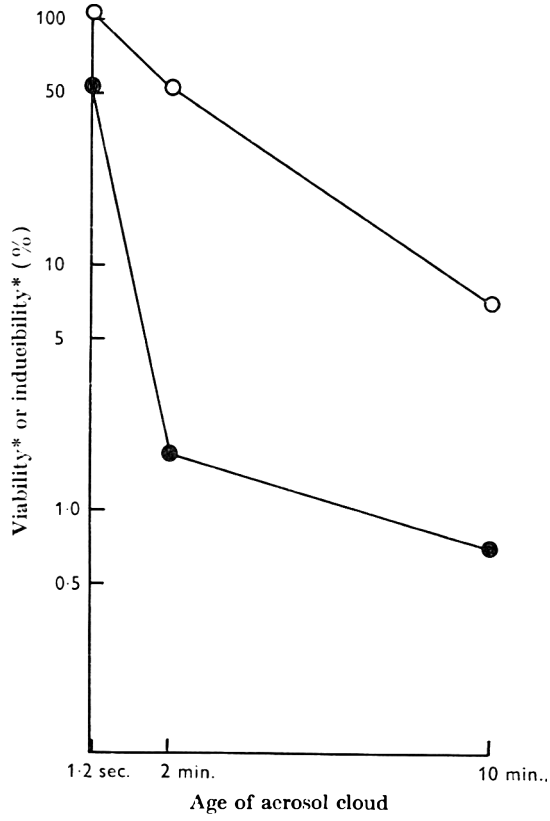

Fig. 4

Fig. 3. The effect of relative humidity on the death rate (over the period 5–30 min.) of populations recovered from aerosols of *Escherichia coli*, strain B.

Fig. 4. The viability (—○—○—) and induced galactosidase (—●—●—) levels of organisms of *Escherichia coli* strain B, recovered from an aerosol cloud held at a relative humidity of 91 %. * For unsprayed organisms the nominal galactosidase activity resulting from induction in a standard test = 100 % and the nominal viability = 100 %.

galactosidase activity against holding time, amounting to only 5% over 30 min. Although statistically significant this change is trivial and might be an artifact caused by the increased accessibility of the enzyme to the test substrate in the thoroughly dried bacteria. Combined results also gave a non-significant ($P = 11\%$) regression in the plot of galactosidase activity against relative humidity.

Ability of Escherichia coli to synthesize protein following recovery from aerosols

Damage to protein synthesizing mechanisms might be lethal in organisms recovered from aerosols. The importance of such damage could be assessed if a relationship could be established between the relative extent of changes of viability and changes in the ability of organisms recovered from aerosols to synthesize protein in response to a specific stimulus. β-Galactosidase was again chosen as a model protein and a β-galactosidase inducer as the stimulus. The ability of *Escherichia coli* to synthesize β-galactosidase immediately after recovery from aerosols decreased so rapidly with increasing aerosol age that the induced enzyme value proved too low to measure on organisms recovered from bacterial clouds more than 10 min. after generation. Fig. 4 illustrates the results of one typical experiment. Table 5 summarizes the results of 13 experiments and gives some evidence that induced galactosidase values decreased relatively faster than viability as the holding time in the aerosol increased. This decrease was obvious between 0 and 1·2 sec. and between 1·2 sec. and 2 min. Unfortunately the confidence belt of the 10 min. data (0·14) was widened by an anomalous result for one of the thirteen observations where the induced galactosidase value was relatively greater than the viability.

Table 5. *Summary of evidence for a correlation between changes in the ability of Escherichia coli to synthesize β-galactosidase immediately after recovery from aerosols and changes in viability*

Age of aerosal cloud	*Induced galactosidase value viability Mean (95% fiducial limits)
1·2 sec.	0·59 (0·43–0·79)
2 min.	0·30 (0·22–0·40)
10 min.	0·14 (0·04–0·51)

* Mean of 13 determinations at relative humidities from 49 to 99%. For non-aerosolized organisms the nominal galactosidase activity resulting from induction in a standard test = 100%, and the nominal viability = 100%.

DISCUSSION

Total bacterial numbers in biological aerosols have been estimated by methods involving dyes (e.g. Dunklin & Puck, 1948; Henderson, 1952; Wolfe, 1961), radiophosphorus (Harper & Morton, 1952; Harper *et al.* 1958), radiosulphur (Miller, Schcrff, Prepoli & Idoine, 1961), spores (Harper *et al.* 1958) and photoelectric measurements (e.g. Fisher, Katz, Lieberman & Alexander, 1955; Ferry, Brown & Damon, 1958). The [^{14}C]-tracer technique has the advantage over these methods that a stable and easily determined tracer was incorporated within the bacteria which were actually being studied, without appreciably altering either medium, growth condition or spray fluid. The instability of *Bacillus globigii* spores was unexpected since their resistance to other stresses, including storage in various

environments, and to heat, is well known. No explanation can be offered for the anomalous behaviour of these spores.

Most of the work described in this paper was concerned with recovered aerosols having viabilities of from 5 to 100%. Populations of lower viability were avoided since death processes in highly stressed, and thus perhaps, selected, organisms might not be representative of those causing death of the bulk of a population in the aerosol. The plot of log survival versus time for bacterial aerosols may commonly be divided into two or more distinct regions. The death rate of *Escherichia coli* strain B, under the conditions described here, can be represented by an initial (0–5 min.) rapid decay followed by a slower logarithmic decay over 5–30 min. Data for the survival of organisms recovered from aerosols 5 min. after generation provide a measure of the initial rapid decay and demonstrate the appearance of troughs in the plot of survival *versus* relative humidity (Fig. 1). Comparatively narrow zones of relative humidity at which dried bacteria are unstable have been found in other experimental systems, e.g. freeze-dried organisms of *Serratia marcescens* die most rapidly at a water content corresponding to equilibration with water vapour at a relative humidity of approximately 90% (Monk, Elbert, Stevens & McCaffrey, 1956; Monk & McCaffrey, 1957; Bateman, McCaffrey, O'Connor & Monk, 1961). Davis & Bateman (1960) observed a similar phenomenon with freeze-dried, washed suspensions of *E. coli*. Aerosols of several strains of *E. coli* display sharp troughs in the plot of survival versus relative humidity in an atmosphere of nitrogen (Cox, 1966); for *E. coli* strain B these troughs occurred at relative humidities of 100, 97, 85 and 50%.

The dependence of the later (5–30 min.) phase of decay upon relative humidity (Fig. 3) is quite different from the initial decay process. The completely different responses of the processes represented by the two parts of the decay curve to changes of relative humidity suggests that at least two sequential lethal processes occur.

Experimental difficulties inherent in the study of the small populations which may be recovered from bacterial aerosols may be avoided when bacterial suspensions are dried on filter membranes to simulate the aerosolized state. Webb and colleagues (see review by Webb, 1965) studied the effect of drying on several biochemical systems in bacteria by using such membranes. However, lethal processes in the aerosol may not parallel those on membrane filters, since death and dehydration rates of bacteria on filter membranes are generally much lower than in aerosols. Also, bacteria on a membrane filter are not subject to mechanical stresses as in an aerosol and are in contact with a solid surface, in contrast to the aerosol environment. Nevertheless, the present results with β-galactosidase stability and induction in aerosolized bacteria are in general agreement with Webb's (1965) findings.

If β-galactosidase is typical of bacterial proteins then although there is no extensive destruction of the protein itself in the aerosol or immediately after recovery, loss of protein-synthesizing ability either leads to death or is an expression of the lethal lesion. Since organisms recovered from aerosol clouds 1·2 sec. after generation of the aerosol are substantially viable (70–100%) yet show a severe decrease in their ability to synthesize protein in response to a specific stimulus, this decrease must either be reversible or not immediately lethal. Study of protein synthesis in

organisms immediately after recovery from aerosols might indicate the site of the actual lethal process.

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Reducing Compounds and the Growth of *Phytophthora infestans*

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SUMMARY

An investigation into the effect of light on the growth of *Phytophthora infestans* revealed that whereas exposure to light caused diminished growth on pea medium, growth on several other media was not decreased. This diminished growth was an indirect effect of light caused by an alteration of the medium. All the *P. infestans* tested reacted similarly, but most other fungi examined were unaffected. Germination of zoospores of *P. infestans* appeared to be especially sensitive. This inhibitory effect was not removed by heat and peroxides were not, therefore, considered as the immediate cause of the inhibition. Nevertheless, following exposure to light, peroxide formation and the appearance of the inhibitory effect were closely associated. Addition of several reducing compounds to pea medium overcame the effect of exposure to light on zoospore germination. Mycelial growth was also improved by similar additions to a defined medium. This was particularly marked following the addition of glutathione (10^{-3} M) and L-ascorbic acid (1·0 g./l.). The decrease in reducing potential during illumination might, in the light of experiments conducted with defined media, explain the observed effects of illumination.

INTRODUCTION

Hirst (1953) detected a marked diurnal periodicity in the occurrence of *Phytophthora infestans* in the atmosphere and suggested that this diurnal fluctuation depended on hygroscopic changes during drying to liberate sporangia. This suggestion was in part based on the report by Crosier (1934) that the sporulation process in *P. infestans* was unaffected by light. The peak spore concentration of *P. infestans* spores occurred shortly before noon and coincided with a similar peak in the numbers of *Pseudoperonospore humuli* (hop downy mildew) spores (Hirst, 1953). However, with hop downy mildew, Yarwood (1937) suggested that the diurnal cycle of sporulation was basically dependent on the alteration of light and darkness. In view of these observations, and of the close taxonomic relation between the downy mildews and *P. infestans*, it was considered of interest to investigate the effect of light on the development of *P. infestans*. As compared to work with the downy mildews, such an investigation offers the advantage that *P. infestans* can be cultured *in vitro*.

METHODS

Media. Pea medium was used throughout. It was obtained by macerating 160 g. 'Birds Eye' frozen peas in 1 l. water for 3 min. in a Waring Blendor, and filtering the homogenate through muslin. When required as a solid medium, 2% (w/v) agar

was then added to the liquid. Where a defined medium was used, 0·05 % (w/v) L-β-asparagine and 0·2 % (w/v) vitamin-free Casamino acids were added to the basal medium described by Hall (1959). All the media were autoclaved at 115° for 10 min.

Conditions of illumination. Solidified medium was exposed to light in 6 in. × $\frac{5}{8}$ in. Pyrex test tubes, and liquid medium in 8 oz. soft-glass medicine bottles (United Glass Blowers Ltd.). These bottles were laid flat, providing a layer of medium approximately $\frac{1}{4}$ in. deep. The cultures were placed in a light-proof chamber 2 ft. 6 in. beneath two fluorescent lamps (Osram 40 W. Daylight MCF/U), at which level the light intensity was 130 f.c. These lamps provided light of wavelengths between 3900 Å and 7400 Å, and little, if any, ultraviolet radiation was emitted. A temperature of 21° ± 1° was maintained within the chamber by a fan which circulated air beneath the lamps. To obtain conditions of darkness, tubes and bottles were covered with aluminium foil, although this decreased the temperature in the cultures by 0·8° compared with the temperature within exposed cultures.

Inoculum. Mycelial discs were cut from the edges of the cultures grown on the basal medium described by Hall (1959), to which only 0·05 % (w/v) L-β-asparagine was added. To obtain zoospores, sporangia were washed with water from cultures on pea medium slopes which had been similarly treated 48 hr earlier. When kept at 10° for 3 hr, zoospores readily emerged from these sporangia.

Measurements. All cultures were incubated in darkness at 12°. Measurements were made after incubation only for 6 days when pea medium was used, but after 14 days when the defined medium was used. Colony length was measured in all treatments inoculated with mycelial discs, and in treatments inoculated with zoospores the degree of germination was assessed on a scale in which 5 (the normal maximum) represented the resulting mycelial growth on unilluminated pea medium at 6 days.

RESULTS

The effect of pre-exposure of organic media on the growth of Phytophthora infestans

A preliminary experiment showed that continuous illumination decreased the growth of *Phytophthora infestans* when grown on pea medium, but not when grown on defined medium. To investigate whether this inhibition on illuminated pea medium was a direct or indirect effect of light, tubes of both pea and defined media were exposed to light before inoculation for periods of 144, 72, 24 and 0 hr, and then inoculated with mycelial discs of isolate D (see Fig. 1).

Illumination of the defined medium before inoculation had little effect on growth (see Fig. 1), but illumination of the pea medium for only 24 hr before inoculation decreased colony length; longer periods of illumination did not cause a greater decrease of growth. The decreased growth of *P. infestans* on illuminated pea medium therefore appeared to be an indirect effect of light caused by an alteration of the medium.

To determine if exposure of other organic media to light also decreased the growth of *Phytophthora infestans*, tubes of media prepared from vegetable juice, frozen beans, oatmeal or dried peas, were exposed to continuous light for 3 days before inoculation with mycelial discs. Although the growth rate on all these media was less than half the rate on pea medium, pre-illumination of all four media did not affect growth,

which indicated that the light-sensitive factor present in frozen peas was not present in other organic material, including dried peas.

Effect of pre-exposure on the growth of Phytophthora infestans isolates

In previous experiments only *Phytophthora infestans* isolate D was used, but to ensure that the results obtained were not atypical, seven other isolates (Table 1) were tested for their response to pea medium pre-illuminated for 24 hr. A comparison between isolates was possible after measuring colony length. The results in Table 1 demonstrate that pre-illumination of the pea medium restricted the growth of all the isolates tested, although the degree of inhibition varied between isolates.

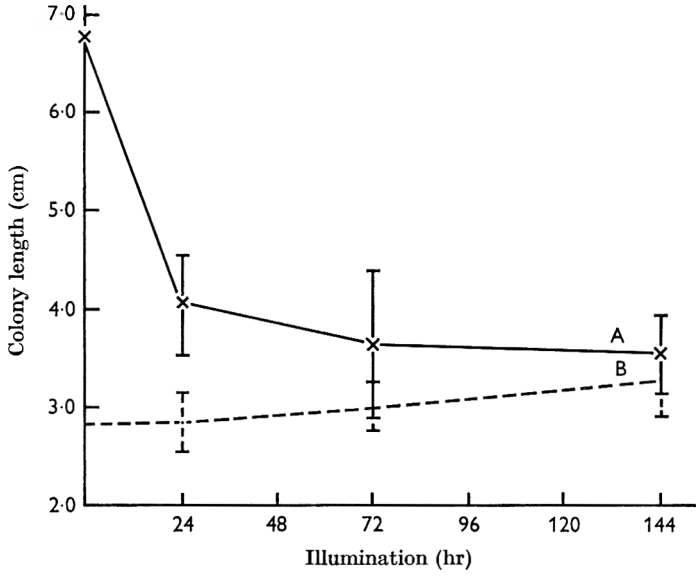

Fig. 1. Effect on the growth of *Phytophthora infestans* of media illuminated before inoculation for periods up to 144 hr. A, Pea medium; B, defined medium.

Table 1. *Response of eight isolates of Phytophthora infestans to illuminated pea medium.*

Isolate	Age (years)	Potato variety	Non-illuminated	Illuminated	% inhibition
A	6	Unknown	40·50*	31·75*	22
B	4	Unknown	49·75	31·00	38
C	2	Pentland Beauty	51·00	37·50	27
D	2	Golden Wonder	47·75	32·25	33
E	1	Majestic	39·25	22·75	41
F	1	Majestic	32·25	0·00	100
G	0	King Edward	51·75	32·25	38
H	0	Orion	52·00	32·00	39

L.S.D. two treatment totals. $P < 0{\cdot}001 = 6{\cdot}75$.
* Total colony length in cm., from eight replicates.

Sensitivity of various other fungi

To determine whether the growth of other fungi was inhibited by illuminated pea medium, the response of several fungi was tested on illuminated medium which had been re-autoclaved before inoculation to destroy any peroxides present (see below). Only *Phytophthora palmivora* was inhibited by illuminated pea medium; growth of *Acremoniella* sp., *Alternaria tenuis*, *Botrytis cinerea*, *Fusarium* sp., *Phytophthora cryptogea*, *P. heveae* and *Pythium* sp. remained unaltered by exposure of the medium to light.

Influence of inoculum source on the inhibitory effect of illuminated pea medium

To investigate the effect of the inoculum on the inhibitory effect of illuminated medium, mycelial discs of *Phytophthora infestans* isolate D and two zoospore concentrations were inoculated into tubes of illuminated and non-illuminated pea medium. The results are shown in Pl. 1, fig. 1. When zoospores were used as inoculum, the inhibitory effect was especially pronounced, largely because few spores germinated on illuminated medium. Zoospore germination appeared, therefore, to provide a more sensitive measure of the inhibitory effect of illuminated pea medium.

Effect of autoclaving on the inhibition caused by illuminated pea medium

Exposure of organic matter to light causes oxidation by processes which have been discussed in a series of papers edited by Lundberg (1961). In autoclaved pea medium this oxidation is non-enzymic and might result in the formation of peroxides. A marked similarity between the inhibition caused by the addition of hydrogen peroxide to organic media and the inhibition caused by ultraviolet and visible light was reported by both Wyss, Bennett Clark, Hass & Stone (1948) and Weinhold & Hendrix (1963). If the inhibitory effect of illuminated pea medium was caused by thermolabile peroxides, re-autoclaving illuminated medium might be expected to remove this inhibition.

Mycelial discs of *Phytophthora infestans* isolate D were inoculated into tubes of pea medium which had received one of the following treatments: (*a*) Tubes illuminated for 24 hr and re-autoclaved (115° for 10 min.). (*b*) Tubes illuminated for 24 hr but not re-autoclaved. (*c*) Tubes not illuminated but re-autoclaved before inoculation. (*d*) Tubes not illuminated and not re-autoclaved (control).

An analysis of colony length after incubation for 6 days is given in Table 2. This experiment not only showed the effect of illumination, but also showed that re-autoclaving both illuminated and non-illuminated media decreased growth. The non-significance of the interaction term suggested, however, that the inhibitory effect was unaltered by reheating and that peroxides did not directly cause this inhibition; it is possible that peroxides may in some way have been protected by the medium.

The nature of the alteration caused by the exposure of pea medium to light

The presence of peroxides in pea medium may cause the oxidation of metabolites required for normal growth by *Phytophthora infestans*, in which case peroxide formation might be associated with the appearance of the inhibitory effect following illumination. For this reason peroxide concentration and zoospore germination

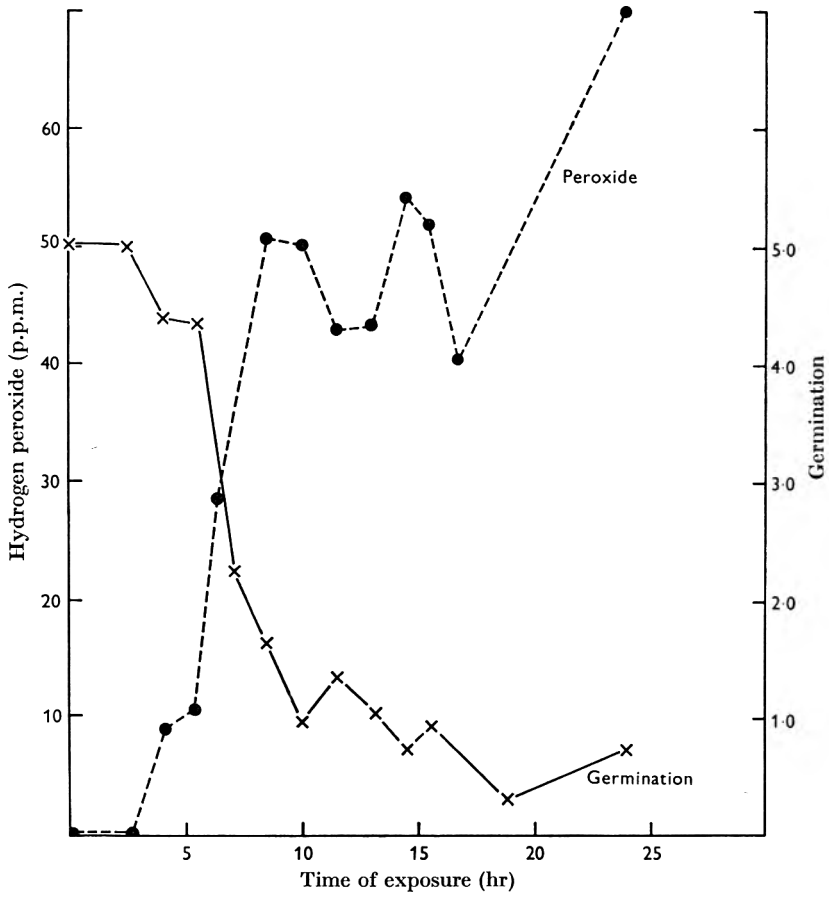

Fig. 2. The effect of up to 30 hr illumination of pea liquid on peroxide formation and zoospore germination. Peroxide concentration is expressed in terms of p.p.m. (v/v) hydrogen peroxide, and germination is assessed on a 0–5 scale.

Table 2. *Analysis of variance of colony length showing the effect of heat on the inhibition caused by illuminated pea medium*

Source of variation	N	M.S.	V.R.	*P*
A. Re-autoclaving *vs.* not re-autoclaved	1	0·42	14·00	< 0·01
B. Illuminated *vs.* non-illuminated	1	5·85	195·00	< 0·001
C. Interaction re-autoclaving × illumination	1	0·10	3·33	N.S.
Error	24	0·03	—	—

were measured for liquid pea medium at intervals of illumination for up to 30 hr. Peroxide concentration was estimated by a technique based on that described by Koch, Stern & Ferrari (1958). A sample (0·25 ml.) of the illuminated pea liquid, 20 ml. 90% ethanol, 0·2 ml. conc. HCl, and 0·05 ml. 5% (w/v) ferrous ammonium

sulphate in 3 % HCl were shaken together for 30 sec., 1·0 ml. 20 % (w/v) ammonium thiocyanate was then added, and after 3 min. the extinction of the solution at 480 mμ was measured, using a Beckman S.P. 600 spectrophotometer. The extinction produced by the sample was compared with that produced by the same volume of liquid pea medium withdrawn at the same time but autoclaved at 105° for 5 min. The difference in extinction at 480 mμ (E_{480}) between the sample and the autoclaved blank was taken as a measure of the peroxide present in the sample, and expressed in terms of p.p.m. hydrogen peroxide by reference to a standard graph of E_{480} against hydrogen peroxide.

There was a 4-hr period before any peroxide was detected; thereafter the peroxide concentration rapidly increased, reaching 50 p.p.m. after 10-hr illumination (Fig. 2). With longer illumination times the concentration of peroxide remained fairly constant, and in another experiment a peroxide concentration equivalent to 50–60 p.p.m. hydrogen peroxide was maintained for at least 144 hr.

The liquid pea medium not required for each peroxide determination was used to prepare tubes of solid media. Each tube was inoculated with a loopful of zoospore suspension of *Phytophthora infestans* isolate F, already shown to be extremely sensitive to illuminated pea medium. A small inhibitory effect on germination was observed after only 4-hr illumination (Fig. 2), and the degree of germination then declined rapidly, the inhibitory effect reaching a maximum after 10-hr illumination. The inhibitory effect on germination appeared, therefore, after between 5- and 10-hr illumination and its appearance coincided with a rapid increase in peroxide concentration.

The addition of reducing compounds to pea medium

In addition to the formation of peroxides, other chemical changes are likely following the irradiation of organic matter. Arnow (1936) discussed the effects of u.v. irradiation on proteins and amino acids, and suggested that several amino acids may be altered on exposure to sunlight. Sulphydryl (–SH) groups may be oxidized by u.v. irradiation (Woodward, 1933).

If the inhibitory effect of illuminated pea medium were caused by the oxidation of substances within the medium, then the addition of reducing substances might possibly overcome the effect of illumination. Therefore, the compounds listed in Table 3 were added to liquid pea medium before, or after, it was exposed to light for 48 hr. All media were adjusted to pH 6·8 before autoclaving and solidified with agar in sloped tubes. Each tube was then inoculated with a loopful of zoospore suspension (10^5 zoospores/ml.). When germination was assessed the scale had to be extended beyond 5 because, in certain cases, it was better than that on the non-illuminated pea medium control.

L-Ascorbic acid, L-cysteine HCl, and thioglycollic (mercapto-acetic) acid not only prevented the inhibitory effect when added to the liquid before illumination, but actually improved germination as compared with that on the non-illuminated control (Table 3). All three compounds also overcame the effect of exposure when added to the liquid after illumination.

When added before exposure, glutathione partially overcame the inhibitory effect, but DL-alanine, L-cystine, DL-methionine and glycollic acid were ineffective no matter when added to the liquid.

Table 3. *Zoospore germination following the addition of several compounds to pea medium before, and after, 48-hr illumination*

Compound	Formula	Conc. (M)	Germination: Added before illumination	Germination: Added after illumination
Thioglycollic acid	$SH.CH_2.COOH$	10^{-3}	10·0 ± 0·00	8·00 ± 0·00
Glycollic acid	$OH.CH_2.COOH$	10^{-3}	0·13 ± 0·08	0·26 ± 0·17
L-Cysteine hydrochloride	$SH.CH_2.CH(COO.HCl).NH_2$	10^{-3}	5·70 ± 2·01	4·05 ± 2·31
DL-Alanine	$CH_3.CH(NH_2).COOH$	10^{-3}	0·10 ± 0·05	0·27 ± 0·10
L-Cystine	$S.CH_2.CHNH_2.COOH$ / $S.CH_2.CHNH_2.COOH$	10^{-3}	0·78 ± 0·58	0·72 ± 0·43
DL-Methionine	$CH_3S.CH_2.CH_2.CHNH_2.COOH$	10^{-3}	0·60 ± 0·27	0·15 ± 0·05
Glutathione	$CO.NHCH.CH_2.SH$; CH_2; CH_2; $CH.NH_2.COOH$; CO; NH; $CH_2.COOH$	10^{-3}	3·10 ± 0·10	0·70 ± 0·32
Ascorbic acid		10^{-2}	5·80 ± 0·50	4·90 ± 0·25
Non-illuminated pea medium. Control		—	5·00 ± 0·00	—
Illuminated pea medium. Control		—	—	0·15 ± 0·08

Table 4. *Growth of Phytophthora infestans as influenced by compounds in the presence, or absence, of ascorbic acid*

Compound (all at 10^{-3} M)	Ascorbic acid not added	Ascorbic acid added (1·0 g./l.)
Thioglycollic acid	2·10*	2·20*
Glycollic acid	1·30	1·60
L-Cysteine hydrochloride	2·00	2·80
DL-Alanine	1·00	1·40
Serine	1·00	1·40
L-Cysteine	1·00	2·40
DL-Methionine	1·20	1·50
Cystathionine	1·10	1·50
Glutathione (reduced)	1·20	4·40
None	1·20	1·80

L.S.D. two treatment values 0·35 ($P < 0·001$).
* Figures are the mean colony length (cm.) of six replicates after 7-day incubation.

The addition of sulphydryl compounds to a defined medium

Compounds which contained a –SH group overcame the inhibitory effect of illuminated pea medium. Replacement of the –SH group with H or OH, or oxidation to S–S, rendered these compounds ineffective. Ascorbic acid also overcame the inhibition, which indicated that perhaps *Phytophthora infestans* required the presence of reducing groups for growth. To investigate this point further the compounds listed in Table 4 were added to a basal medium which contained (%, w/v) KH_2PO_4, 0·05; $MgSO_4.7H_2O$, 0·025; D-glucose, 2·5; L-β-asparagine, 0·05; 1 ml. of the vitamin solution described by Pontecorvo (1953); distilled water. All compounds were added at a concentration of 10^{-3} M and the pH was adjusted to 6·0 before autoclaving at 115° for 10 min. The effect of each compound on the growth of *P. infestans* was also tested in the presence of L-ascorbic acid (1 g./l.). Each tube was inoculated with a mycelial disc and colony length was measured after incubation for 7 days.

The results (Table 4) show that L-ascorbic acid, L-cysteine HCl, and thioglycollic acid increased growth as compared with that on the control medium. This effect of L-cysteine HCl and thioglycollic acid on growth was lost by the replacement of the –SH group with either H or OH. Glutathione, which was possibly altered during autoclaving (Isherwood, 1959), had no effect on growth when added alone, but in the presence of L-ascorbic acid it considerably increased growth (Pl. 1, fig. 2). L-cystine, which contains sulphur in a form readily reduced to –SH, increased growth only in the presence of ascorbic acid, but DL-methionine and DL-cystathionine, which contain sulphur in a form not easily reduced, had no effect on growth even in the presence of L-ascorbic acid.

Clearly, the interaction between –SH-containing compounds and ascorbic acid was dependent on the –SH compound used. Glutathione interacted strongly with L-ascorbic acid, but thioglycollic acid did not interact. Cysteine appeared to interact only slightly; to see whether this small interaction was the result of the concentrations used, a factorial experiment was made, using four concentrations (0·0, 0·01, 0·1 and 1·0 g./l.) of both cysteine and ascorbic acid. The best growth was obtained with 0·1 g./l. L-cysteine HCl + 1·0 g./l. L-ascorbic acid, which were approximately the concentrations used previously. On the other hand, when added together at 1·0 g./l., L-ascorbic acid and L-cystiene HCl appeared to have a toxic effect on growth.

DISCUSSION

The evidence presented in this paper shows that the decreased growth of *Phytophthora infestans* on illuminated solid pea medium resulted not from a direct effect of light on the fungus, but from an indirect effect which caused an alteration of the medium. This alteration was complete after illumination for only 12 hr. Work by Bedford (1927), Woodrow, Bailey & Fulmer (1927), Wyss *et al.* (1948) using ultraviolet radiation and Weinhold & Hendrix (1963) using light also showed that effects of irradiation on micro-organisms resulted from an alteration of the organic media used. Whereas illuminated pea medium agar inhibited all the *P. infestans* isolates tested, the majority of other fungi tested were unaffected; this suggests that perhaps the metabolism of *P. infestans* is in some way different from that of even taxonomically similar species. Both Wyss *et al.* (1948) and Weinhold & Hendrix (1963)

reported that the type of inoculum had a marked effect on the magnitude of the inhibition caused by ultraviolet and light irradiation. In the present experiments zoospore germination was more sensitive to light-illuminated medium than was mycelial growth, suggesting that the effect of light was especially pronounced during the early stages of growth.

Work by Bedford (1927), Wyss *et al.* (1948) & Weinhold & Hendrix (1963) has indicated that the inhibitory effect of ultraviolet and light-irradiated medium may be caused by the presence of peroxides. Autoclaving did not remove the inhibitory effect of illuminated pea medium agar; and peroxides, therefore, seemed not to be the immediate cause of the decreased growth of *Phytophthora infestans*. However, the close association between peroxide formation and the appearance of the inhibitory effect suggested that peroxides were, in some way, responsible for the decreased growth.

Geise (1955) presented evidence which indicated a difference between hydrogen peroxide and ultraviolet irradiation in their effects on cell division in protozoa, and Bennett Clark, Wyss, & Stone (1950) suggested that certain effects of ultraviolet irradiation might result from the inactivation of sulphydryl groups, and not from the formation of peroxides.

Not only did –SH-containing compounds and L-ascorbic acid overcome the inhibitory effect of illuminated pea medium but they also markedly increased the growth of *Phytophthora infestans* when added to a defined medium. The importance of the –SH group was emphasized by the fact that replacement of this group in sulphydryl compounds by H, OH, or –SS rendered the compounds ineffective. However, the fact that ascorbic acid also increased growth made it likely that it was the reducing properties of these compounds that were important (Hodgson, 1958). In view of the apparent requirement by *P. infestans* for reducing conditions, the difference in reducing potential between illuminated and non-illuminated medium might explain the effect of illumination, although the changes which caused this difference remained obscure.

Besides pointing the importance of reducing compounds in the metabolism of *Phytophthora infestans* this investigation has shown a point of practical value, namely that the addition of glutathione 0·3 g./l. and ascorbic acid 1·0 g./l. to the basal medium described earlier, considerably enhanced the growth of the fungus and brought it within the range of the best of the undefined media. Because these compounds are oxidized in light, cultures grown in this medium should be kept in darkness.

On the basis of the results presented in this paper, it may be argued that the growth of *Phytophthora infestans* within the potato depends on the maintenance of reducing conditions within host cells. Because the parasitism of *P. infestans* resembles that of obligate parasites (Brown, 1955) it is perhaps of interest to note that Sahai & Shaw (1961) detected increased amounts of ascorbic acid and glutathione on a rust-infected susceptible cereal variety. This caused a rise in the reducing potential (Kaul & Shaw, 1960) which led Shaw (1963) to conclude that high reducing potentials were needed within host cells to enable growth and development of the rust fungus.

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EXPLANATION OF PLATE

Fig. 1. Inhibition caused by illuminated pea medium and the influence of inoculum source. A. Mycelial disc; B, $32{\cdot}7 \times 10^2$ zoospores; C, $1{\cdot}3 \pm 10^2$ zoospores. U, Not illuminated.

Fig. 2. The effect of the sulphydryl group and ascorbic acid on the growth of *Phytophthora infestans*. A, Thioglycollic acid; B, L-cysteine HCl; C, L-cystine; D, glycollic acid; E, glutathione (reduced); X, L-ascorbic acid; Y, no ascorbic acid (control).

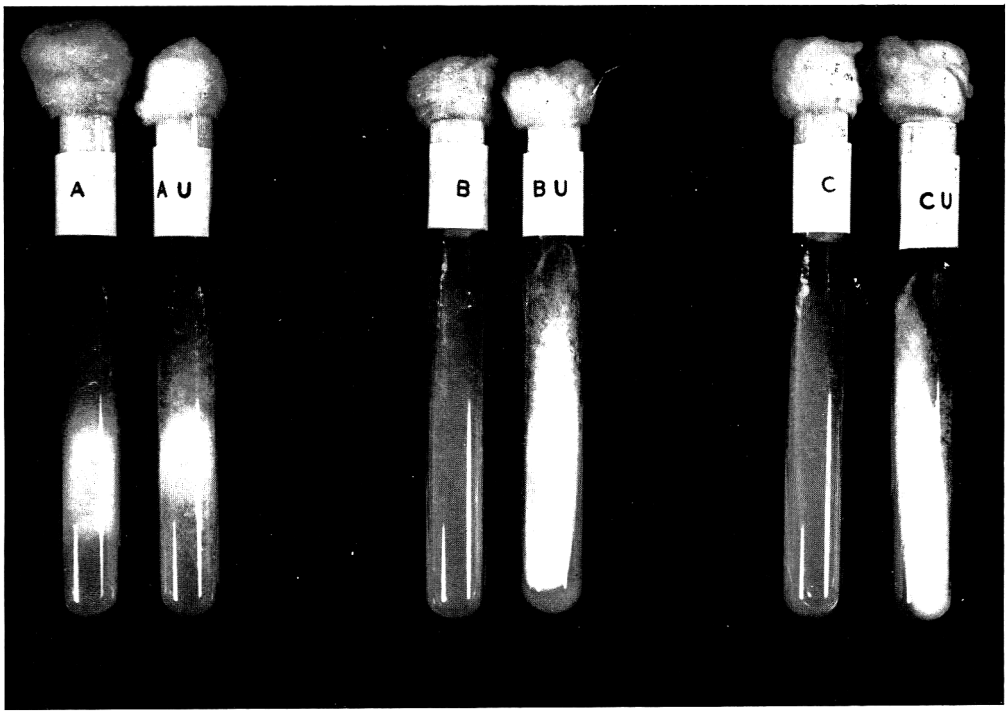

Fig. 1

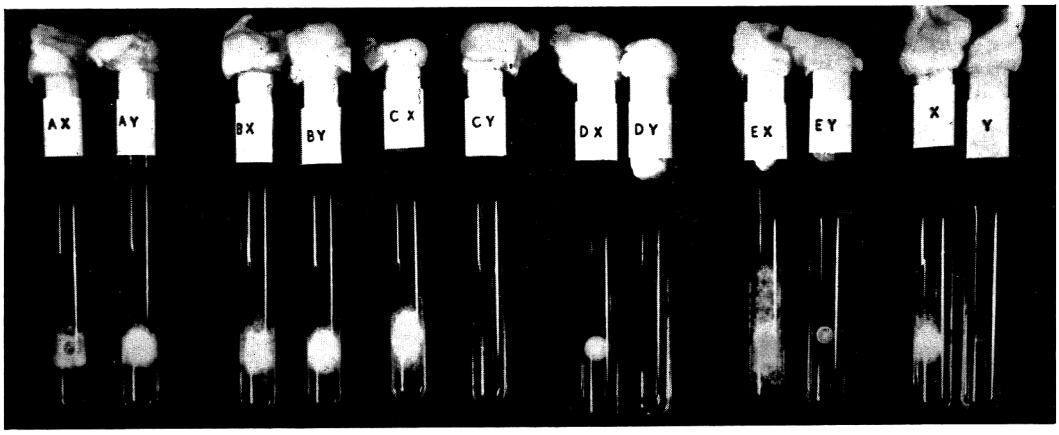

Fig. 2

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Characteristics of Bovine Mammillitis Virus

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SUMMARY

A virus isolated from cattle in Scotland does not seem to have been described previously as existing in Europe. The virus has been provisionally designated bovine mammillitis virus (BMV). It grows in tissue cultures, particularly of bovine origin, giving rise to large multinucleate cells which have type A inclusions in many of their nuclei. Baby mice injected with it develop skin lesions, but only a limited number of passages can be made. The BMV particle has a nucleocapsid of about 80 mμ diameter which, in complete particles, is surrounded by a loose envelope. The virus is sensitive to ether and chloroform, and contains DNA with a base composition of 64% G+C and a band-width molecular weight of 34×10^6. BMV shows antigenic similarity, in neutralization tests and double-diffusion tests in agar, to the group II viruses (prototype Allerton) of lumpy skin disease (Alexander, Plowright & Haig, 1957). All these features place it in the family of the herpesviruses.

INTRODUCTION

Milking cows on several farms in the West of Scotland were affected with ulcers on their teats during the autumn of 1963. From one a virus was isolated in tissue cultures. Infected culture fluid produced lesions similar to those of the naturally occurring disease when injected into cattle, and from these the virus was re-isolated (Martin, Martin & Lauder, 1964). The clinical syndrome did not appear to have been described previously but might have been confused with cowpox. The virus seemed a new agent with general features similar to those of the Herpesvirus group; and also to resemble in particular the group II viruses (prototype Allerton) described in association with one type of lumpy skin disease of cattle in Africa (Alexander, Plowright & Haig, 1957). These findings are reported in this paper.

The virus has been provisionally named bovine mammillitis virus (BMV).

METHODS

Viruses. BMV was isolated initially in bovine lymph node cells, but subsequent passages were made in a variety of cells including calf kidney (CK), bovine conjunctiva (DBC), lamb testis (LT) and the BHK 21/C13 line of baby hamster kidney cells.

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The Allerton strain of the group II lumpy skin disease viruses of Alexander *et al.* (1957) was received from the Kenya Veterinary Research Laboratories, Kabete, as the second calf kidney passage and was passaged further in CK or BHK 21/C13 cells.

All virus titrations were carried out in tubes or bottles, generally using four replicate cultures per ten-fold dilution. Titres are expressed as 50 % cytopathogenic doses (Cp.D 50) per ml.

Media. Eagle's basal medium with 2 × concentration of vitamins and amino acids and supplemented with tryptose phosphate broth (10 %, v/v), or Hanks's balanced salt solution with lactalbumin hydrolysate (0·5 %, w/v) and yeast extract (0·1 %, w/v), were generally used for growing and maintaining cells. Unheated calf serum (10 %, v/v) was incorporated in all media, but was omitted when growing virus, to avoid any possible neutralization with different batches of serum. The diluent for viruses and sera also consisted of Eagle's medium plus tryptose phosphate, without calf serum.

Antisera. The antisera against BMV and Allerton virus used in the neutralization and precipitation tests were produced in guinea pigs. These were given two intradermal injections of virus, as tissue culture fluid of the 7th CK cell passage, at an interval of 1 month and bled 10 days after the second injection. Antisera against other viruses came from sources given in the Acknowledgements, were prepared in rabbits unless otherwise indicated, and were stated to have the following neutralizing titres (reciprocal of end-point dilution) against 100 Cp.D 50 of the homologous virus: pseudorabies antiserum, > 1000; equine herpes type 1 and type 2 antiserum, both > 1000; B virus antiserum (from monkey), 100; infectious bovine rhinotracheitis (IBR) antiserum, 512; malignant catarrhal fever (MCF) antiserum (from ox), 64; herpes simplex antiserum (from guinea pig), 32. The varicella human acute-phase serum had a complement-fixing titre of < 8; while the convalescent-phase serum had a titre of ⩾ 512.

Neutralization and precipitation tests. Neutralization tests were performed in tubes. Serial tenfold dilutions of virus were made. Generally, four tubes were used for each dilution. A constant 1/5 or 1/10 dilution of antiserum, inactivated at 56° for 30 min., was mixed with an equal volume of each dilution of virus and allowed to interact either at 37° for 60 min. or at 4° overnight. Each tube was inoculated with 0·2 ml. of virus + serum mixture, given 1 ml. of medium without calf serum, and incubated at 37°. Tests were read after 5 days.

Double-diffusion tests were done in agar on glass slides, using 2 ml. of Difco 'Bacto' agar per 3 in. × 1 in. slide. The concentration of agar was 0·7 % (w/v) in 0·14 M-NaCl buffered with 0·1 M-phosphate, pH 7·0 (PBS). Cups 3 or 5 mm. in diameter were cut in the agar with cork-borers and filled to the brim with reactants. The edge-to-edge distance between cups was 4–6 mm. Slides were placed in humidified boxes at room temperature, and tests were usually read after 3 days. The antigen preparations for gel-diffusion tests were made from BMV or Allerton virus grown in BHK 21/C13 cells at 34° for 3 days. The cells were scraped off into the medium and spun down at 1500 rev./min. for 10 min. The deposit was resuspended in PBS at a concentration of $\sim 10^8$ cells/ml. and disrupted by ultrasonic vibration in a Dawe Soniclean bath. Concentrates were stored at −20°. Antisera were heated at 56° for 30 min. before use. The anti-BMV serum contained low levels of precipitating antibody against a calf serum component and was therefore mixed

before use with an equal volume of calf serum. The antiserum prepared against Allerton virus formed no precipitate with calf serum.

DNA extraction. The method used was based on that developed previously for herpes viruses (Russell & Crawford, 1964). Infected cell suspensions were treated with sodium dodecyl sulphate (1 %) for 2 min. at 25° and then made 1 N with sodium perchlorate and deproteinized by shaking with chloroform + isoamyl alcohol (24:1) as described by Marmur (1961). The DNA was then collected on a glass rod after addition of 2 volumes of ethanol and redissolved in standard saline citrate (Marmur, 1961).

Density gradient centrifugation. Equilibrium density gradient centrifugation was used to determine the base composition and molecular weight of the DNA (Meselson, Stahl & Vinograd, 1957). The DNA was centrifuged in CsCl (density 1·72 g./ml.) containing Tris buffer (0·05 M, pH 8·5) for 1 day at 44,770 rev./min. followed by 2 days at 31,410 rev./min. in a Spinco model E ultracentrifuge. Ultraviolet absorption photographs were taken and scanned with a Joyce–Loebl microdensitometer to determine the shape and positions of the bands. A derivative of the band width squared was plotted against relative DNA concentration and the molecular weight calculated from the slope of the plot (Thomas & Berns, 1961). The base composition was calculated from the position of the bands relative to marker DNA of known density (Sueoka, 1961).

Ether and chloroform sensitivity. Ether sensitivity tests were performed after the method of Andrewes & Horstmann (1949). Chloroform sensitivity was estimated by mixing 1 part of chloroform with 2 parts of infective tissue culture fluid. The mixture and the control were shaken for 10 min. at room temperature, centrifuged lightly and the supernatant inoculated on to monolayers.

Electron microscopy. Cultures of foetal bovine conjunctiva cells were infected with BMV and harvested 5 days later. Both the cells and the supernatant culture fluid were prepared for examination in the electron microscope. The tissue culture fluid was centrifuged at 800–1000 rev./min. to remove cell debris, then at 100,000*g* for 90 min. The pellet obtained was resuspended in a drop of fluid; a droplet was placed on a specimen grid, which was then washed with distilled water and inverted on to a solution of potassium phosphotungstate (PTA), 1 % (v/v), pH 7, the excess stain being drained off on filter paper before examination.

Cell pellets were obtained by scraping infected monolayers from bottles into a small volume of medium and centrifuging at 800–1000 rev./min. for 5 min. After washing in buffered saline, the cell pellet was fixed in buffered osmium tetroxide (1 % (v/v), pH 7·4) for 45 min. at 4°. The fixed cells were then washed quickly in water, dehydrated with increasing concentrations of ethanol, and embedded in prepolymerized methacrylate (9:1 mixture of *n*-butyl and methyl methacrylate). Thin sections were cut with a Huxley-type microtome and stained with lead hydroxide (Millonig, 1961). All examinations were made in a Siemens Elmiskop 1 at magnifications of ×20,000 and ×40,000.

RESULTS

Tissue culture

A small volume of fluid aspirated from the lesions on one cow produced morphological changes when inoculated on to monolayers of calf lymph node, DBC, or BHK 21/C13 cells. The appearance of control and infected DBC cultures is shown in Pl. 1, figs. 1, 2. After 24–48 hr incubation at 37°, discrete foci of cytopathic change appeared which became confluent as they enlarged and increased in number. They consisted of large cell masses of bizarre shape, often with long cytoplasmic processes, apparently formed by the fusion of many cells. Large, refractile, globular cells could be seen, loosely attached to the remaining cell sheet or floating freely in the medium. The end process, which followed in 2–4 days from the onset, was complete disruption and detachment of the cell monolayer.

Coverslip preparations of uninfected and BMV-infected DBC cells were fixed with methanol and stained with haematoxylin and eosin (Pl. 1, figs. 3, 4). Infected cultures showed large syncytia with varying numbers of nuclei, sometimes as many as 30–40 in one giant cell. Many nuclei contained single, homogeneous, eosinophilic inclusions separated from the marginated chromatin by a clear zone. The virus grew and produced large syncytia in several different cell types, such as CK, DBC and LT cells, reaching levels of 10^4–10^5 Cp.D50/ml. in the culture fluid. Such fluids, mixed with an equal volume of buffered glycerol (50%, v/v) and stored for 4 months at 4°, showed a loss of $10^{0.5}$ Cp.D50/ml., whereas samples stored without glycerol lost $10^{1.0}$ Cp.D50/ml. Storage at −20° was satisfactory for at least 4 months with or without added glycerol.

BMV was sensitive to ether and chloroform. With control virus titres of $10^{4.0}$–$10^{5.5}$ Cp.D50 per ml. of culture fluid, no infectivity could be detected in the treated preparations, even when tested undiluted.

Animal inoculation

Experimentally produced lesions in cattle resembled those of the natural disease (Martin *et al.* 1964; Martin, Martin, Hay & Lauder, 1966). Skin lesions were produced in guinea pigs and baby mice. In the former, on inoculation of BMV culture fluid into the skin of the back or of the hind-footpads, an inflamed area developed at the site of inoculation, followed by superficial exfoliation. White mice 1–3 days old could be infected readily with BMV, whereas those more than about 7 days old failed to develop lesions. On subcutaneous or intraperitoneal inoculation of infective fluid of the fifth to the tenth culture passage into newborn mice, inflamed patches appeared on the skin of the head and trunk after 4–7 days. The lesions were particularly severe around the eyes, nose and on the ears of some mice, presenting red or bluish areas of alopecia. Similar spots were sometimes also seen on the toes, legs or tail. At the first passage most of the mice developed skin lesions, lost weight and died within 10 days. In the next few passages, made with 10% mouse carcase suspension, deaths generally occurred by the fifth day. It has so far not been possible, however, to continue mouse-to-mouse transmission of the virus beyond about the 5th passage.

Chick embryos inoculated by either the intravenous, yolk sac, or chorioallantoic route did not die; nor were obvious lesions produced in the embryos.

Serology

BMV was allowed to react, in a neutralization test, with antisera against various members of the Herpesvirus group. No neutralization was obtained with any antiserum other than the homologous serum and that against Allerton virus (Table 1). In a further test, with equine herpes, types 1 and 2, malignant catarrhal fever (MCF), B virus antisera, and varicella acute and convalescent sera, there was no neutralization of BMV. In cross-neutralization tests with BMV and Allerton virus and their respective antisera, neutralization occurred in the homologous and in both heterologous virus+serum mixtures (Table 2).

Table 1. *Neutralization tests with BMV and various antisera*

*Control (normal rabbit serum)	Antisera						
	Herpes simplex	‡Herpes simplex	MCF	IBR	Pseudo-rabies	Allerton	BMV
5·8†	6·0	5·5	6·0	5·6	6·0	4·0	3·0

* All sera diluted 1/10, except ‡ diluted 1/5.
† Titre of BMV ($\log_{10}$ Cp.D 50/ml.).

Table 2. *Cross-neutralization of BMV and Allerton virus*

Virus	Rabbit normal serum	Rabbit Allerton antiserum	Guinea-pig Allerton antiserum	Guinea-pig BMV antiserum
Allerton	4*	1·5	$\leqslant$ 0·5	1·6
BMV	$\geqslant$ 6	2·3	$\leqslant$ 0·5	2·3

* Log_{10} Cp.D 50/ml.

In double-diffusion tests in agar, BMV and Allerton virus concentrates gave two bands of precipitate with both the homologous and the heterologous antiserum. No such bands were given by these antisera with a herpes simplex (HFEM strain) virus concentrate similarly prepared from infected BHK21/C13 cells and shown to react with a rabbit antiserum to herpes simplex. The BMV and Allerton virus concentrates did not react with this herpes antiserum. There was complete fusion of the respective bands formed by BMV and Allerton antiserum with a given virus preparation, indicating the similarity of the antigen–antibody systems concerned. Each virus completely absorbed the precipitins from both antisera.

Electron microscopy

Negatively stained PTA preparations of fluid from infected cultures showed particles which appeared circular in profile and had hollow capsomeres arranged in cubic symmetry. These particles measured about 80 mμ in diameter. Some were surrounded by a membrane which was readily distorted and even ruptured during preparation (Pl. 1, fig. 5). The diameter of completely enveloped particles varied widely and some measured up to 250 mμ.

In sections of infected cells, virus particles were seen both in the cytoplasm and in the swollen nuclei. The organization of the cytoplasm appeared to be disrupted. The complete particles resembled those of herpes simplex virus in having a dense

nucleoid surrounded by a double ring, and were approximately 100 mμ in diameter. Intranuclear virus particles were bounded by a single electron-opaque ring, in contrast to cytoplasmic particles which possessed two such rings (Pl. 1, fig. 6).

Virus DNA

The DNA from cells infected with BMV or Allerton virus was compared with respect to base composition and molecular weight (Table 3). Within the precision of the methods used, the DNA's of the two viruses were identical. The base composition of 64–65 % guanine plus cytosine is lower than the figure of 71 % G+C previously found for another bovine herpesvirus, IBR virus (Russell & Crawford, 1964). The molecular weight, as estimated from band width in equilibrium density gradients, is similar to that found for other viruses of the herpes group ($\sim 32 \times 10^6$). This value is a minimum estimate and other methods of molecular weight determination might be expected to give higher values.

Table 3. *Base composition and molecular weight of BMV and Allerton virus DNA*

DNA	Base composition (% G+C)	Molecular weight (NaDNA)
BMV	64	34×10^6
Allerton virus	65	32×10^6

DISCUSSION

Among mammalian viruses, the herpesviruses are characterized by having DNA as their genetic material and emerging from the cell nucleus with their capsid enclosed in an envelope. BMV possesses these two properties and this suggests that it is a member of the herpesvirus family. This classification is supported by several attributes in which this virus resembles the other family members: it multiplies in a variety of cultured cells, giving rise to syncytia in which many nuclei contain type A inclusions (Andrewes *et al.* 1961); it is readily inactivated by ether and chloroform; its DNA has a molecular weight similar to that of other herpesviruses; and its virion has the typical herpesvirus structure in negatively stained preparations and in sections of infected cells. BMV is therefore identified as another bovine member of the family of herpesviruses. Like other bovine herpesviruses, it produces syncytia and intranuclear inclusions in cultured cells. It resembles IBR virus (Armstrong, Pereira & Andrewes, 1961), but differs from most lines of MCF virus (Plowright, Macadam & Armstrong, 1965) in its relative ease of cultivation and its release in significant amount from infected cells into the culture fluid. The serological reactions of BMV separate it clearly from these and other herpesviruses, with the exception of the Allerton prototype strain of the group II viruses of lumpy skin disease (Alexander *et al.* 1957), from which it cannot be distinguished in standard neutralization and agar-precipitation tests.

The viruses of group II, of which BMV appears to be an errant member, have hitherto been found only in Africa, in cattle with lumpy skin disease. They are represented by 11 isolates from four different outbreaks in the Republic of South Africa, including the Elsie s River, Pentrich Grange, and Allerton strains (Alexander *et al.*

1957; Weiss, 1960); by one isolate from Ruanda-Urundi (Huygelen, 1960); and by one, the Kiel–Hansen strain, from Kenya. Now BMV, this antigenically identical, or closely related, virus, similar in cultural and other characteristics, has been isolated in Scotland, but from cattle with a different clinical syndrome. The virus does not appear to have been described before in Europe, and the question of how it came to be on this island so far from its relatives in Africa is a matter for conjecture. It may have been present in Britain for a long time, either as a relatively avirulent strain or associated with a clinical syndrome mistaken for cowpox. Alternatively, it may have derived from one of the causative agents of lumpy skin disease, introduced in the recent past from Africa, but for some reason provoking a different host response. It is possible, although there is no proof at present, that these group II viruses can exist as harmless commensals which occasionally produce virulent mutants of varying pathogenic capacity.

Until now, the group II viruses have been thought to be associated only with clinical cases of lumpy skin disease, though Huygelen and his colleagues (1960) recorded udder lesions in cows in Ruanda-Urundi. It is now clear that they can be associated with at least one other syndrome, that of bovine mammillitis (Martin *et al.* 1966). Whether these viruses may also be present in unaffected and perhaps relatively resistant cattle populations has yet to be determined. It may happen that viruses of BMV or of Allerton type will be discovered in other European countries in which neither kind of disease has been reported. With the aim of distinguishing, within group II, between BMV and lumpy skin disease viruses and so perhaps coming closer to answering some of these questions, it is hoped to make a more detailed comparison of BMV and Allerton virus, particularly with respect to the disease which they cause in laboratory animals, to any differences in their histopathology, and to the possibility of serological differentiation within the group.

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EXPLANATION OF PLATE

Figs. 1–4 are photomicrographs of 5-day monolayer cultures of DBC cells: Figs. 1 and 2 are unfixed and unstained; figs. 3 and 4 are methanol-fixed and stained with haematoxylin and eosin. The mark in figs. 5 and 6 measures 100 mμ.

Fig. 1. Uninoculated culture (× 65).

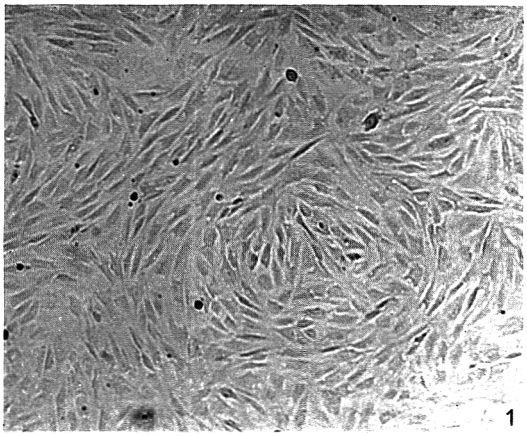
Fig. 2. Culture infected 24 hr. previously with BMV, showing formation of syncytia (× 65).

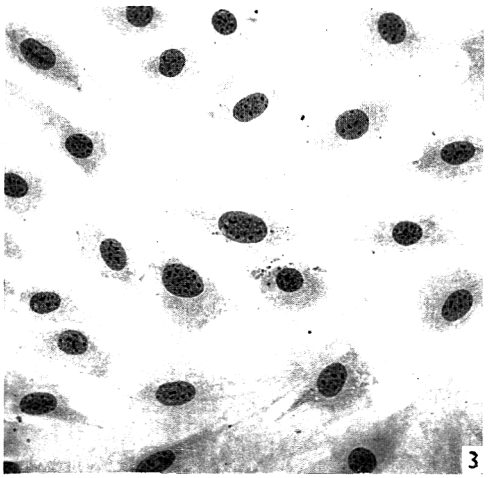
Fig. 3. Uninfected DBC cells (× 250).

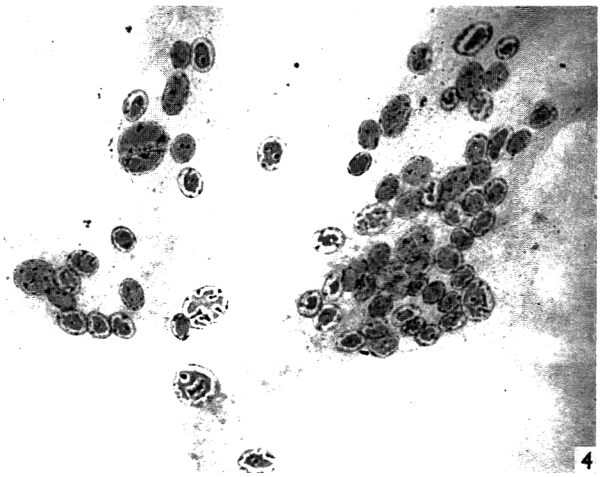
Fig. 4. Syncytia of BMV-infected DBC cells, showing type-A intranuclear inclusions in varying stages of development (× 250).

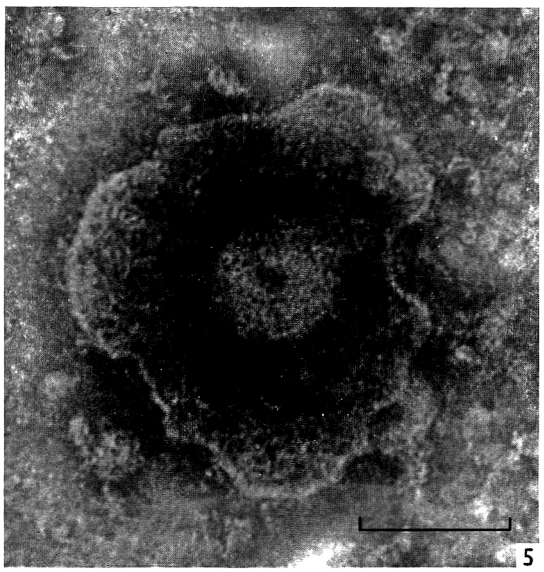
Fig. 5. Complete BMV particle in concentrate of infective culture fluid, negatively stained with PTA, showing capsid and large, loose envelope (× 200,000).

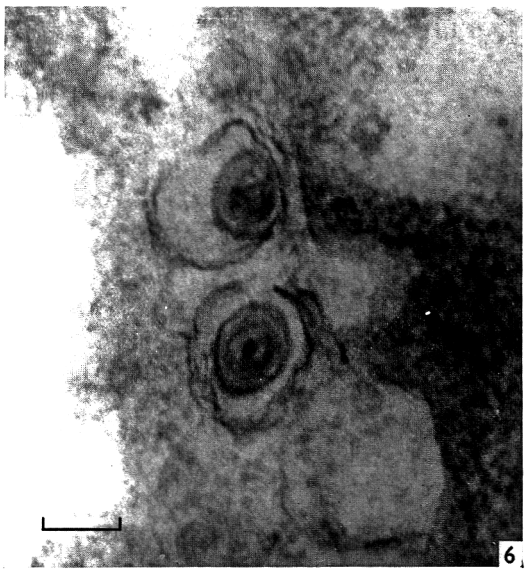
Fig. 6. Two BMV particles within cytoplasmic vesicles in 2-day-infected cell. Note nucleoid and double ring. (× 100,000.)











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The Nutritional Requirements of some Pasteurella Species

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SUMMARY

The nutritional requirements for growth on agar media at 28° and at 37° were investigated for strains representative of *Pasteurella pseudotuberculosis*, *P. pestis* and Pasteurella strain 'X' (*Yersinia enterocolitica*). At 28°, strains of *P. pseudotuberculosis* either required no growth factors or were dependent on thiamine or pantothenate. Most strains of *P. pestis* required cystine, methionine and phenylalanine but some strains could dispense with methionine or phenylalanine while others required additional factors. Strains of Pasteurella 'X' either were thiamine-dependent or required no factors. At 37° all strains showed additional requirements. Most strains of *P. pseudotuberculosis* could then grow with any 3 of the 4 factors glutamic acid, thiamine, cystine and pantothenate; other strains specifically required all 4 factors and nicotinamide. Strains of *P. pestis* grew reliably on media supplemented with cystine, methionine, phenylalanine, glycine, valine, isoleucine, glutamic acid and thiamine when incubated in CO_2-enriched air. All strains of Pasteurella 'X' required thiamine and either cystine or methionine; some substrains showed additional requirements. Virulent and avirulent representative strains of *P. pestis* had similar calcium requirements for growth on defined media.

INTRODUCTION

Auxotrophic markers have proved among the most useful of those used in studying the genetics of micro-organisms. With knowledge of the minimum nutritional requirements of parent organisms one can hope to derive auxotrophic mutant strains and devise highly selective media capable of detecting rare recombinational events involving the genetic determinants of auxotrophy. As a preliminary to a study of the genetics of *Pasteurella pseudotuberculosis* we investigated the requirements of this species, as reported here. Since this species has many properties in common with *P. pestis* it was of interest to observe whether the resemblances between the two extended to the nutritional requirements. Accordingly we have re-examined some aspects of the nutrition of *P. pestis* in parallel with *P. pseudotuberculosis*. In addition we also investigated strains of Pasteurella 'X' (Knapp & Thal, 1963), a species which, as yet, has not received intensive study but seems to have close relationship to *P. pseudotuberculosis*. Recently Mollaret & Lucas (1965) used the name *Yersinia enterocolitica* for Pasteurella 'X' and have given references to other synonyms.

Unlike previous investigators who have examined the nutrition of pasteurellas (Rao, 1939, 1940; Berkman, 1942; Doudoroff, 1943; Herbert, 1949; Hills & Spurr, 1952; Higuchi & Carlin, 1958; Brownlow & Wessman, 1960), we attempted to

define nutritional factors which permit reliable growth of organisms isolated on agar surfaces rather than of inocula in liquid media. We chose to do so, first because solidified media have greater application to genetic studies and, secondly to ensure that we were observing the requirements of the majority population of strains rather than those of meiotrophic minorities, which in liquid cultures might overgrow and obscure the response of the majority.

METHODS

Organisms. Pasteurella pseudotuberculosis. Representatives of each of the 5 known serological types A, B, C, D, E (Thal, 1954) were examined in detail. They were the strains PB1/+(type A), 16II(B), 43III(C), 32IV(D) and 25V(E). We included also the strain PB1/− which is a VW− derivative of PB1/+ (Burrows & Bacon, 1960) and the strains MD31 and Nielson which are of interest in being VW+ strains of the relatively rare serotypes C and D, respectively. The strain Nielson is an example of a VW+ isolation from a human infection. Apart from MD31 (see below), the above and other strains mentioned in the text were from our own culture collection.

Pasteurella pestis. The virulent strain MP6 and the avirulent (VW−) strain TS were used to identify requirements for growth at 37°. A culture of strain MP6 recently passaged in mice was used to ensure minimum contamination of the culture with spontaneously arising avirulent derivatives. Seventy-three additional strains from our collection were examined for their ability to grow at 28° on a defined medium which we had previously developed for use with strain TS at this temperature (Burrows & Bacon, 1954). Strains which differed nutritionally from this strain are referred to individually in the text.

Pasteurella 'X'. Five strains were investigated. Two we received from Dr E. Thal (Statens Veterinarmedicinska Anstalt, Stockholm), labelled 'Winblad Pp-X 346' and 'Tungelsta Pp-X 370', which we refer to as strains XA and XB, respectively. Three other strains X66, X69 and X70 were kindly provided (together with the *P. pseudotuberculosis* strain MD31 above) by Dr R. R. Brubaker, (Fort Detrick, Frederick, Maryland, U.S.A.).

Media. BAB: blood agar base no. 2 (Oxo Limited) supplemented with $Na_2S_2O_5$ 0·1 g./l. was used as the complete medium. The different minimal media used for the identification of growth requirements were as follows.

MA: (g./l. distilled water) K_2HPO_4, 10·5; KH_2PO_4, 4·5; $(NH_4)_2SO_4$, 1·0; sodium citrate, 0·5; $MgSO_4.7H_2O$, 0·1; agar (Oxoid No. 3), 15; adjusted to pH 7·2 and autoclaved at 115° for 15 min.; glucose, 2·0, added after autoclaving.

MAS: this was medium MA to which was added $Na_2S_2O_5$, 0·1, after autoclaving.

MASGF: this was medium MAS+(mM) glycine, 1·0; DL-isoleucine, 0·5; DL-valine, 0·2; L-cystine, 0·2; DL-methionine, 0·2; DL-phenylalanine, 0·2. The concentrations given are those of the L-isomer of racemic mixtures.

Growth factors tested (μM final concentration in media): the 20 amino acids, 200; the purines adenine, guanine, xanthine, hypoxanthine, 50; the pyrimidines cytosine, thymine, uracil, 50; haemin, 10; the vitamins inositol, pimelic acid, 100; choline chloride, 20; nicotinamide, *p*-aminobenzoic acid, 5; pyridoxine, 2; Ca pantothenate, riboflavine, thiamine, 1; folic acid, 0·1; biotin, 0·01.

The customary precautions about purity of chemicals and cleanliness of glassware were observed throughout. Disposable plastic Petri dishes containing 20 ml. medium agar were used in all experiments. Growth factors were added as separate sterile solutions to the melted agar media just before plates were poured.

Suspensions of organisms. All strains were stored as dried pellets (Stamp, 1947). For use pellets were streaked on BAB plates, incubated for 2 days at 28° to confirm their purity and the resulting growth subcultured to BAB slopes. These were incubated for 17 hr at 28°, the organisms suspended in phosphate buffer (0·033 M, pH 7·0) and diluted (*c.* 10^6-fold) in buffer to give 2×10^3 organisms/ml. Of the final dilution, 0·1 ml. samples were spread over the surfaces of complete agar media and of the defined minimal media under test.

Incubation. Effort was made to ensure correct incubator temperatures. For experiments at 28° small variations in temperature did not influence the results, but for those conducted near 37° the temperature was critical. For experiments at 37° an incubator fitted with an air-circulating fan and housing a recording thermograph was used. Its thermostat was adjusted to give a minimum temperature of 37°; this setting gave a maximum of 38°. Plates incubated in CO_2-enriched atmospheres were housed in sealable cylindrical containers, 25 cm. diam, and 25 cm. deep, and gassed with a mixture of 95 % (v/v) air + 5 % (v/w) CO_2 for 5 min. before sealing and placing in the incubator. The containers were re-gassed daily. All plates were examined daily and scored for macroscopically visible colonies over an 8-day period of incubation.

Criteria of growth. Defined media were considered satisfactory when they permitted reliable growth of the sparsely seeded organisms into macroscopically visible colonies (1·0 mm. diam.) within 3 days of incubation (4 days for the slower-growing *Pasteurella pestis*) and yielded colony numbers similar to those given by equal samples of suspensions plated on the complete medium.

Abbreviations. Growth factor names are abbreviated in the tables according to current usage in publications on microbial genetics:

ala	alanine	*ile*	isoleucine	*ser*	serine
asp	aspartic acid	*met*	methionine	*thi*	thiamine
cys	cystine	*nic*	nicotinamide	*thio*	thiosulphate
glu	glutamic acid	*pan*	pantothenate	*thr*	threonine
gly	glycine	*phe*	phenylalanine	*try*	tryptophan
				val	valine

RESULTS

Nutritional requirements for growth at 28°

Pasteurella pseudotuberculosis. In preliminary tests all strains grew on medium MASGF, a defined medium which we had used extensively for the growth of *Pasteurella pestis* at 28°. Simplification of this medium by the omission of all amino acids and of bisulphite did not impair its efficiency as a plating medium for strains of *P. pseudotuberculosis* of types A, B or E, which thus were independent of any growth factors. These strains plated on medium MA produced colonies 1 mm. in diameter within 3 days and gave viable counts equal to, or frequently exceeding, those on complete medium. The strains 43III, MD31 and 32IV grew slowly and

inconsistently on medium MA but supplementation of this medium with thiamine or pantothenate permitted their growth at a rate similar to that shown by the above non-exacting strains. The single additions of valine, glutamic acid or methionine to medium MA accelerated the growth of strains 43 III and 32 IV but less effectively than the single additions of thiamine or pantothenate. The ability of these strains to grow on medium MASGF, which does not contain these vitamins, indicated that they were replaceable by the amino acid supplements contained in this medium. The type D strain Nielson resembled the strains of types A, B and E in having no growth factor requirements, unlike the other type D strain, 32 IV. Cultures of strain 25 V contained a minority population which showed a thiamine- or pantothenate-dependence.

Thus for *P. pseudotuberculosis* medium MA supplemented with thiamine (or pantothenate) was a medium suitable for the growth of all tested strains and for the identification and characterization of auxotrophic derivatives of them. In other studies we have used this medium successfully for all these purposes.

Pasteurella pestis. The requirements of representatives of this species (strains TS and MP 6 and derivatives of them) had already been defined. Essential factors were cystine, phenylalanine and methionine, with glycine, valine and isoleucine as stimulatory factors (Burrows & Bacon, 1954). To ensure full viable counts on agar surfaces from small inocula the addition of bisulphite, or haemin (Herbert, 1949) is necessary, even on complete media. In this study we examined the behaviour of several other strains to identify those requiring additional factors and those for which cystine, phenylalanine and/or methionine were dispensible. Strains having additional requirements were characterized by conventional auxanographic techniques. The majority of strains (49 of 73 tested) were nutritionally similar to strains TS and MP 6; those which differed are shown in Table 1. Although strains independent of methionine or of phenylalanine were observed no strain appeared capable of dispensing with cystine and thus to be able to utilize SO_4^{2-} as a sole source of sulphur. (We would note here that thiosulphate can effectively replace any stated requirement for cystine throughout this paper.)

Table 1. *Strains of Pasteurella pestis differing nutritionally from P. pestis strain* TS *at 28°*

P. pestis strain TS requires cystine + methionine + phenylalanine

Strains not requiring:	
methionine:	A 28, WCDC Ppl
phenylalanine:	A 36, Harbin, H III 50, M 38, M 39, M 40, Yokohama
Strains additionally requiring:	
arginine:	Bryans, Elizabeth, NM 499668, O 19, O 24, WCDC Ppl, WCDC Pp5, WCDC Pp6
leucine	O 23
proline	A 16, A 29
tryptophan:	H 27 L
uracil:	Butembo, Elizabeth, EVGS, Hurni.

Pasteurella 'X'. Strain XB and the majority components of cultures of XA and X 66 grew freely on medium MA and were therefore independent of growth factors. Tests with the minority component of strain XA (which we called strain XAb) showed

it to be dependent on thiamine or valine. The finding that valine could replace thiamine was unexpected and led us to test the purity of our valine by examining its ability to support the growth of two thiamine-dependent mutant strains of *P. pseudotuberculosis* and two of *Salmonella typhi*. None of these 4 mutants could use valine as an alternative to thiamine; all grew freely on medium MA + thiamine and on medium MA + thiamine + valine. We concluded that our valine was free from detectable thiamine and that it could in fact replace thiamine as a growth factor for strain XAb. In a further test each of 12 samples of valine from different sources showed growth factor activity for this strain. A sample of norvaline was however inhibitory and prevented growth even in the presence of valine or thiamine. Strain x70 resembled strain XAb in requiring thiamine or valine although here thiamine was the preferred factor. The minority component x66b of strain x66 required cystine + thiamine but seemed unable to use valine as a replacement for thiamine. We were informed by Dr R. R. Brubaker that strain x69 was tryptophan-dependent and this we confirmed; it had no other requirements.

The growth factor requirements of the representatives of the 3 Pasteurella species for growth at 28° are summarized in Table 2.

Table 2. *Nutritional requirements for growth of Pasteurella spp. on agar media at 28°*

			Growth factors						
Species	Strain	Type	*try*	*cys*	*met*	*phe*	*val*	*thi*	*pan*
P. pseudotuberculosis	PB 1/+	A VW+	.	.	.	.	.	.	.
	PB 1/−	A VW−	.	.	.	.	.	.	.
	16 II	B VW−	.	.	.	.	.	.	.
	MD 31	C VW+	.	.	.	.	.	+	+
	43 III	C VW−	.	.	.	.	.	+	+
	Nielson	D VW+	.	.	.	.	.	.	.
	32 IV	D VW−	.	.	.	.	.	+/+	
	25 V	E VW−	.	.	.	.	.	.	.
P. pestis	MP 6	VW+	.	+	+	+	.	.	.
	TS	VW−	.	+	+	+	.	.	.
Pasteurella 'X'	XA		.	.	.	.	.	.	.
	XA*b*		.	.	.	.	+/+		.
	XB		.	.	.	.	.	.	.
	x66		.	.	.	.	.	.	.
	x66*b*		.	+	.	.	.	+	.
	x69		+	.	.	.	.	.	.
	x70		.	.	.	.	+/+		.

+ = growth factor required; . = growth factor not required; +/+ = alternative factors.

Nutritional requirements for growth at 37°

Pasteurella pseudotuberculosis. The strains PB 1/+, PB 1/−, 16 II, Nielson, MD 31 and 25 V behaved similarly and may be considered together. None could grow on medium MA or on medium MAS at 37°, but all grew to some extent, although not reproducibly or uniformly, on medium MASGF. Addition of pantothenate or of thiamine to this medium permitted reliable growth within 4 days. Omission of individual factors contained in medium MASGF + pantothenate again resulted in inconsistent growth, suggesting either that all components were essential or that

some factors were inhibitory in the absence of others. At this point it seemed simpler to determine what additions to medium MA were necessary for growth. No single addition of the factors present in medium MASGF was effective. Addition of bisulphite (to give medium MAS) permitted some slow growth on the further addition of pantothenate or thiamine, but not on the addition of other factors singly. The further addition of cystine to medium MAS + pantothenate or thiamine improved the response of all strains whereas similar addition of methionine was inhibitory. Although no growth occurred on medium MAS + cystine the plated organisms retained their viability throughout 8 days of incubation and were capable of giving full colony numbers when subsequently re-incubated at 28°.

On a basal medium of MAS + cystine + pantothenate, thiamine and glutamic acid were effective supplements to promote early and reliable growth of all strains. Tests of medium MAS supplemented with all permutations of these 4 factors showed none to be effective when added singly, thiamine + glutamic acid to be the best of paired additions, and any triplet to be similarly effective to any other triplet and to all 4 factors together. These findings are shown in Table 3. Similar experiments with strains PB 1/+ and PB 1/− incubated in CO_2-enriched air gave results comparable with those obtained above.

Table 3. *Growth responses of some strains of Pasteurella pseudotuberculosis at 37°*

Basal medium was MAS. Organisms were plated at an estimated concentration of 200 organisms/plate; the plates were incubated at 37° in air. Numbers of colonies are given only for those media on which colonies predominantly exceeded 1 mm. diam. For other media: s = predominantly small colonies (*c.* 0·5 mm. diam.); m = predominantly minute colonies (*c.* 0·2 mm. diam.); . = no visible colonies. The figures give the means of duplicate plates in each of three experiments.

	Strain														
	PB 1/+			PB 1/−			16 II			Nielson			25 v		
	Time of incubation (days)														
	2	4	8	2	4	8	2	4	8	2	4	8	2	4	8
Medium supplements	no. of colonies														
Nil	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
pan	.	.	m	.	m	m	.	s	s	.	.	s	.	.	m
cys	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
thi	.	.	s	.	.	s	.	m	s	.	.	s	.	.	s
glu	.	.	m	.	.	m	.	m	m	.	.	m	.	.	m
pan cys	.	.	s	.	m	s	.	s	95	.	m	126	.	m	m
pan thi	.	.	s	.	m	s	.	s	142	.	.	130	.	s	136
pan glu	.	m	s	.	s	s	.	s	s	.	.	s	.	s	s
cys thi	.	117	121	.	141	149	.	.	122	.	.	128	.	.	122
cys glu	.	.	s	.	.	s	.	.	.	.	.	m	.	.	.
thi glu	.	154	154	s	173	193	.	165	165	.	173	177	.	173	173
pan cys thi	.	107	109	s	99	101	s	144	145	.	124	124	s	107	135
pan cys glu	.	122	131	s	146	151	s	140	161	.	76	116	s	115	122
pan thi glu	.	172	172	m	190	193	.	142	142	.	145	145	.	168	169
cys thi glu	.	168	168	m	192	192	.	129	154	.	161	161	.	154	159
pan cys thi glu	.	157	165	s	145	146	s	131	144	.	125	135	s	157	165
(Complete medium)	159	162	162	177	177	177	146	146	145	165	165	165	153	153	153

On a basal medium of MAS supplemented with thiamine, glutamic acid, cystine and pantothenate, we looked for a growth-stimulatory effect of calcium and of bicarbonate on the VW+ strains PB1/+ and Nielson. Comparable strains of *Pasteurella pestis* are reported to be selectively stimulated by these factors (Surgalla, Andrews & Baugh, 1964). However, we did not detect such effect; additions of $CaCl_2$ over the range 0·0025 M to 0·02 M showed progressively greater inhibition of growth with all strains, while $NaHCO_3$ added over the same range was without detectable effect. The medium was not altered in its growth-producing efficiency by substituting glycerol as C-source in place of glucose, or urea as N-source in place of ammonium sulphate. Thiosulphate adequately replaced cystine as a source of sulphur for all strains, but slightly decreased the growth rate of strain PB1/+. The mixture glycerol, ammonium sulphate and cystine tended to give more robust colonies after the complete 8 days of incubation than did other permutations of the alternative C-, N- and S-sources.

The optimum pH value for growth on medium MAS + thiamine + glutamic acid + cystine + pantothenate medium was between 7·0 and 7·2. The minimum tolerated pH value was 6·8 and the maximum 9·0.

Pasteurella pseudotuberculosis strains 43 III and 32 IV behaved differently from those described above but similarly to each other. On the medium developed for the above strains few or no colonies developed within 8 days. Supplementation of this medium with aspartic acid, or with nicotinamide, permitted all plated organisms to produce visible colonies within 4 or 3 days, respectively. They therefore showed an alternative requirement for one or other of the factors aspartic acid or nicotinamide. Tests of our aspartic acid with two nicotinamide-dependent mutants of strain PB1/+ showed that it was not detectably contaminated with nicotinamide. Thus a medium consisting of MAS + thiamine + glutamic acid + cystine + pantothenate + nicotinamide supported reliable growth of strains 43 III and 32 IV; omission of each factor singly showed each to be essential.

Pasteurella pestis. It was known that strains of this species had additional requirements for growth at 37° and consequently would not grow on medium MASGF. Supplementation of this medium with biotin + pantothenate, with serine + threonine or with proline (factors which past workers have concluded to be essential for the growth of this organism at 37°), or with all these factors together, did not permit any colony formation. The further addition of glutamic acid to a medium containing all the above supplements sometimes allowed growth of a small fraction of the total plated organisms. Consistently reliable growth of both the strains TS and MP6 eventually was achieved on a medium comprising MASGF in which cystine was replaced by thiosulphate, with glutamic acid and thiamine added, followed by incubation in CO_2-enriched air.

The requirement for additional CO_2 was removed when the medium was supplemented with liver extract, with mouse blood or with casein hydrolysate. The activity of the last supplement suggested that an amino acid or acids could replace CO_2 for growth and led to the identification of serine + threonine as an effective pair. It was necessary, however, to use serine at 0·02 mM, i.e. one-tenth of the concentration routinely used, while keeping threonine at 0·2 mM. Growth did not occur in air when these amino acids were both used at 0·2 mM but in the presence of additional CO_2 the higher concentration of serine was not inhibitory. Later it was shown that

the two amino acids could themselves be replaced by homoserine 0·2 mM. We would note here, however, that on some occasions poor growth was obtained with the above supplements whereas, in the same experiments, full growth resulted on unsupplemented plates incubated in CO_2-enriched air; we therefore regard the latter condition as being the more reliable. Not infrequently pantothenate appeared to be able to replace thiamine, but we did not find conditions where such replacement was consistently effective and therefore regard thiamine as the more reliable growth factor.

We concluded from this part of our study that the most reliable defined medium for the growth of our representative strains of *Pasteurella pestis* was that consisting of medium MAS supplemented with the thiosulphate, glycine, valine, isoleucine, methionine, phenylalanine, glutamic acid, thiamine, pH 6·8 (see later), with incubation in CO_2-enriched air. Whereas for growth in air glycine and glutamic acid appeared to be essential, their omission from media incubated with CO_2-enrichment resulted only in delayed initiation of growth. Omission of any other factor prevented growth.

On media containing serine + threonine reasonable growth occurred only over the range from pH 6·4 to 7·0 in air. For growth on the same medium in CO_2-enriched air the pH value was less critical and high counts were obtained within 3 days over the tested range pH 6·0 to 7·2 (Table 4). On media not containing serine and threonine the tolerated pH values for growth in CO_2-enriched air were 6·4 minimum and 8·2 maximum; on all the tested media the optimum pH value appeared to be near to 6·8. It is probable that much of the inconsistent behaviour we experienced with

Table 4. *Influence of pH value on the growth of Pasteurella pestis strain TS on defined agar medium at 37°*

Defined medium was MAS + thiosulphate + glycine + valine + isoleucine + methionine + phenylalanine + glutamic acid + serine (0·02 mM) + threonine + thiamine. The medium was adjusted from its initial pH 7·2 to the required values by adding N-HCl. Plates were seeded with about 200 organisms, divided into two sets of which one was incubated in air and the other in air enriched with CO_2 (5 %, v/v) at 37°. The figures are the means of duplicate plates in each of two experiments.

	Gas phase					
	Air				95 % (v/v) air + 5 % (v/v) CO_2	
	Time of incubation (days)					
Defined medium at pH	3	4	6	8	3	8
	Colony numbers					
7·2	0	20	65	78	166	166
7·0	54	141	161	165	189	189
6·8	53	150	162	168	230	230
6·6	37	91	149	153	223	224
6·4	33	128	160	161	236	236
6·2	0	13	65	82	195	195
6·0	0	3	42	52	182	193
BAB complete medium	200	200	200	200	204	204

Pasteurella pestis in experiments before the recognition of the beneficial effects of CO_2 resulted from the use of defined media at pH 7·2 which is seen to be near the upper limit of tolerable pH value for growth in air.

It has been observed that virulent strains of *Pasteurella pestis* have an apparent dependence on Ca ions, not shown by VW − avirulent strains, since the former are inhibited when plated on complete media supplemented with Mg ion + oxalate, whereas the latter grow freely, at 37° (Higuchi & Smith, 1961; Brubaker & Surgalla, 1962). As we had not observed our representative strains to differ in growth factor requirements in this study it seemed of interest to examine their relative responses to Ca-deficiency on the defined medium now available. This contained Mg at 0·4 mM but no Ca other than that contaminating the ingredients. Of these the agar would have contributed the bulk of the Ca in the medium and, according to data supplied by the manufacturers, would have yielded a Ca concentration of 0·13 mM. We anticipated therefore that additions of Na oxalate in excess of this concentration would selectively inhibit the virulent representative strain MP6 if this, in fact, had a requirement for Ca in excess of that of the VW − strain. No differential inhibition was however observed over a range of additions of Na oxalate from 0·25 mM to 20 mM. Titration of the optimum addition of Ca to the medium showed this to be 4 mM, and of Mg 8 mM, for both strains. The single addition of Ca 20 mM

Table 5. *Comparative behaviour of Pasteurella pestis strains* TS (*VW*−) *and* MP6 (*VW*+) *on defined media variously supplemented with oxalate, Mg and Ca*

Complete medium was BAB. Defined medium was MAS + thiosulphate + glycine + valine + isoleucine + methionine + phenylalanine + glutamic acid + thiamine; pH 6·8. Supplements: Ox = Na oxalate, Mg = $MgCl_2$, Ca = $CaCl_2$ each to give 20 mM except where specified. To permit easier enumeration of colonies on the white opaque media resulting from the double supplementation with Ox and Ca, triphenyl tetrazolium chloride was added to all plates to a final concentration of 0·005 %. Plates were incubated at 37° in 95 % (v/v) air + 5 % (v/v) CO_2 and counted daily. Counts are expressed as % of the number of colonies developing on unsupplemented medium BAB. The figures give the means of duplicate plates in each of three experiments.

		Strain					
		TS			MP6		
		Time of incubation (days)					
		3	4	8	3	4	8
Medium	Supplement(s)	Colony numbers (%)					
BAB		100	100	100	100	100	100
	Ox + Mg	109	109	109	3	6	48
Defined		64	89	95	69	104	109
	Ox 2·5 mM	37	81	93	74	106	125
	Ox 5 mM	45	62	86	57	83	107
	Ox 10 mM	38	57	64	59	67	105
	Mg	35	76	94	76	80	115
	Ca	0	10	74	0	21	74
	Ox + Mg	26	96	114	16	51*	98
	Ox + Ca	75	87	105	90	120	124
	Mg + Ca	21	29	33	19	32	39
	Ox + Mg + Ca	11	91	98	0	59	95

* All of 10 colonies taken at random proved virulent for mice.

inhibited both strains similarly; Mg at this concentration showed no marked inhibition of either. The combination of oxalate + Mg both at 20 mM (as used in the differential medium of Higuchi & Smith) showed some selective inhibition of the virulent strain but not to an extent which could be of diagnostic value (Table 5).

Pasteurella 'X'. Like the other two species, strains of Pasteurella 'X' were nutritionally more exacting at 37° than at 28°. All showed a requirement for thiamine; at 37° valine was not an effective alternative to this vitamin. These strains also showed impaired sulphur metabolism. Strain XA specifically required methionine, strains x66 and x70 cystine; the remaining strains XB and x69 required either methionine or cystine. We observed a requirement for alanine by strains x66 and x69 which we had not observed with any other Pasteurella strain in this work. The substrain x66b in addition required glutamic acid + pantothenate + nicotinamide and thus resembled the strains 43 III and 32 IV of *P. pseudotuberculosis*. Unlike these however substrain x66b could not use aspartic acid as an alternative to nicotinamide. Provision of additional CO_2 did not alter the requirements of the strains of Pasteurella 'X'.

Table 6. *Nutritional requirements for growth of pasteurellas on agar media at 37°*

			Growth factors												
Species	Strain	Type	*ala*	*try*	*cys*	*met*	*phe*	*gly*	*ile*	*val*	*glu*	*thi*	*pan*	*nic*	CO_2
*P. pseudotuberculosis**	PB 1/+	A VW+	.	.	.	.	.	.	.	.	+	+	.	.	.
	PB 1/−	A VW−	.	.	.	.	.	.	.	.	+	+	.	.	.
	16 II	B VW−	.	.	.	.	.	.	.	.	+	+	.	.	.
	MD 31	C VW+	.	.	.	.	.	.	.	.	+	+	.	.	.
	43 III	C VW−	.	.	+	.	.	.	.	.	+	+	+	+	.
	Nielson	D VW+	.	.	.	.	.	.	.	.	+	+	.	.	.
	32 IV	D VW−	.	.	+	.	.	.	.	.	+	+	+	+	.
	25 V	E VW−	.	.	.	.	.	.	.	.	+	+	.	.	.
P. pestis	MP 6	VW+	.	.	+	+	+	+	+	+	+	+	.	.	+
	TS	VW−	.	.	+	+	+	+	+	+	+	+	.	.	+
Pasteurella 'X'	XA		.	.	.	+	.	.	.	.	.	+	.	.	.
	XA b		.	.	.	+	.	.	.	.	.	+	.	.	.
	XB		.	.	+	/	+	.	.	.	.	.	+	.	.	.
	x66		+	.	+	.	.	.	.	.	.	+	.	.	.
	x66b		+	.	+	.	.	.	.	.	+	+	+	+	.
	x69		+	+	+	/	+	.	.	.	.	.	+	.	.	.
	x70		.	.	+	.	.	.	.	.	.	+	.	.	.

+ = growth factor required; . = growth factor not required; +/+ = alternative factors.

* Strains of *P. pseudotuberculosis*, other than 43 III and 32 IV, grew well in the presence of any 3 of the 4 factors: *glu*, *thi*, *pan*, *cys*. Of paired additions *glu* + *thi* was preferred.

The optimum pH value determined for the growth of strain XA on MA + methionine + thiamine was about pH 7·8, but little difference in growth rate or in colony numbers was seen over the range pH 7·2 to 8·0. Although colonies developed less rapidly at higher pH values full colony numbers were obtained throughout the range pH 7·2 to 9·0. In contrast, no colonies developed on media with initial pH values of 6·8 or lower.

The growth factor requirements of the representatives of the 3 Pasteurella species for growth at 37° are summarized in Table 6.

DISCUSSION

The strains of *Pasteurella pseudotuberculosis* examined differed from those of *P. pestis* for growth at 28° in having no growth factor requirements, or a simple requirement for thiamine or pantothenate. This was so for 75 of 76 strains from our collection; the exception was *P. pseudotuberculosis* strain 74R which was dependent on additional, unidentified factor(s). It is however not difficult to select strains of *P. pestis* which nutritionally resemble strains of *P. pseudotuberculosis* in having no amino acid requirements by being phenylalanine- and methionine-independent and, like their parents, capable of utilizing thiosulphate in place of cystine. Such strains are still distinguishable from *P. pseudotuberculosis* by their inability to use SO_4^{2-} as a sole source of sulphur (Englesberg, 1952). It is of interest to note that amino acid independent strains of *P. pestis* isolated by us retain the high virulence characteristic of this species.

From our survey of the requirements of strains of *Pasteurella pestis* for growth at 28° it is notable that those classified by Devignat (1951) as 'var. *mediaevalis*' (on criteria of geographical distribution, ability to ferment glycerol and inability to reduce nitrate) were all phenylalanine-independent. In this respect they differed from strains classified in the varieties '*antiqua*' (glycerol +, nitrate+) and '*orientalis*' (glycerol −, nitrate+). Possibly the distinctive behaviour of the virulent strain Yokohama in the studies of Wessman, Miller & Surgalla (1958) was associated with its independence of phenylalanine. A second notable point is that whereas Rao (1939) identified proline as an essential factor for the growth of *P. pestis*, strains with this requirement seem to be rare; only two such strains were identified among the 73 examined here. Arginine-dependence seems to be more common among strains of recent isolation in North America (strains prefixed WCDC and NM) than among those isolated elsewhere.

Experiments conducted at 28° in the present work generally were reproducible and gave clear indications of the essential requirements for growth with all strains. This was by no means so for those conducted at 37°, particularly with *Pasteurella pestis*. One reason for the conflicting results with this organism became clear when the influence of CO_2-enrichment was discovered. We were then able to show that growth or no growth on some defined media depended on the chance presence or absence of cultures being grown for other purposes in the incubator. Similarly, when plates of defined media were housed in containers (initially to prevent desiccation during prolonged incubation) growth could occur when complete medium control plates were housed in the same containers but not otherwise. By using CO_2-enriched atmospheres and media at pH 6·8 we removed most of the irregular behaviour earlier experienced with this organism. Strains of the other two species showed no requirement for additional CO_2. Nevertheless, they were CO_2-dependent as shown by their delayed growth and poor viability on incubation at 37° in air from which CO_2 had been removed by absorption with soda-lime, even when plated on complete media.

All three Pasteurella species were similar in having additional requirements for growth at 37° over those required at 28°, as was first shown for *Pasteurella pestis* by Hills & Spurr (1952). Impaired thiamine-synthesis was noted with all representatives. The interchangeability of aspartic acid and nicotinamide for the growth of

some strains of *P. pseudotuberculosis* accords with the findings of Ortega & Brown (1960) and of Albertson & Moat (1965) that the biosynthesis of nicotinic acid involves condensation of aspartic acid with a 3C compound.

On agar solidified media our virulent and avirulent strains of *Pasteurella pestis* showed equal dependence on added CO_2 for growth; this contrasts with the results of others with liquid media where only virulent strains showed such dependence (Delwiche, Fukui, Andrews & Surgalla, 1959; Surgalla, Andrews & Baugh, 1964). In studies with cell-free extracts Baugh, Lanham & Surgalla (1964) found that materials from virulent and from avirulent (VW −) organisms equally were capable of fixing CO_2 by carboxylation of phospho-enolpyruvate to give oxalacetate and thence aspartate. In tests which we have not detailed here neither of these products was capable of replacing CO_2 for growth in air under our conditions. In similar contrast, we did not detect any marked difference in the calcium requirements of our two representatives whereas with liquid media, Higuchi, Kupferberg & Smith (1959) noted a higher requirement on the part of virulent strains. Gadgil (1964), however, in studies with a virulent strain growing in liquid media observed a complex response to Mg and Ca additions which could not simply be explained in terms of the availability of Ca for growth. The selective inhibition of virulent strains on the addition of Mg oxalate to complete agar media (Higuchi & Smith, 1961; Brubaker & Surgalla, 1962), which is attributed to Ca-deficiency, may have another basis.

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Observations on the Adsorption of Caulobacter Bacteriophages containing Ribonucleic Acid

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SUMMARY

Caulobacter bacteriophages containing ribonucleic acid have been found to attach to pili of their hosts. Only when the phage is actually lytic for a given host can the phage+pilus complexes be observed; non-specific complexes are not formed. Removal of the pili from the host by shear treatment before phage adsorption resulted in effective inhibition of phage adsorption. While swarmer caulobacters frequently possessed pili which were located at the flagellated pole of the organism, stalked caulobacters were almost always devoid of these structures. Adsorption experiments with segregated populations of caulobacters indicated that stalked caulobacters adsorbed RNA phages very poorly, while in swarmer populations and unsegregated cultures the RNA phages were efficiently adsorbed.

INTRODUCTION

The demonstration by Crawford & Gesteland (1964) that R17, a male specific bacteriophage containing ribonucleic acid (RNA), attached to pili of F^+ and Hfr strains of *Escherichia coli* drew attention to the processes of RNA-phage adsorption and infection (Brinton, Gemski & Carnahan, 1964; Bradley, 1965; Brinton, 1965; Valentine, Wedel & Ippen, 1965; Edgell & Ginoza, 1965). Among the bacteriophages lytic for Caulobacter species isolated by Schmidt & Stanier (1965) are several small spherical viruses which contain RNA. It would be of interest to know whether pili to which RNA 'caulophages' can attach occur in caulobacters. The present paper reports an investigation to determine whether the process of RNA-phage adsorption in these stalked bacteria is similar to that observed with *E. coli* + RNA-phage systems, and how stalk formation in caulobacters affects RNA-phage adsorption.

METHODS

Bacteriophages and hosts. Bacteriophages ΦCb8r, ΦCb12r, and ΦCb23r, and bacterial strains *Caulobacter bacteroides* CB 8 and CB 11a, *C. crescentus* CB 2 and CB 15, and *C. fusiformis* CB 27 and CB 29 were used in this work. These RNA-phages were isolated and described by Schmidt & Stanier (1965); the Caulobacter strains originated from the collection of Dr Jeanne Stove Poindexter.

Media. For the propagation of host strains, preparation of phage lysates, and adsorption experiments, the standard complex caulobacter medium (PYE) was used (Poindexter, 1964).

Lysate preparation and partial purification of bacteriophages. Lysates were pre-

pared by eluting phages from confluently lysed PYE agar overlayers. The lysates were concentrated and partially purified by two cycles of differential centrifugation (Schmidt & Stanier, 1965).

Preparation for electron microscopy. PYE broth cultures of Caulobacter strains were grown at 30° without shaking to the late exponential phase of growth. Two ml. of the culture were sedimented by centrifugation at 7000 *g*, and to the bacterial pellet were added 2 ml. of a phage preparation containing 10^{10} to 10^{11} plaque-forming units (p.f.u.). The bacterial pellet was gently resuspended and phage adsorption allowed to proceed at room temperature for 10–20 min. The adsorption mixture was centrifuged at 7000 *g* and the supernatant fluid discarded. The pellet was gently resuspended in distilled water (1 ml.) and prepared for electron microscope observation by the negative staining method (Brenner & Horne, 1959; Bradley, 1962). The stains used were 2% sodium phosphotungstate (pH 7·0), 2% sodium tungstate (pH 7·2) and 1% uranyl acetate (pH 4·0). Specimens were placed on Formvar-coated carbon-stabilized grids and examined with a Siemens Elmiskop I electron microscope operated at 60 kV.

Shear treatment. The blending procedure for removal of bacterial pili (Brinton, 1965; Valentine *et al.* 1965) was used with *Caulobacter bacteroides* CB 11a and *C. fusiformis* CB 27. The bacterial cultures were subjected repeatedly to 2 min. periods of blending at 4° by using a pre-cooled MSE Atomix blender at maximum speed. Each shear treatment was followed by centrifugation at 7000 *g*; the bacterial pellet was then resuspended in fresh medium. After the final centrifugation, the sheared bacteria were resuspended in PYE broth to the volume of the original culture and used in phage adsorption experiments.

Segregation of Caulobacter cultures. The two morphologically distinct types of caulobacter cells, swarmers and those with stalks, can be segregated to obtain relatively homogeneous populations. The centrifugal method of Stove & Stanier (1962) was used to obtain segregated populations of *C. bacteroides* CB 11a and *C. fusiformis* CB 27. Four successive centrifugations, employing a Servall Superspeed with SS–34 rotor at gravitational fields of 1000 to 1500 *g*, gave adequate segregation of these strains.

Measurement of adsorption. Caulobacter cultures containing about 5×10^9 colony-forming units/ml. in PYE broth supplemented with 0·004 M-$MgSO_4$ were pre-incubated for 5 min. at 30° on a slow rotary shaker. Phage was then added at a multiplicity of infection of 0·01. At succeeding time intervals, samples were diluted 1/100 in chloroform-saturated PYE broth to stop further adsorption and to eliminate phages which had adsorbed to host caulobacters. Unadsorbed phages were then determined as plaque-forming units by appropriate dilution and plating by the agar overlayer method (Adams, 1959). For calculation of % unadsorbed phage, the initial phage titre of the adsorption mixture was determined at zero time with omission of the chloroform treatment.

RESULTS

Lytic activity

The host ranges of the caulobacter RNA-phages with the hosts used in this study are shown in Table 1. There was no overlap of host specificity among these caulophages; they are also serologically distinct (Schmidt & Stanier, 1965).

Observation of phage + pilus complexes

An electron microscope survey for formation of phage + pilus complexes was made on the RNA-phages in the presence of the various Caulobacter strains. This survey included specific phage + host systems, and also phage + caulobacter mixtures in which the phages did not cause lysis of the Caulobacter strains. In all cases tested, the caulobacter RNA-phages able to cause lysis of a given strain were found to form complexes with pili emanating from the host caulobacters (Table 2). In the non-specific RNA-phage + caulobacter mixtures, no phage + pilus complexes were observed. No exception to the correlation between ability to cause lysis and formation of phage + pilus complexes has so far been found.

Table 1. *Lytic activity of caulobacter RNA-phages*

	Caulophage		
	ΦCb8r	ΦCb12r	ΦCb23r
Host strain	Lytic activity		
C. crescentus CB 2	−	+	−
C. crescentus CB 15*	−	+	−
C. bacteroides CB 8	+	−	−
C. bacteroides CB 11a*	+	−	−
C. fusiformis CB 27*	−	−	+
C. fusifornis CB 29	−	−	+

* Host strain used in preparation of specific phage lysate.

Table 2. *Formation of RNA-phage + pilus complexes in caulobacters*

	Caulophage		
	ΦCb8r	ΦCb12r	ΦCb23r
Caulobacter	Presence (+) or Absence (−) of phage + pilus complex		
C. crescentus CB 2	−	+	−
C. crescentus CB 15	−	+	−
C. bacteroides CB 8	+	−	−
C. bacteroides CB 11a	+	−	−
C. fusiformis CB 27	−	−	+
C. fusiformis CB 29	−	−	+

Electron micrographs of phage + pilus complexes in which the pili originate from swarmer caulobacters are shown in Pl. 1, figs. 1–3. In all three Caulobacter species found to possess pili, these filamentous structures emanated from the flagellated pole of the swarmer; it is this pole which eventually gives rise to stalk development (Poindexter, 1964; Schmidt & Stanier, 1966). The pili were not found at the opposite non-flagellated pole, nor did they occur at peritrichous sites of origin. The pili occurred on most swarmers of *Caulobacter fusiformis*, but were observed rather infrequently in *C. crescentus* and *C. bacteroides*. Stalked caulobacters are almost always devoid of pili. In the very few instances where pili were seen on stalked caulobacters, these structures emanated from the terminal end of the stalk (Pl. 2, fig. 4). The

occurrence of pili on a stalked caulobacter was noted only twice, although many hundreds of stalked caulobacters have been observed in the presence of their specific RNA-phage, and with numerous phage+pili complexes occurring on swarmers present in the same preparation.

In *Caulobacter fusiformis*, the swarmers usually had several pili; up to 8 pili on one caulobacter were observed (Pl. 1, fig. 1). *Caulobacter crescentus* and *C. bacteroides* seldom possessed more than 4 pili/swarmer; more frequently only one pilus or none at all were observed (Pl. 1, figs. 2, 3).

The pili are several microns in length. The usual observable diameter is approximately 40 Å in *Caulobacter crescentus* CB 2 (Pl. 2, fig. 5) and in *C. fusiformis* CB 27. No substructure was evident. Occasionally, when several pili were present on a *C. fusiformis* organism, wider pili with apparent diameters of 75–80 A were observed. These wider forms seemed to have a core penetrated by the negative stain (Pl. 2, fig. 6). The diameter of the pili of *C. bacteroides* was not reliably determined.

Table 3. *RNA-phage adsorption with sheared and normal caulobacter cultures*

Expt. no.	Caulophage	Caulobacter host	Shear treatment (2 min.)	Phage remaining unadsorbed at 7 min. (% unadsorbed)	Phage remaining unadsorbed at 15 min. (% unadsorbed)
1	ΦCb8r	CB 11a	—	53	20
			×3	100	99
2	ΦCb32r	CB 27	—	43	17
			×3	85	75
3	ΦCb23r	CB 27	—	54	23
			×4	100	97

Adsorption of caulophages to sheared hosts

To determine whether RNA-phage adsorption in caulobacters is dependent on the presence of shear-sensitive structures as has been demonstrated for the adsorption of male-specific RNA-phages of *Escherichia coli* (Brinton, 1965; Valentine *et al.* 1965), adsorption experiments with sheared and untreated caulobacter cultures were made. Two systems, *Caulobacter bacteroides* CB 11a and ΦCb8r, and *C. fusiformis* CB 27 and ΦCb23r, were examined. The results are shown in Table 3. In both systems, host cultures which had undergone shear treatment just before the adsorption experiment were greatly decreased in their capacity to absorb the RNA-phages. With adequate shear treatment, phage adsorption was negligible.

Phage adsorption after chloramphenicol treatment of caulobacters

Edgell & Ginoza (1965) showed that R17, a male-specific RNA-phage of *Escherichia coli*, has an adsorption requirement for metabolically active host organisms. Pre-treatment of the host with chloramphenicol or other metabolic inhibitors prevented irreversible adsorption of phage R17. An analogous situation appears to exist with caulobacter RNA-phage adsorption. Growing cultures of *Caulobacter fusiformis* CB 27 and *C. bacteroides* CB 11a were exposed to chloramphenicol (150 μg./ml.)

for 2 hr, during which time increase in turbidity of the cultures ceased. Phage-adsorption experiments were made with chloramphenicol-inhibited cultures and with control cultures. In both phage systems pre-treatment of the host caulobacters with chloramphenicol prevented phage adsorption.

Adsorption of caulophages in segregated host populations

Because stalked caulobacters had so rarely been observed to possess pili it was of interest to determine how RNA-phage adsorption in populations of stalked caulobacters would be affected by the natural absence of pili. Segregated populations of *Caulobacter fusiformis* CB27 were more than 90% homogeneous at the beginning of the adsorption experiment; however, division of stalked caulobacters, giving daughter swarmers did occur during the experiment. Division was not prevented by the addition of a metabolic inhibitor because of the adsorption requirement for metabolically active host caulobacters. Despite the increasing numbers of swarmers, the adsorption of caulophage ΦCb23r was sharply decreased in the stalked caulobacter populations, as compared to that in swarmer populations and in control unsegregated cultures of *C. fusiformis* CB27 (Table 4). Similar results were obtained with caulophage ΦCb8r and segregated *C. bacteroides* CB11a populations.

Table 4. *RNA-phage adsorption with segregated Caulobacter fusiformis host populations*

	Unadsorbed caulophage (ΦCb23r) at time	
	7 min	15 min
Host caulobacters	% unadsorbed phage	
Unsegregated control	61	33
Stalked caulobacters	95	82
Swarmers	45	25

DISCUSSION

The attachment of caulobacter RNA-phages to pili of their hosts and the dependence of their adsorption upon the presence of these pili are features consistent with the situation described for male-specific RNA-phages of *Escherichia coli* (Crawford & Gesteland, 1964; Brinton, 1965; Valentine *et al.* 1965). Bradley (1965) reported a similar observation for attachment sites of an RNA-phage of *Pseudomonas aeruginosa*. Thus for all known RNA-phage systems the phage+pilus association is a general characteristic. Brinton (1965) suggested that bacterial pili participate in the conjugation process. It becomes evident that bacteria of the Caulobacter group should be investigated for ability to conjugate, based on the presence in these organisms of RNA-phage+pilus complexes such as occur in *E. coli* and *P. aeruginosa*, which are both known to possess sexuality.

The data presented here do not eliminate the possibility that receptor sites for the caulobacter RNA-phages apart from the pili might exist on the surface of the organisms. However, the sensitivity of RNA-caulophage adsorption to shearing treatment of the host, and the poor phage adsorption with stalked caulobacter

populations which largely lack pili, implicate the pili as significant phage receptors. The relationship of the phage+pilus association in the actual infection of the caulobacters with viral RNA remains obscure.

Bradley (1965) reported that the pili of *Pseudomonas aeruginosa* to which RNA-phages attach are polar, as found for the pili of the three Caulobacter species studied here. Both the genera *Pseudomonas* and *Caulobacter* are characterized by polar flagellation. The diameter of the *P. aeruginosa* pilus is about 40 Å (Bradley, 1966), similar to the diameter observed for most pili of *Caulobacter fusiformis* and *C. crescentus*. The F pili of *E. coli* are wider, 85 Å, and have peritrichous sites of origin (Brinton, 1965). There is a possibility that the few wider pili of *C. fusiformis* which have been observed were actually two pili intertwined, since they have been found only where several pili emanated from the pole of a swarmer and where entanglement of pili was notable. The uneven nature of the negative stain frequently results in apparent variation in the diameter of a pilus. Sodium tungstate was used for negatively-stained preparations of caulophages ΦCb8r and ΦCb12r since these phages disintegrate in sodium phosphotungstate. The phage+pilus complexes were not observable in preparations stained with uranyl acetate, although the phages remained intact.

It had been previously noted in one-step growth experiments that RNA-phages did not multiply in segregated populations of stalked host caulobacters (unpublished data). The very infrequent occurrence of pili on stalked caulobacters, and the poor adsorption of RNA-phages to the stalked caulobacters provide a possible basis for explanation of this observation. The stalked caulobacters may represent a form relatively resistant to RNA-phages which are, however, able to infect the piliated swarmer before it has undergone stalk development.

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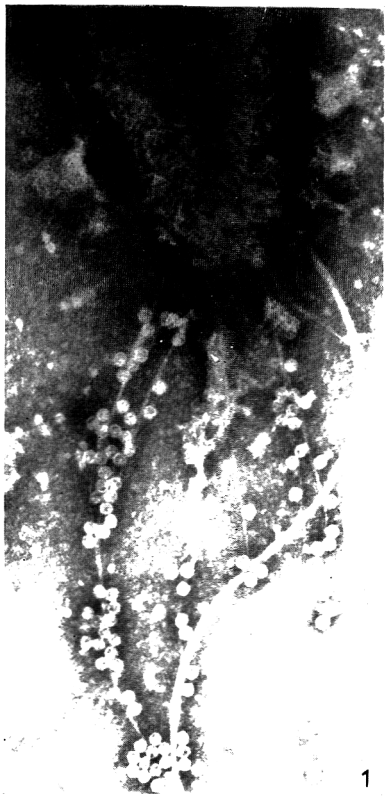

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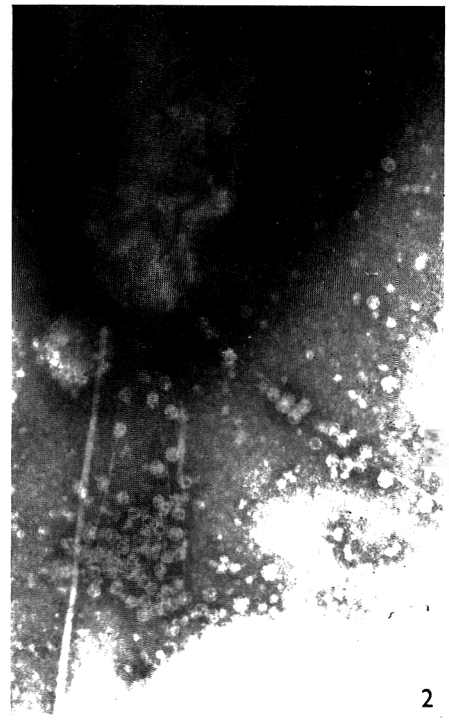

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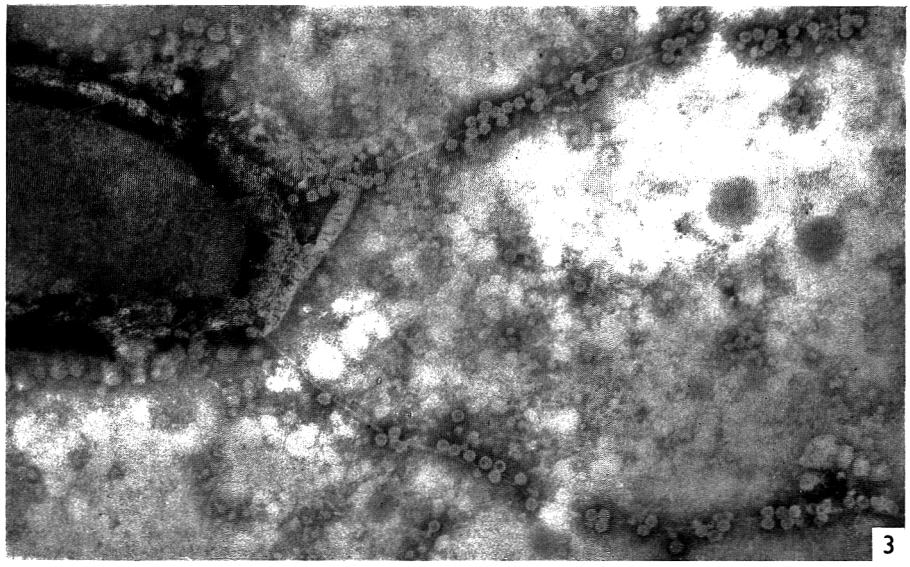

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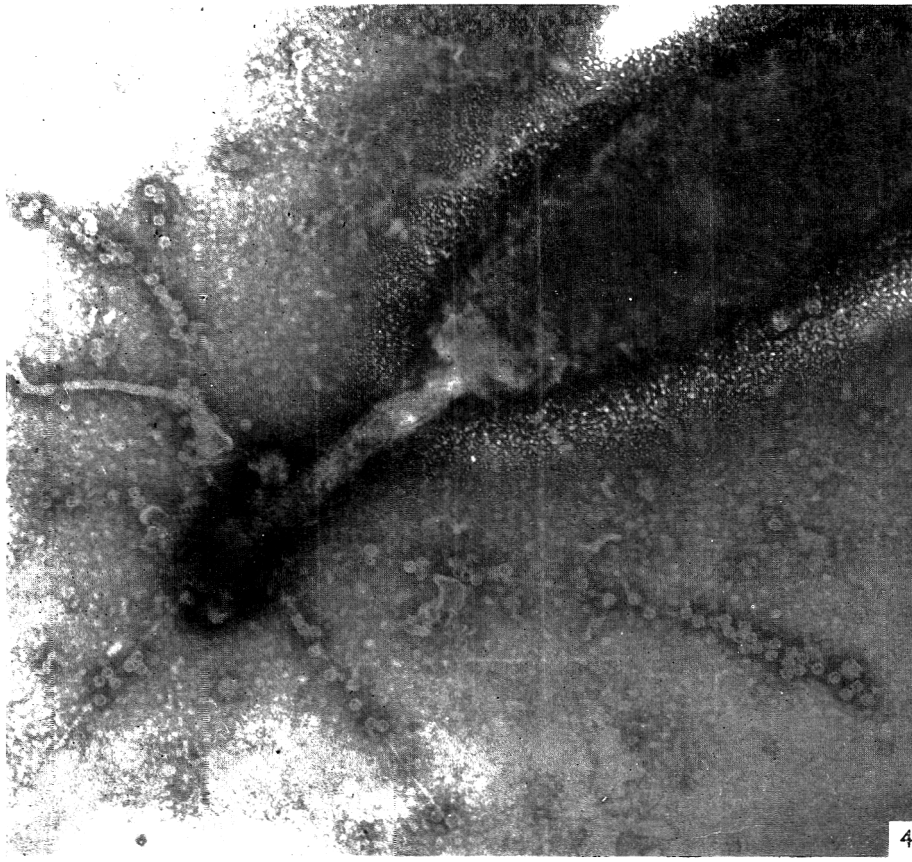
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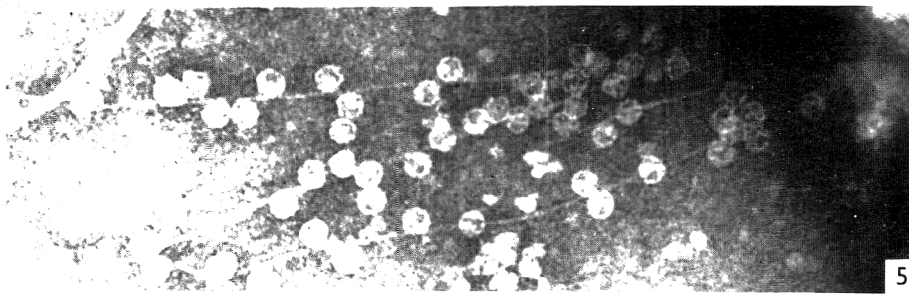
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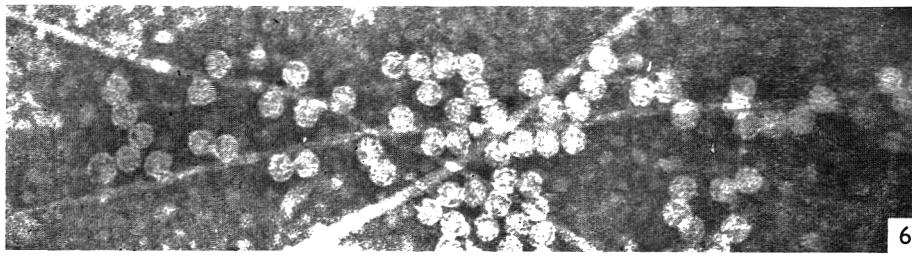
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EXPLANATION OF PLATES

PLATE 1

Figs. 1–3. Caulobacter swarmers with RNA-phage attachment to pili. Sodium tungstate stain. × 68,000.

Fig. 1. *C. fusiformis* CB27 and caulophage ΦC*f*23r.

Fig. 2. *C. crescentus* CB2 and caulophage ΦCb12r.

Fig. 3. *C. bacteroides* CB11a and caulophage ΦCb8r.

PLATE 2

Fig. 4. *C. fusiformis* CB27 stalked organisms and caulophage ΦCb23r. Sodium tungstate stain. × 68,000.

Fig. 5. Pili of *C. crescentus* CB2 and caulophage ΦCb12r. Sodium tungstate stain. × 144,000.

Fig. 6. Pili and flagellum of *C. fusiformis* CB27 and caulophage ΦCb23r. Sodium tungstate stain. × 144,000.

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Some Criteria for the Recognition of *Nocardia madurae* (Vincent) Blanchard

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SUMMARY

A combination of characters for the description and identification of strains of *Nocardia madurae*, based on the examination of 44 strains, is presented. Although nearly all the 44 strains were received from medical laboratories, a few were isolated from soil. Some of the distinguishing properties of *N. madurae* are compared with those of *N. asteroides*, *N. caviae*, *N. brasiliensis*, *N. pelletieri* and *Streptomyces somaliensis*.

INTRODUCTION

During a comparative study of strains of mycobacteria, nocardias, and streptomycetes Gordon & Mihm (1962*a*) obtained as many strains as possible of *Nocardia asteroides* (Eppinger) Blanchard from the larger culture collections and collections of investigators interested in the species. Old stock strains, new isolates, and a number of their variants were examined. Properties common to all these strains were used to describe *N. asteroides* and to differentiate it from the other species represented in the collection which formed the basis of that study. As far as possible, this procedure was repeated in searching for criteria by which other species could be recognized.

Although a group of apparently useful properties for delineating *Nocardia madurae* (Vincent) Blanchard was found early in the study, progress in accumulating strains, particularly freshly isolated ones, to confirm the value of the distinguishing characteristics was very slow. Eventually enough strains of *N. madurae* were acquired from medical laboratories and from soil to provide reasonable assurance that the pattern of reactions presented here would be useful in the separation of *N. madurae* from other nocardias and from the streptomycetes. These reactions by which *N. madurae* can be recognized are compared with the reactions of *N. asteroides* (Eppinger) Blanchard, *N. brasiliensis* (Lindenberg) Castellani & Chalmers, *N. caviae* (Erikson) Gordon & Mihm, *N. pelletieri* (Laveran) Pinoy, and *Streptomyces somaliensis* (Brumpt) Waksman & Henrici. The patterns of reactions by which *N. caviae*, *N. pelletieri* and *S. somaliensis* are characterized, however, are based on very few strains and may be unreliable.

METHODS

Organisms. The strains listed in Tables 1, 3 and 4 and by Gordon & Mihm (1962*a*, *b*) were compared with each other and also with the other strains of mycobacteria, nocardias and streptomycetes in this collection.

Tests

The strains were examined according to the procedures described by Gordon (1966) with the following additions:

Hydrolysis of aesculin. The cultures were inoculated into aesculin broth (aesculin, 1 g.; ferric citrate, 0·5 g.; peptone, 10 g.; NaCl, 5 g.; water, 1000 ml.: Cowan & Steel, 1965), incubated at 28°, and observed for growth and blackening of the medium at 2, 4 and 6 weeks. A tube of the same broth without aesculin was also inoculated with each culture and used as a control.

Resistance to lysozyme. A solution of 0·1 g. lysozyme (Nutritional Biochemicals Corp., Cleveland, Ohio) in 60–65 ml. of sterile 0·01 N-HCl in a 100 ml. volumetric flask was boiled over an open flame for 20 min., and then plugged with sterile cotton-wool, cooled to room temperature, and brought to 100 ml. by the addition of sterile 0·01 N-HCl. Five ml. of the lysozyme solution was mixed with 95 ml. of sterile glycerol broth (peptone, 5 g.; beef extract, 3 g.; glycerol, 70 ml.; water, 1000 ml.; pH 7·0) and dispensed in 2·5 ml. amounts in sterile plugged tubes. A loopful of a 14- to 28-day culture in glucose or glycerol broth was inoculated into a tube of lysozyme broth and into a control tube of glycerol broth. The two tubes were observed for growth after 14 and 28 days of incubation at 28°.

Serial transfer in carbohydrate broth. Flasks of arabinose, mannitol, rhamnose and xylose broths were prepared by adding 10 ml. of a 10% (w/v) aqueous solution of each carbohydrate, sterilized by autoclaving separately, to 90 ml. of sterile broth containing $(NH_4)_2HPO_4$, 1 g.; KCl, 0·2 g.; $MgSO_4.7H_2O$, 0·2 g.; distilled water, 1000 ml. (Ayers, Rupp & Johnson, 1919). The broth was adjusted to pH 7·0 before the addition of 15 ml. of a 0·04% (w/v) solution of bromcresol purple. Each flask was heavily inoculated and incubated at 37°. After 2 or 3 months the remainder of the inoculum and the growth, if any, were pipetted to another flask of the same broth.

RESULTS

Nocardia madurae (*Vincent*) *Blanchard*

Microscopically, cultures on glycerol agar of the 44 strains of *Nocardia madurae* listed in Table 1 formed filaments which did not retain carbol fuchsin. The undisturbed colonies on Bennett's agar and on soil extract agar were densely or loosely filamentous. Under the conditions of the examination, 20 of the 44 strains produced aerial hyphae. The aerial hyphae of six of the 20 strains were long, with short curling side branches that segmented into short chains of spores; the aerial hyphae of the remaining 14 strains were sparse and varied from short and straight to long and branching.

After 2 weeks of incubation at 28° on Bennett's agar, the growth of some cultures was whitish, shiny, spreading, and either flat, coarsely wrinkled or folded. The whitish growth of some cultures contained a scattering of rose-coloured dots, and the crests of the folds of other whitish cultures were rose-coloured. The growth of other strains was beige, spreading, and flat or coarsely wrinkled; the growth of strains 780 and 1253 was reddish orange. Most of the strains did not form aerial hyphae visible to the unaided eye; the cultures of a few strains were, however, thickly coated with aerial hyphae; and others were sparsely coated. Brown, soluble

Table 1. *Strains identified as Nocardia madurae (Vincent) Blanchard*

Laboratory no.	Name when received, source, strain name or number
N 1070, N 5654	*Nocardia madurae* (Vincent) Blanchard; NCTC (1070, madura foot; 5654, Persia)
407	*N. madurae*; Julia M. Coffey, N.Y. State Dept. of Health (47294); J. B. Fischer; N. F. Conant
431	*N. madurae*; Isabel Christison, Duke Univ.; N. F. Conant (2177); A. González Ochoa (415)
507	*N. madurae*; P. Thibault, Inst. Pasteur, Paris (Tunis)
554	*N. madurae*; E. N. Azarowicz, Univ. of Calif. (62); C. Halde
614	*N. madurae*; E. N. Azarowicz (26)
633	*N. madurae*; A. González Ochoa, Inst. de Salubridad y Enfermedades Tropicales, Mexico (412, mycetoma of the foot)
635, 636, 791, 793	*N. madurae*; A. González Ochoa (614, mycetoma of the foot; 420, mycetoma of the back; 485; 1004)
686	*N. madurae*; L. Ajello, Communicable Disease Center, Chamblee, Ga. (A-528); J. Lamb (mycetoma)
706	*N. madurae*; C. W. Emmons, Nat. Inst. of Health, Bethesda, Md. (9979); J. Lamb (mycetoma)
742	*N. madurae*; J. D. Schneidau, Jun., Tulane Univ. (339, madura foot)
751–753	*N. madurae*; J. E. Mackinnon, Inst. de Higine, Montevideo (IHM 769, Chile; IHM 869, North Africa; IHM 1154, India)
754	*N. madurae*; J. E. Mackinnon (IHM 1390); A. González Ochoa (case 444, madura foot)
776, 777	*N. madurae*; Jacqueline Walker, London School of Hygiene and Tropical Medicine (A. 260, A. 261; mycetoma pedis)
1189, 1190	*N. madurae*; A. E. deArêa Leão, Inst. Oswaldo Cruz, Rio de Janeiro (1078, 2593)
1234	*N. madurae*; G. Altmann, Tel Hashomer Government Hosp., Israel (TH 5, mycetoma pedis)
*	*N. madurae*; Mary P. Lechevalier, Rutgers Univ. (P 56, Sal 1; soil)
780	*N. pellctieri*; Jacqueline Walker (A. 292 (9972))
609	*N. pseudomadurae*; E. N. Azarowicz (4)
W3632A	*Nocardia* sp.; G. Altmann, Military Hosp. No. 5, Israel (madura foot)
510	*Nocardia* sp.; P. Thibault (madura foot)
623	*Nocardia* sp.; E. N. Azarowicz (47)
637	*Nocardia* sp.; A. González Ochoa (486)
743	*Nocardia* sp.; Lucille K. Georg, Communicable Disease Center, Chamblee, Ga.; I. W. Kuhl (madura foot)
1136	*Nocardia* sp.; Joan M. Mihm, Rutgers Univ. (B-2, soil)
1210	*Nocardia* sp.; J. B. Fischer, Ontario Dept. of Health, Toronto (356, gluteal abscess)
571	*Proactinomyces pseudomadurae* Baldacci; N. M. McClung, Univ. of Kansas (3); Centraalbur. v. Schimmelcultures
1087, 1091	*Streptomyces albus*; H. D. Tresner, Am. Cyanamid Co., Pearl River, N.Y. (AO-927-M-811, AO-928)
852	*S. madurae* (Vincent) González Ochoa & Sandoval; L. Briceño Iragorry, Univ. Central de Venezuela (FMC N. 107, Sablac; mycetoma of the foot)
972	*S. madurae*; Mildred Feo, Univ. Central de Venezuela (FMC 96); D. Borelli (mycetoma of the foot)
971	*S. pelletieri* (Laveran) Waksman & Henrici; Mildred Feo (FMC 92); A. González Ochoa (thoracic mycetoma)
1092	*S. madurae*; A. González Ochoa (4088, mycetoma of the foot)
1253, 1260	*S. madurae*; F. Mariat, Inst. Pasteur, Paris (7087 B, 812)

* Two strains not added to this collection.

pigment was produced by some cultures, and a purple, soluble pigment by others. The appearance of the strains isolated from soil did not differ from that of strains from other sources. Because the growth of strains 571 and 609, received as *Nocardia*

pseudomadurae and *Proactinomyces pseudomadurae*, was whitish and beige, respectively, the pink and red pigments originally ascribed to them by Baldacci (1944) were presumably lost during cultivation *in vitro*.

The following combination of physiological properties (Table 2) was found to differentiate all 44 strains of *Nocardia madurae* from the other strains in our

Table 2. *Comparison of physiological characteristics (% positive strains)*

Property	*Nocardia madurae* (44 strains)	*Nocardia pelletieri* (15 strains)	*Streptomyces somaliensis* (22 strains)
Decomposition of			
Adenine	0	0	0
Casein	100	100	100
Hypoxanthine	100	100	0
Tyrosine	86	100	100
Urea	0	0	0
Xanthine	0	0	0
Growth at			
50°	0	7	0
45°	36	67	55
40°	98	100	91
35°	100	100	100
28°	100	100	100
10°	0	13	9
Survival at 50° for 8 hr	100	100	50
Nitrite from nitrate	100	100	0
Resistance to lysozyme	5	0	0
Utilization of			
Citrate	80	7	18
Malate	84	53	37
Succinate	80	0	45
Hydrolysis of			
Aesulin	100	0	0
Starch	100	13	41
Acid from			
Adonitol	91	0	0
Arabinose	100	0	0
Erythritol	0	0	0
Galactose	84	7	0
Glucose	100	100	50
Inositol	61	0	0
Lactose	55	0	0
Maltose	55	0	64
Mannitol	100	7	0
Mannose	89	0	5
Melibiose	0	0	0
α-*m*-D-glucoside	0	0	0
Raffinose	0	0	0
Rhamnose	100	0	0
Sorbitol	0	0	0
Trehalose	96	93	0
Xylose	100	0	0

collection: decomposed casein and hypoxanthine; did not attack adenine, urea or xanthine; grew at 40°; survived 50° for 8 hr; reduced nitrate to nitrite; were sensitive to lysozyme; hydrolysed aesculin and starch; acid formed from arabinose,

glucose, mannitol, rhamnose, trehalose and xylose; unable to produce acid from erythritol, melibiose, α-*m*-D-glucoside, raffinose or sorbitol. Utilization of citrate, malate and succinate; decomposition of tyrosine; and the formation of acid from adonitol, galactose and mannose were less dependable characters of *N. madurae*.

Table 3. *Strains identified as Nocardia pelletieri (Laveran) Pinoy*

Laboratory no.	Name when received, source, strain name or number
677	*Nocardia africana* Pijper & Pullinger; E. N. Azarowicz, Univ. of Calif. (152); Inst. Pasteur (84)
408	*N. pelletieri* (Laveran) Pinoy; Julia M. Coffey, N.Y. State Dept. of Health (47293)
513, 514	*N. pelletieri*; P. Thibault, Inst. Pasteur, Paris
610	*N. pelletieri*; E. N. Azarowicz, Univ. of Calif. (10)
687	*N. pelletieri*; L. Ajello, Communicable Disease Center, Chamblee, Ga. (436); N.F. Conant (989)
705	*N. pelletieri*; C. W. Emmons, Nat. Inst. of Health, Bethesda, Md. (9971); E. C. Smith (Naudi, mycetoma)
741	*N. pelletieri*; J. D. Schneidau, Jun., Tulane Univ. (305); C. Lacaz (293)
755	*N. pelletieri*; J. E. Mackinnon, Inst. de Higiene, Montevideo (IHM 614); Lab. de Parasitologie, Faculté de Médecine, Paris
756	*N. pelletieri*; J. E. Mackinnon (IHM 615); A. Pijper (*Actinomyces africanus*)
757	*N. pelletieri*; J. E. Mackinnon (IHM 1342, Abbot's)
778, 781	*N. pelletieri*; Jacqueline Walker, London School of Hygiene and Tropical Medicine (A. 290, A. 293); E. C. Smith (mycetoma of the arm, mycetoma of the foot)
779	*N. pelletieri*; Jacqueline Walker (A. 291, Merucci's)
N 4162	*Streptomyces pelletieri* (Laveran) González Ochoa & Sandoval; NCTC (4162); J. T. Duncan; E. C. Smith (Naudi)

Nocardia pelletieri (Laveran) Pinoy

Cultures on glycerol agar of the 15 strains of *Nocardia pelletieri* in our collection (Table 3) were filamentous and not acid-fast. Their colonies on soil extract agar and Bennett's agar were loosely or densely filamentous and did not exhibit aerial hyphae. One strain (757) on tyrosine and other agars, however, showed irregularly branching aerial hyphae.

Macroscopically the cultures after 2 weeks of incubation on Bennett's agar were pink, bright red, or dark red with a brownish, almost metallic tinge. Some were flat or coarsely wrinkled and spreading; others resembled a scattering of red-stained bread crumbs on the surface of the slant; and some formed a reddish brown soluble pigment.

During the cultivation of stock cultures of *Nocardia pelletieri* on Waksman's 'yeast dextrose' agar, beige-coloured variants developed spontaneously from strains 755 and 779.

The following pattern of physiological reactions of the strains of *Nocardia pelletieri*, separated them from the other strains in this collection: disintegrated casein, hypoxanthine and tyrosine; did not decompose adenine, urea or xanthine; grew at 40°; survived at 50° for 8 hr; reduced nitrate to nitrite; were sensitive to lysozyme; did not hydrolyse aesculin; formed acid from glucose and trehalose; did not produce acid from the remaining carbohydrates listed in Table 2.

Several unsuccessful attempts were made to obtain variants of strains of *Nocardia pelletieri* capable of forming acid from arabinose, mannitol, rhamnose and xylose

and thus to resemble strains of *N. madurae*. In one attempt, cultures of *N. pelletieri* were transferred serially in arabinose, mannitol, rhamnose or xylose broths for 18 months. Although several of the resulting cultures lost their bright red pigment and became yellowish or beige in colour, none of them produced acid from any of the four carbohydrates.

Streptomyces somaliensis (Brumpt) Waksman & Henrici

Cultures on glycerol agar of the 22 strains of *Streptomyces somaliensis* listed in Table 4 were filamentous and not acid-fast. The colonies of the 22 strains on soil extract agar and Bennett's agar were loosely or densely filamentous. Straight aerial hyphae which varied in length and branching were formed sparsely or abundantly by 13 of the 22 strains. Sporulation was not observed.

Table 4. *Strains identified as Streptomyces somaliensis (Brumpt) Waksman & Henrici*

Laboratory no.	Name when received, source, strain name or number
632	*Actinomyces somaliensis* (Brumpt) St John-Brooks; A. González Ochoa, Inst. de Salubridad y Enfermedades, Mexico, D. F. (1064); J. T. Duncan
N3026	*Streptomyces pelletieri* (Laveran) Waksman & Henrici; NCTC (3026); J. T. Duncan; E. C. Smith (mycetoma)
719	*S. somaliensis* (Brumpt) Waksman & Henrici; G. Altmann, Tel Hashomer Hosp., Israel (TH/4, mycetoma pedis)
1267–1269	*S. somaliensis*; F. Mariat, Inst. Pasteur, Paris (313, 314, 315); J. Walker (A. 311, A. 310, A. 312)
1270–1276	*S. somaliensis*; F. Mariat (383, 386, 387, 702, 733, 755, 761)
1278–1282, 1298	*S. somaliensis*; F. Mariat (764A, 764B, 395, 765, 754, 746)
1295	*S. somaliensis*; J. E. Mackinnon, Inst. de Higiene, Montevideo (IHM 1290)
1296, 1297	*S. somaliensis*; J. E. Mackinnon (1531, 1532); I. G. Murray (1071, 1070)

The macroscopic appearance of the cultures of *Streptomyces somaliensis* grown on Bennett's agar for 2 weeks was not uniform. The growth was flat, finely or coarsely wrinkled, or warty; pigments were whitish, yellowish, beige, beige with a scattering of dark brown dots on the surface, or cocoa brown. Only four cultures had a coating of whitish aerial hyphae visible to the unaided eye; a few cultures formed a brown soluble pigment.

Physiological characteristics for recognizing the 22 strains of *Streptomyces somaliensis* (Table 2) were the clearing of casein and tyrosine; inability to decompose adenine, hypoxanthine, urea or xanthine; growth at 40°; failure to reduce nitrate to nitrite or to hydrolyse aesculin; sensitivity to lysozyme; and, with the exception of glucose and maltose, lack of acid production from carbohydrates. Eleven of the 22 strains formed acid from glucose, and 14 strains formed acid from maltose. Among the properties listed in Table 2, only decomposition of hypoxanthine, reduction of nitrate to nitrite, and acid formation from trehalose differentiated the strains of *Nocardia pelletieri* from those of *S. somaliensis*.

Comparison of six species

The more useful properties for recognizing *Nocardia madurae*, *N. pelletieri* and *Streptomyces somaliensis*, presented in Table 2, are compared with those of *N. asteroides*, *N. caviae* and *N. brasiliensis* (Gordon & Mihm, 1962*a*, *b*) in Table 5.

Although acid-fastness was a variable character of *N. asteroides*, *N. caviae* and *N. brasiliensis*, acid-fastness, when observed, was most helpful in identifying strains of these three species. Resistance to lysozyme was a reliable property of *N. asteroides*, *N. caviae*, *N. brasiliensis* and also of *S. lavendulae* and *S. rimosus*. Strains of *N. madurae*, *N. pelletieri*, *S. somaliensis*, *S. griseus* and *S. fradiae* were sensitive to lysozyme. Strains of *N. asteroides*, *N. caviae* and *N. brasiliensis* were also separated from those of *N. madurae*, *N. pelletieri* and *S. somaliensis* by decomposition of urea, and from *N. pelletieri* and *S. somaliensis* by hydrolysis of aesculin.

Table 5. *Some criteria for identifying six species of Nocardia and Streptomyces*

	% Positive strains.					
Property	*Nocardia asteroides* (142 strains)	*Nocardia caviae* (21 strains)	*Nocardia brasiliensis* (62 strains)	*Nocardia madurae* (44 strains)	*Nocardia pelletieri* (15 strains)	*Streptomyces somaliensis* (22 strains)
Acid-fastness	51	76	80	0	0	0
Decomposition of						
Casein	0	0	98	100	100	100
Hypoxanthine	4	100	94	100	100	0
Tyrosine	2	0	100	86	100	100
Urea	97	100	100	0	0	0
Xanthine	0	100	0	0	0	0
Survival at 50° for 8 hr	93	86	0	100	100	50
Nitrite from nitrate	88	100	90	100	100	0
Resistance to lysozyme*	99	100	100	5	0	0
Hydrolysis of aesculin	100†	100	100†	100	0	0
Acid from						
Adonitol	0	0	0	91	0	0
Arabinose	0	0	0	100	0	0
Glucose	97	100	97	100	100	50
Inositol	3	100	100	61	0	0
Mannitol	1	90	94	100	7	0
Rhamnose	32	5	0	100	0	0
Trehalose	—	—	—	96	93	0
Xylose	0	5	0	100	0	0

* 21 strains of *Streptomyces lavendulae* were resistant to lysozyme; 29 of 30 strains of *S. rimosus* were resistant; 23 strains of *S. fradiae* and 25 strains of *S. griseus* were not resistant.

† 50 strains examined.

Under the conditions of examination, the 142 strains of *Nocardia asteroides* in this collection (Table 5) were differentiated from *N. caviae* and *N. brasiliensis* by their inability to dissolve casein, hypoxanthine, tyrosine or xanthine; survival at 50° for 8 hr, and failure to form acid from inositol or mannitol. Although the strains of *N. caviae* resembled the strains of *N. asteroides* in many respects, *N. caviae* differed from *N. asteroides* in decomposing hypoxanthine and xanthine and in the production of acid from inositol and mannitol. The strains of *N. brasiliensis* attacked casein, hypoxanthine and tyrosine but not the crystals of xanthine; did not survive 50° for 8 hr; and formed acid from inositol and mannitol.

DISCUSSION

Nocardia madurae, *N. pelletieri* and *Streptomyces somaliensis* have been accepted as separate species by many investigators, and the distinctiveness of the three aggregates has been demonstrated by different workers in different ways. Disagreement on certain properties of the strains due to the use of different media and methods has occurred, but in my opinion this disagreement is relatively unimportant, because media and procedures are constantly changing. If today's workers recognize a taxonomic unit by different means, the investigators of the future will also recognize the same unit by procedures as yet unknown.

Confirmation is offered here (Table 3) for the assignment of *Nocardia africana* Pijper & Pullinger (1927) to the synonymy of *N. pelletieri* by González Ochoa & Vázquez Hoyos (1953), Conant *et al.* (1954), Mackinnon & Artagaveytia-Allende (1956) and Mariat (1958). An assignment of *Proactinomyces pseudomadurae* Baldacci (1944) to the synonymy of *N. madurae* (Table 1) by other workers was not found, although Mariat (1962) recognized rose- and red-pigmented strains from southern Europe and northern Africa as *N. madurae*.

While investigators apparently agree on the specific status of *Nocardia madurae*, *N. pelletieri* and *Streptomyces somaliensis*, the generic location of these three species is still unsettled. Waksman & Henrici (1948) transferred *N. pelletieri* (Laveran, 1906) Pinoy (1912) and *N. somaliensis* (Brumpt, 1906) Chalmers & Christopherson (1916) to the genus *Streptomyces*, and González Ochoa & Sandoval (1955) transferred *N. madurae* (Vincent, 1894) Blanchard (1896) to *Streptomyces*. These assignments, based on morphology, were accepted by Mackinnon & Artagaveytia-Allende (1956), Mariat (1958, 1962), Lynch & Moghraby (1961), Emmons, Binford & Utz (1963), and others.

Study of a goodly number of strains of nocardias and streptomycetes from a variety of sources revealed, however, that under the conditions of examination the characteristics of acid-fastness, fragmentation of the substrate hyphae, abundance of aerial hyphae, and segmentation of the aerial hyphae into chains of spores, used for the separation of the genera *Nocardia* and *Streptomyces*, were variable for *N. asteroides*; and that the properties of fragmentation of the substrate hyphae and formation of aerial hyphae and spores were variable for the streptomycetes (Gordon & Mihm, 1962*a*). Physiological criteria to separate the two genera were not found. The observation by Sohler, Romano & Nickerson (1958) that resistance to lysozyme could not be used for the separation of nocardias from streptomycetes, a retraction of an earlier report by Romano & Sohler (1956), is confirmed here (Table 5).

A division of the genera *Nocardia* and *Streptomyces* based on the chemical composition of the cell wall or of whole cells was offered by Romano & Nickerson (1956), Sohler, Romano & Nickerson (1958), Cummins & Harris (1958), Becker, Lechevalier, Gordon & Lechevalier (1964), Becker, Lechevalier & Lechevalier (1965), Yamaguchi (1965), and Murray & Proctor (1965). These workers demonstrated the presence of *meso*-diaminopimelic acid and arabinose in the cell-walls or in the whole cells of the nocardias and *LL*-diaminopimelic acid without arabinose in the streptomycetes. Of the strains listed in Tables 1, 3 and 4, 10 of *N. madurae*, three of *N. pelletieri*, and two of *S. somaliensis* were among those examined by Becker *et al.*

(1964). The remaining strains of *S. somaliensis* were also found to possess LL-diaminopimelic acid (H. A. & M. P. Lechevalier, unpublished data). The resulting generic separation was adopted in this report, but not wholeheartedly.

The statement of Cowan & Steel (1965) that 'The different kinds of bacteria are not separated by sharp divisions but by slight and subtle differences in characters so that they seem to blend into each other and resemble a spectrum', applies to the nocardias and streptomycetes. Morphologically and physiologically, various species of *Nocardia* and *Streptomyces* seem to form a spectrum, with *N. asteroides* at one end and *N. brasiliensis*, *N. madurae*, *N. pelletieri*, *S. somaliensis*, *S. lavendulae* and *S. griseus* following in that order. Any generic line dividing this spectrum will seem somewhat artificial and arbitrary, because it will assign related species to different genera.

The hierarchial system, the basis of our communication, is not well suited to the naming of the taxonomic units comprising a spectrum. As knowledge of an increasing number of strains and of an increasing number of their characters accumulates, dissatisfaction with the assignment of each strain to a genus and species may become generally widespread and result in a different, internationally adopted, framework of communication (Cowan, 1965). For the present, however, the need of communication dictates the assignment of a generic and a specific name to each taxonomic unit. Currently the *meso-* or LL-form of diaminopimelic acid and the presence or absence of arabinose in the cells or cell walls appear to be more stable properties than the morphological characteristics of these strains and therefore have been used here for generic separation. Future investigations, perhaps like those of Lanéele, Asselineau & Castelnuovo (1965) on the lipids of the cells may provide other, more acceptable, generic demarcation in the spectrum of the nocardias and streptomycetes.

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The Relationship of F type Piliation and F phage Sensitivity to Drug Resistance Transfer in R^+F^- *Escherichia coli* K12

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SUMMARY

Resistance factors (R factors), of the kind which confer sensitivity to F specific phage as well as promoting conjugation in enterobacteria determine the production of a pilus similar to the specific pilus seen on F^+ bacteria. R factors, however, unlike F, also determine the production of a repressor of function: this means that in an established R^+ culture, only a small proportion of the bacteria can conjugate, be infected with F phage, or produce the pilus. Under conditions where repression is lifted, the three characters, conjugation, F phage sensitivity and production of the pilus, are coordinately de-repressed.

INTRODUCTION

The presence of the sex factor, F, in a bacterial strain can be recognized by three effects: the bacteria are able to conjugate, they are sensitive to certain phages, and they produce a special type of fimbria (Crawford & Gesteland, 1964), or pilus (Brinton, Gemski & Carnahan, 1964), which can be seen to act as the receptor for one group, at least, of F specific phages. The evidence suggests that the particular action of the F factor in this connexion is to determine the production of the pilus, which in addition to acting as phage receptor (Edgell & Ginoza, 1965), is involved in conjugation (see Brinton, 1965).

Resistance factors (or R factors) resemble F in promoting cell unions which are able to lead to their own transfer from one bacterium to another (see Watanabe, 1963*a*). However, while virtually every bacterium in an F^+ culture may be able to conjugate, R factors determine the synthesis of a cytoplasmic repressor which limits their own function, so that once the R factor is established in a strain, the ability to conjugate is expressed in only a minority of the bacteria (Meynell & Datta, 1965). If the R factor is transferred to recipient R^- bacteria which have no preformed repressor in their cytoplasm, a period follows during which its conjugating function is uninhibited, before its own repressor gene has had time to restore the repression. High frequency transfer (HFT) systems can therefore be

produced by initiating the rapid spread of an R factor through a large R^- recipient population from a small number of R^+ bacteria (Watanabe, 1963*b*).

One large class of R factors is closely related to F (Meynell & Datta, 1966*a*). These have been named i^+ (Egawa & Hirota, 1962), or fi^- (for 'fertility inhibition'; Watanabe *et al.* 1964), because they inhibit the expression of F; so that after acquiring an R factor, an F^+ culture is no longer able to conjugate with its accustomed frequency nor visibly lysed by F specific phage. R factors belonging to this class are not only conjugation factors, but actually confer sensitivity to F phage (Meynell & Datta, 1966*a*); they must therefore, like F, determine the production of the specific F phage receptor, since it is lack of this receptor which is responsible for the insensitivity of F^- bacteria* (Engelhardt & Zinder, 1964). Evidently the gene determining synthesis of the specific phage receptor is present in fi^+ R factors as well as in F. Phage sensitivity in R^+ cultures is repressed in the same way as conjugating ability, so that the number of bacteria which can be infected with F phage is very small in established R^+ cultures and greatly increased in HFT systems with bacteria which have newly acquired the factor (Meynell & Datta, 1965). Since the production of the phage receptor by R or by F is subject to repression by R, it is probable that the fi^+ character results from an extension to F of the repression which the R factor ordinarily exerts on itself. In this way, F and fi^+ R factors appear as conjugation factors determining synthesis of the same surface structure and subject to the same regulatory mechanism.

The specific phage receptor on F^+ bacteria is a particular kind of pilus present in small numbers on the bacterium, slightly broader and often very much longer than the common pili (Type I: Brinton, 1965) which closely cover the surface of either an F^+ or an F^- bacterium. It was therefore important to see whether the same morphological structure acted as phage receptor on R^+F^- bacteria. Bacteria with attached phage particles could not be seen in established R^+ cultures, which was not surprising since these cultures contain so few phage-sensitive bacteria. However, in HFT systems where conjugation and phage infection were greatly increased, bacteria were easily found which showed pili similar to those on F^+ bacteria and to which phage particles attached all along their length. The same thing was seen with each of three different R factors. In the present paper, we report the results of tests designed to compare the proportions of bacteria able to conjugate, to be infected by F phage and to produce the specific pilus, in R^+F^- cultures grown under various conditions and de-repressed to different extents.

METHODS

Bacteria. These were all derivatives of *Escherichia coli* strain K12, and are listed in Table 1. The R factors were examined in the F^- strains RC709, RC711, RC12, RC24; and strains HfrH and HfrC were used as control bacteria carrying the F factor, and for assay of the phage.

R factors. These were R1, R124 (Meynell & Datta, 1966*a*) and R237, which confers resistance to ampicillin, streptomycin, chloramphenicol and sulphonamide and which was received by courtesy of Dr E. S. Anderson (Enteric Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9). All were shown to be fi^+.

F specific phage. The phage used was MS2 (Davis, Strauss & Sinsheimer, 1961), one of the group of spherical RNA phages. Preparations grown on strain Hfr H and passed through a membrane filter (Millipore, Type HA, pore size 0·45 μ) after preliminary removal of the bacteria by centrifugation showed titres of over 5×10^{11} plaque-forming particles (p.f.p.)/ml.

Table 1. *Escherichia coli K 12 derivatives used*

Strain	Synonym and Characters	Reference
RC 709	J 5-3 ($pro_1^-.met_2^-.lac^+.S^s$) F^- (acridine cured)	Clowes & Rowley (1954)
RC 711	J 6-2 ($pro_2^-.his^-.try^-.lac^-.S^s$) F^- (acridine cured)	Clowes & Rowley (1954)
RC 12	W677 ($thr^-.leu^-.B1^-$: F^-) S^r	Lederberg (1950)
RC 24	129 (W677; S^r) *fim*$^-$	Maccacaro, Colombo & Nardo (1959)
Hfr H	From 58.161 (met^- : F^+) with F integrated near the *thr* locus.	Hayes (1953)
Hfr C	From 58.161 (met^- : F^+) with F integrated near the *pro* locus	Cavalli-Sforza (1950)

Antiphage serum. This was prepared against another F specific phage $\mu 2$ (Dettori, Maccacaro & Piccinin, 1961), but neutralized phage MS2 at the same rate, with a K value of about 2500. Antibacterial antibodies were absorbed from the serum to prevent agglutination during tests of the bacterial suspensions for phage sensitivity; the antiphage activity of the serum remained unaltered.

Media. Nutrient broth was either Oxoid Nutrient Broth no. 2 or a tryptic digest of beef.

TYECa broth consisted of: Oxoid Tryptone, 10 g.; yeast extract, 1 g.; NaCl, 8 g.; dissolved in 1 l. distilled water and adjusted to pH 7·2. After sterilization by autoclaving, glucose was added to 0·15 % (w/v) and $CaCl_2$ to 0·002 M, for Ca^{2+} or other divalent cations are required for phage penetration (Paranchych, 1966), and possibly also for attachment (Valentine & Strand, 1965).

Nutrient agar consisted of Oxoid Nutrient Broth no. 2, solidified with 1·25 % (w/v) Davis N.Z. agar.

Tryptone Difco agar contained: Oxoid Tryptone, 10 g.; NaCl, 8 g.; glucose, 1 g.; Difco Bacto agar, 10 g.; dissolved in 1 l. distilled water and adjusted to pH 7·2.

The mineral salts medium was that of Tatum & Lederberg (1947), used with the omission of asparagine, and solidified with 1·5 % (w/v) Davis N.Z. agar.

Experimental procedure

HFT systems were prepared by using donor and intermediate strains nutritionally distinguishable from the strain chosen to act as recipient. In some cases, a donor and intermediate were used which could also be distinguished from one another. The donor (R^+) and intermediate (R^-) bacteria, taken from nutrient agar plates (which for the R^+ strain contained antibiotic), were grown for about 6 hr in broth. The R^+ culture was then mixed with the R^- culture in the proportions given in Table 2, column 5; 1 ml. of the mixture was then added to 9 ml. fresh broth and incubated overnight. Cultures with various degrees of HFT were made by altering the ratio of donor to intermediate, the broth, and the temperature of incubation; and by using different bacterial strains. Spread of the R factor throughout the intermediate

strain sometimes yielded a culture uniformly drug-resistant and largely released from repression. On the other hand, it should be noted that where the spread was poor, many of the bacteria examined were in fact R^-. While it was possible to get reproducible results by exactly repeating a given experiment, the conditions determining the extent to which the R factor was disseminated through the intermediate are not understood.

For tests of established R^+ cultures, pure cultures of the R^+ strain were incubated overnight.

Next morning, each culture to be tested was diluted 1/20 in TYECa broth and incubated in a water bath at 36·5° (unless otherwise stated). At the end of 2 hr, when the bacterial concentration was about 4×10^8/ml., the following procedures were carried out.

(*a*) Electron microscopy for piliation. A sample of 0·5 ml. of culture for electron microscopy was mixed with 0·5 ml. of phage suspension at 2×10^{11} p.f.p./ml. and incubation was continued for 20 min., when formalin was added to a final concentration of 0·25% (v/v). A second sample was formalinized without the addition of phage. Material was prepared for electron microscopy by modifications of the negative contrast method of Brenner & Horne (1959). In almost all the preparations used for determining the proportion of piliated bacteria, a drop of the culture was dried on a carbon-backed formvar-coated grid. The grid was then stained with uranyl acetate. In a few cultures, this 'dry' method retained so much debris that pili could not be satisfactorily identified. In these cases, a 'wet' method was used; the only difference was that the drop of culture was allowed to remain on the grid for 2–3 min., then stained before finally drying. This gave cleaner grids but fewer bacteria. In both methods, only a fraction remained of the bacteria in the initial drop of culture. 'Wet' and 'dry' preparations of the same culture were compared on two occasions. There were no significant differences in the proportions of piliated bacteria. The material was examined in a Philips EM 200 microscope (80 kV, 25 μ objective aperture). It is not certain that the method of preparation of the specimens produced representative results. Common (type I) pili are known to promote adhesiveness (see Duguid & Wilkinson, 1961). If bacteria with F type pili are either more or less likely to adhere to the grid than those without, sampling would be biased; however, the fact that the proportions were the same whether the grids were prepared by the 'wet' or the 'dry' technique makes such a bias less probable. Some loss of F type pili is likely to occur during preparation, since they are long and detached fragments are not uncommonly seen. Short pili will not be recognized unless they project sideways, otherwise they are obscured by the cell itself. It is not always easy to attribute pili to individual bacteria when these occur in clumps.

(*b*) Assessment of conjugation by resistance transfer. A further sample of 0·5 ml. of the culture under test was added to 4·5 ml. of a 22 hr broth culture of the R^- strain which was to act as recipient. The mixture was incubated for 40 min. and then serial ten-fold dilutions were spread on a set of plates of mineral salts agar appropriately supplemented with nutrients and antibiotics to allow growth of: (i) the donor R^+ strain, (ii) the intermediate strain, (iii) R^+ members of the intermediate strain, (iv) the recipient strain, (v) members of the recipient strain which had received the R factor. Only where donor and intermediate strains were nutritionally

different, could (iii) be distinguished from (i). The frequency of drug-resistance transfer was calculated from the numbers of colonies on these plates, and was expressed either as % resistant bacteria (Table 2, column 8) or % total bacteria (column 9) in the donor culture which transferred resistance. To arrive at these figures, the number of resistant colonies produced by the recipient strain was divided by the number of either resistant colonies or total colonies, produced by bacteria of the donor culture.

Forty minutes was chosen as a suitable time to incubate the mixtures for the following reason. When mixtures of R^+ bacteria just entering the stationary phase and stationary phase R^- bacteria were sampled at intervals and the transfer of

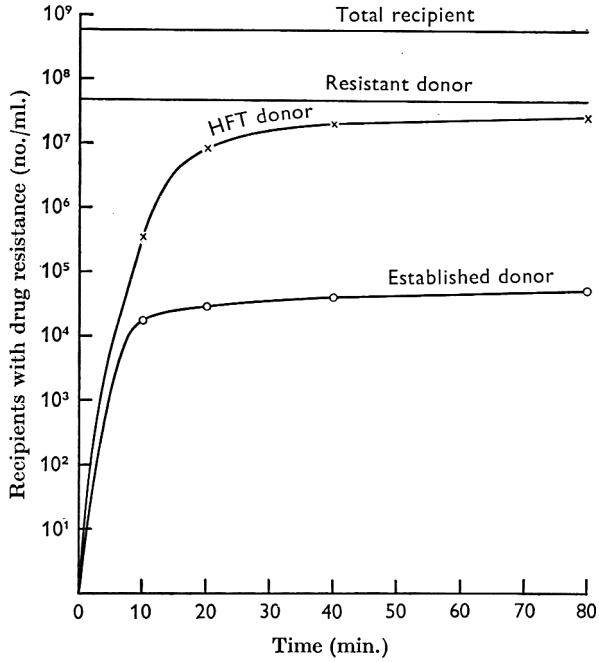

Fig. 1. Resistance transfer. A mixture of one volume of donor culture and nine volumes of recipient culture (see Methods) was incubated at 37°. Samples taken at intervals were assayed for number of bacteria of the recipient culture which had received drug resistance. The two curves show, respectively, the numbers which received resistance from an HFT donor culture and from an established R^+ culture.

resistance plotted graphically, the curve obtained showed an initial rise followed by a levelling off (Fig. 1). While the level of the plateau varied with different R factors, and also with the same factor in different conditions, it was reached by 40 min. in all cases. This suggests that each bacterium able to transmit an R factor does so within a short time of mixing, after which there is no further transfer. The number of transfers sometimes reached, but never exceeded, the number of R^+ bacteria of the donor strain. In these experimental conditions, it appears that each conjugating R^+ bacterium donates R only once, and that there is no spread of the R factor in the stationary phase recipient population.

(*c*) Measurement of proportion of phage-sensitive bacteria. Another sample (2·25 ml.) of the culture under test received 0·25 ml. of phage MS 2 diluted in TYECa

broth to a titre of $3–6 \times 10^{10}$ p.f.p./ml., the ratio of phage to bacteria being thus between 10:1 and 20:1. Incubation was continued for 8 min. to allow adsorption, and 0·25 ml. of the mixture was then transferred to 2·25 ml. of a dilution of antiserum which neutralized about 99·99 % of the phage in 10 min. After a further 10 min., about 10 ml. of TYECa broth were added and the whole amount was poured on a Millipore filter which was sucked almost to dryness before being washed through with another 10 ml. of TYECa broth. The filter was then transferred to 10 ml. TYECa broth in a 250 ml. flask, rinsed well to release the bacteria and discarded. In this way, residual free phage and antibody could be largely eliminated while the bacteria remained relatively concentrated. The released bacterial suspension was immediately assayed for phage to measure the number of bacteria that had become infected; the agar layer method was used with strain Hfr H as indicator on plates of Tryptone Difco agar. The plates were incubated at 42°, since plaques of F specific phage are better developed at this temperature than at 37° (Dettori, Maccacaro & Turri, 1963). Plating was completed by 25 min., for in one-step growth experiments with strain Hfr H, the minimum latent period of the phage was between 30 and 35 min. The infected bacterial suspension was again assayed to observe any increase in the titre of phage after 1·5 hr incubation at 37°. As control on the recovery of infected bacteria from the filter, the number of plaques produced initially by the released bacterial suspension was compared with the number given by simple dilution of the antiserum mixture, using either strain Hfr H or HFT R^+ cultures with a large proportion of phage-sensitive bacteria. These control tests showed that 70–100 % of the infected bacteria (plaque-formers) were recovered from the filter.

Background of non-specific plaques

When the proportion of phage-sensitive bacteria was very low, as in the established R^+ cultures, measurements became inaccurate because of the background phage contaminating the preparations. Attempts to lessen this background of non-specific plaques failed. It was not due to retention of free phage particles by the filter, for although it is reported (Lodish & Zinder, 1965) that in certain media such as 0·15 M-NaCl, over 80 % of phage f2 attached to Millipore filters, only about 1 % is retained from broth. In the present experiments with phage MS2 in TYECa broth, there was no loss of phage in filtrates, and only 0·5–1·5 % was recovered on rinsing the filters in fresh medium. The same held true for antibody treated phage suspensions in which only 0·01 % remained unneutralised, provided the phage stock had previously been filtered. When, however, the phage preparation had simply been treated with chloroform and low-speed centrifugation to remove bacteria, about 20 % of the antibody-surviving plaque-forming particles could be recovered which may have represented phage particles either in clumps or attached to F type pili which are reported to adhere to membrane filters (Valentine & Strand, 1965). The background of plaques in the present experiments appeared to be due to some effect of the presence of bacteria, since the plaque count obtained with a suspension of phage alone was usually lower. The effect was non-specific, however, for the count was just as high with *Bacillus subtilis*, *Staphylococcus aureus* or an aerial micrococcus as it was with *Escherichia coli* K12 F^-R^-; and the number of plaques with *E. coli* K12 F^-R^- was the same whether the bacteria were used live or

after boiling for 30 min., a treatment reported to destroy the ability of F pili to take up the F specific phage f2 (Valentine & Strand, 1965).

RESULTS

Pili similar to those found on F^+ bacteria were seen in HFT preparations of R^+F^- bacteria, including the *fim*$^-$ strain RC 24 which produces no common pili. Plates 1–4 show F^- bacteria carrying R factors, R1, R124 and R237, with strain Hfr H for comparison. F type pili were easily identified for enumeration by their adsorption of F specific phage at high multiplicity, but even in phage-free preparations, the specific pili could be distinguished from common (type I) pili (see Brinton, 1965) by their greater length and diameter or by the presence of end knobs (Lawn, 1966).

Table 2 shows the results of experiments made to examine quantitatively the relationship between F type piliation, as observed directly in the electron microscope; F phage sensitivity, as measured by the proportion of bacteria which could be infected; and the ability to conjugate, as measured by the proportion of bacteria which transferred drug resistance to a recipient within 40 min. Table 2, columns 9–11, shows the % bacteria with the three characters in the different cultures, arranged in ascending order of % bacteria showing F type pili. Low figures are not due only to repression in R^+ bacteria, but in part to poor spread of the R factor through the intermediate strain in the donor culture, so that a considerable proportion of the bacteria examined were still R^-. The distinction can be made from Table 2, column 8, which shows the % of resistant (R^+) bacteria which expressed their conjugating function. Although the R factor did not always spread through the culture, nevertheless those bacteria which were R^+ in mixtures of donor and intermediate were clearly less repressed than bacteria in established R^+ cultures, where no intermediate was present, as can be seen by comparing their respective transfer frequencies given in Table 2, column 8.

The cultures in Expt. 6 were incubated at 39·5°; as the temperature of incubation is increased above 37°, cultures of $R1^+$ bacteria contain progressively fewer bacteria with sensitivity to the phage or ability to transfer R. This is due to failure of function at high temperature, not a failure of the R factor to replicate, as with some temperature-sensitive F-*lac* (Jacob, Brenner & Cuzin, 1963). The effect was much less marked with R124.

The number of pili seen on individual bacteria was very variable. In preparations where many bacteria were piliated, the numbers ranged from one to twelve. However, when only an occasional piliated bacterium was present, it almost never showed more than one or two. This suggested that there were more pili per piliated bacterium in cultures with a high proportion of piliated bacteria than in cultures with a smaller proportion; and also more than the numbers usually reported for F^+ or Hfr cultures (see Brinton, 1965). The impression was supported by the results of an experiment, shown in Fig. 2, in which the numbers of pili on individual bacteria were counted in two HFT R^+ preparations, one showing 96% and the other 6% piliation. Data of Brinton (1965) for an F^+ culture and an Hfr culture containing respectively 50% and 43% piliated bacteria are included for comparison.

No piliated cells were seen in established R^+ cultures, and any estimate of the proportion present would have required the examination of an inordinate number

Table 2. *Capacity of R^+F^- cultures of Escherichia coli κ12 to produce F type pili, transfer drug resistance and support growth of F specific phage*

Expt. (1)	R factor (2)	Donor culture: R^+ donor (3)	Donor culture: R^- intermediate (4)	Ratio (5)	Recipient (6)	No. of bacteria screened for piliation (7)	Resistance transfer: % resistant bacteria transferring R (8)	Resistance transfer: total % bacteria transferring R (9)	Phage infection. % total bacteria infected (10)	Specific pilation. % total bacteria with F type pili (11)
1	None	. HfrH		.	.	100	.	.	68	61
1	None	. Hfrc		.	.	100	.	.	50	46
5	None	. HfrH		.	.	50	.	.	44	86
1	1	RC 711	None	.	RC 709	None	0·009	0·009	0·006	N.E.
1	1	RC 24	None	.	RC 709	None	0·004	0·004	0·005	N.E.
3	1	RC 709	None	.	RC 711	None	0·03	0·03	0·09	N.E.
6	1	RC 709	RC 709	1:50	RC 711	None	< 0·005	< 0·0001	< 0·005	N.E.
6	124	RC 709	RC 709	1:50	RC 711	None	0·29	0·011	0·12	N.E
5	1	RC 711	RC 711	1:50	RC 12	500	3·3	0·08	0·06	< 0·2
5	1	RC 709	RC 711	1:50	RC 12	500	0·7	0·08	0·13	< 0·2
3	1	RC 24	RC 24	1:200	RC 711	300	2·7	0·036	0·17	< 0·3
4	1	RC 711	RC 711	1:50	RC 12	500	0·24	0·009	0·34	< 0·2
4	124	RC 709	RC 711	1:50	RC 12	500	0·49	0·09	1·2	< 0·2*
3	124	RC 709	RC 709	1:100	RC 711	300	11	1·8	N.E.	< 0·3
4	1	RC 709	RC 711	1:50	RC 12	500	0·33	0·057	0·2	0·2
5	124	RC 709	RC 711	1:50	RC 12	500	0·67	0·12	0·49	0·2
3	1	RC 709	RC 709	1:200	RC 711	None	5·6	0·6	1·7	N.E.
4	237	RC 709	RC 711	1:50	RC 12	515	1·7	0·49	4·0	1·1
3	1	RC 709	RC 709	1:5	RC 711	500	4·2	0·9	2·1	2·2
5	237	RC 709	RC 711	1:5	RC 12	600	7·7	2·8	5·0	6
3	237	RC 709	RC 709	1:100	RC 711	100	7·2	2·3	10	14
1	1	RC 709	RC 709	1:10	None	100	N.E.	N.E.	9·6	17
1	1	RC 24	RC 24	1:10	RC 709	100	7·9	5·8	2·2	18
4	124	RC 709	RC 709	1:50	RC 711	100	61	31	18	22
5	124	RC 09	RC 709	1:50	RC 711	100	33	11	9	22
1	1	RC 711	RC 711	1:10	RC 709	100	66	29	5	24
4	237	RC 709	RC 709	1:50	RC 711	100	55	36	15	30
5	1	RC 709	RC 709	1:50	RC 711	50	100	62	22	40
4	1	RC 709	RC 709	1:50	RC 711	100	100	42	13·4	48
5	237	RC 709	RC 709	1:50	RC 711	50	100	50	24	53
2	1	RC 709	RC 709	1:100	RC 711	50	78	77	N.E.	96

* Two loose pili seen.

The experimental technique is described in Methods, The results were obtained in six different experiments, performed on different days, whose numbers are given in column (1). In expt. 6 incubation was at 39·5° instead of the usual 36·5°.

After incubation for 1½ hr the HFT cultures showed an increase in phage titre of between 1000- and 10,000-fold, except in the case of expt. 6, where there was no increase with R1 and only a 250-fold increase with R124.

N.E. = not examined.

of bacteria. Thus, in established cultures, the only comparison possible was between transfer of the R factor and phage infection; and measurements of the proportion of phage-sensitive bacteria become inaccurate as the plaque count decreases to the value of the non-specific background observed in control preparations without any sensitive bacteria.

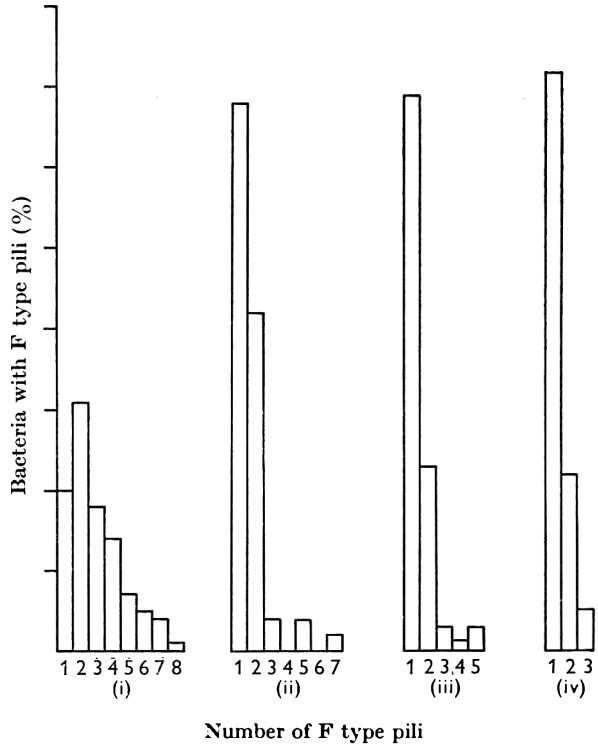

Fig. 2. Numbers of pili on individual piliated bacteria. (i) HFT culture of RC 709 $R1^+$ with 96% piliated bacteria : 100 bacteria examined. (ii) HFT culture of RC 709 $R237^+$ with 6% piliated bacteria: 50 bacteria examined. (iii) Data of Brinton (1965) for F^+ culture with 50% piliated bacteria: 61 bacteria examined. (iv) Data of Brinton (1965) for Hfr culture with 43% piliated bacteria: 72 bacteria examined.

DISCUSSION

The results of the present experiments, made to compare the frequencies with which F type piliation, F phage sensitivity and conjugating ability were expressed in R^+F^- cultures, have shown a high degree of correlation between the three characters. Thus, de-repression for one character was accompanied by de-repression for the other two. The different cultures showed a wide range of expression for all three characters, but the trend was the same for each. In Table 2, the cultures are arranged in ascending order of % of bacteria showing F type pili, but they would have been arranged in much the same order had either of the other two characters been chosen.

It may be seen, nevertheless, that where there were few piliated bacteria (i.e. $< 15\%$), their numbers agreed with the numbers of plaques, whereas, where there

were many (i.e. > 15%), their numbers were closer to the figures for transfer. Correlation between the three characters, as they were examined, might be expected to be imperfect for various reasons. The only one which was directly measured was the F piliation, and even here, complete accuracy cannot be assumed (see Methods). In the second half of Table 2, where the level of piliation is above 15%, the incomplete agreement between the numbers of plaques, on the one hand, and the numbers of bacteria showing F type pili and transferring resistance, on the other, might be attributed to failure of the phage to infect all the bacteria to which it adsorbed. Against this interpretation is the good agreement between plaque counts and the numbers of piliated bacteria in the cultures where few bacteria showed pili. Another explanation might be that, since production of the pilus tends to cause aggregation of the bacteria, the number of plaques underestimates the number of infected bacteria; for some plaques will be produced, not by single infected bacteria, but by clumps of several. Total numbers of bacteria were counted after dilution in the recipient culture, and by spreading the samples on the agar surface, which might be expected to give better dispersion of the bacteria than inoculation in an agar overlay. Marked clumping is observed in HFT systems with *col* I (Stocker, Smith & Ozeki, 1963) where it is attributed to the formation of mating pairs, and lattice formation is well known to occur with F^+ cultures under appropriate conditions. Microscope and electron microscope observations have also confirmed a tendency to clump in HFT R^+ cultures with many piliated bacteria.

When the proportions of bacteria expressing the three characters were low, as in the cultures in the first part of Table 2, the numbers of plaques as well as of piliated bacteria were slightly in excess of the numbers of bacteria apparently able to conjugate. Here the explanation may perhaps lie in the fact that resistance transfer was used as the index of conjugating ability and conjugation may not always inevitably result in actual transfer of the factor. It is conceivable that de-repression may lead not only to the free expression of conjugating ability, but also to a greater probability that the plasmid will be transferred at conjugation.

It seems likely that the specific pilus is involved in conjugation as well as in sensitivity to F phage. This can be tested by using mutants of the F factor defective in either conjugation or phage sensitivity to see whether the two can be dissociated from one another, or whether a decrease in phage-sensitive bacteria is necessarily correlated with a decrease in the frequency of conjugation. In experiments of this kind made by Cuzin (1962), Galucci & Sironi (1964) and Meynell & Datta (1966*b*), no separation was observed and in this way phage sensitivity and the capacity to conjugate appeared to be two different manifestations of the activity of one gene. This may be the gene which determines the synthesis of the pilus. R factors, like F, confer ability to conjugate, sensitivity to F phage and synthesis of the pilus. But they also determine the synthesis of a repressor of function, and, by using R factors instead of F, it is thus possible to demonstrate that the three characters, conjugation, phage sensitivity and production of the pilus, are coordinately de-repressed.

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EXPLANATION OF PLATES

Plates 1–4 are electron micrographs of *Escherichia coli* K12 carrying different conjugation factors and mixed with F. specific phage MS2. Three kinds of appendage can be differentiated: the specific pili are coated with round particles of phage MS2; common (Type I) pili have no adsorbed particles; flagella can be recognised by their sinuous shape, greater width and characteristic ultrastructure. All the preparations were negatively stained with uranyl acetate. The calibration bar in the full plate micrograph represents 1 micron, that in the inset micrograph represents 2000 Å.

PLATE 1

Escherichia coli K12 F⁻R1⁺

PLATE 2

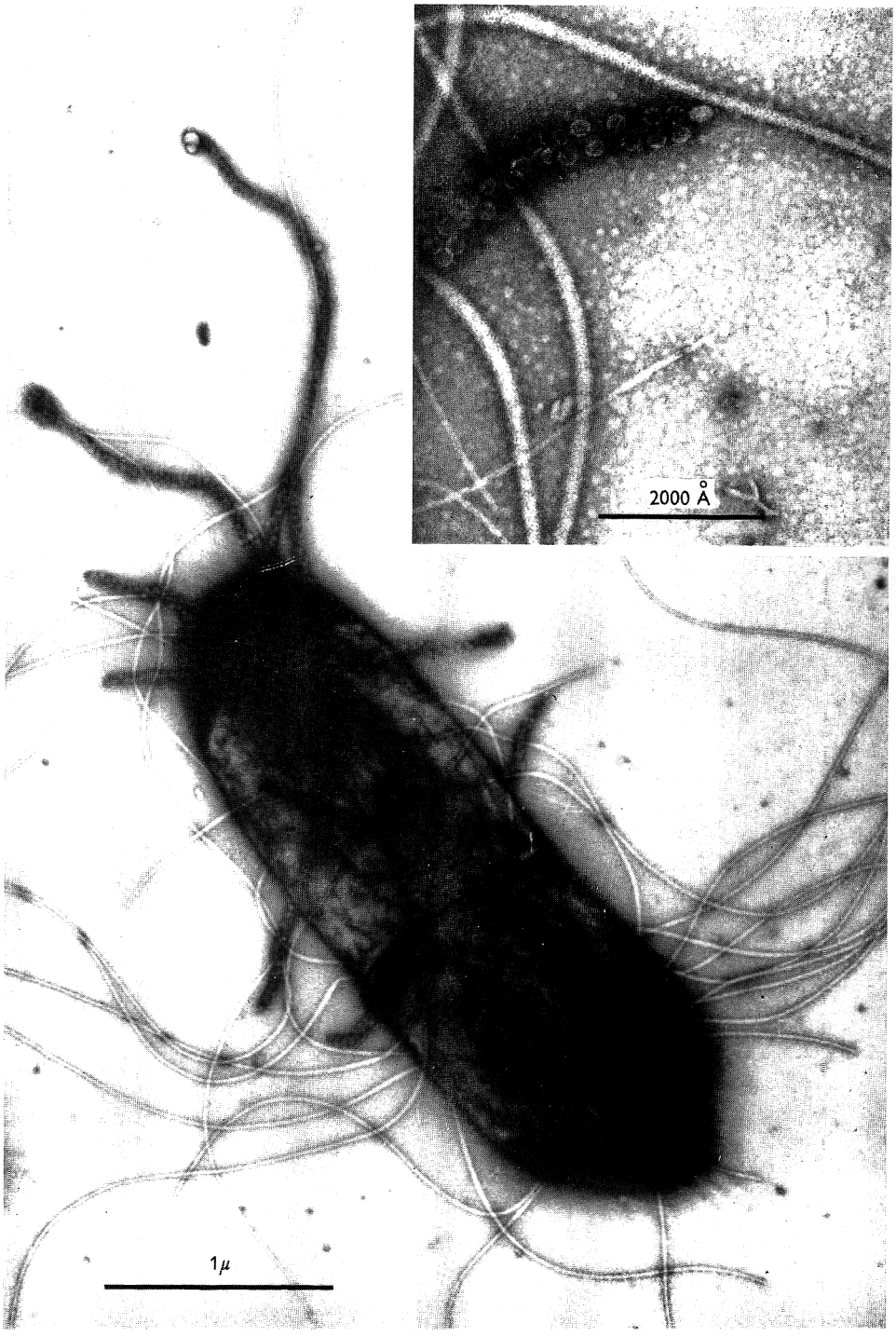
Escherichia coli K12 F⁻R124⁺

PLATE 3

Escherichia coli K12 F⁻R237⁺

PLATE 4

Escherichia coli K12 Hfr H



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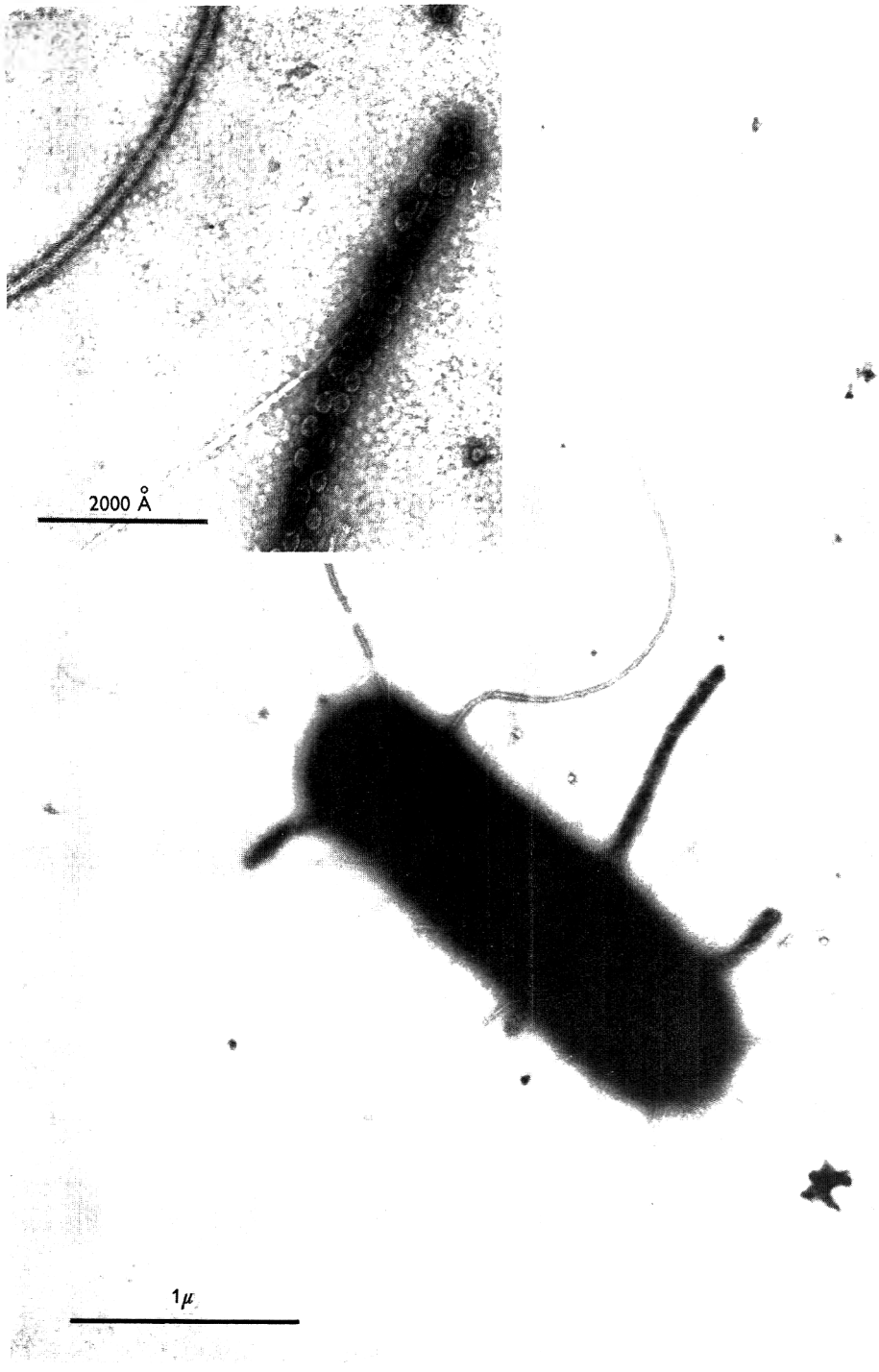

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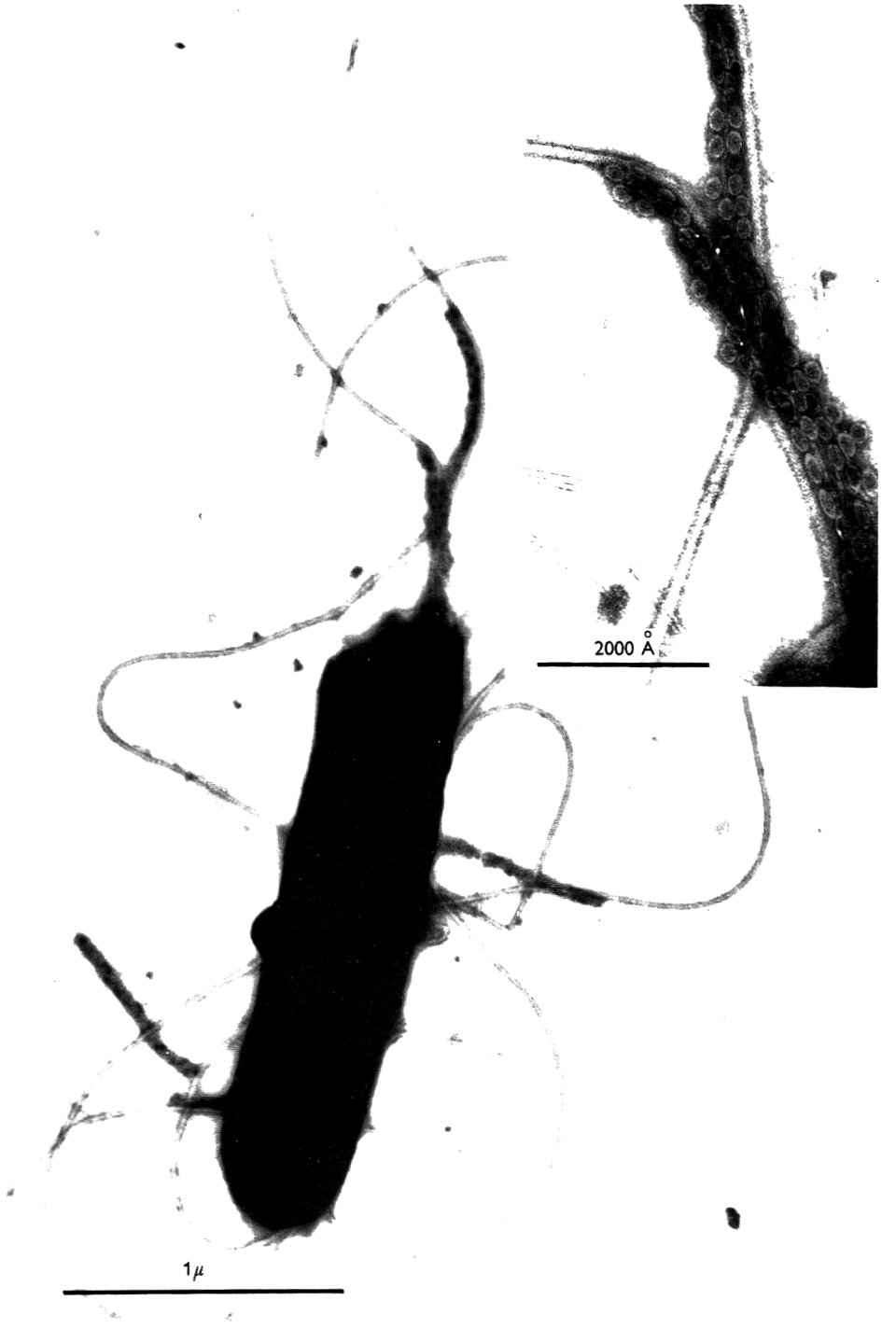

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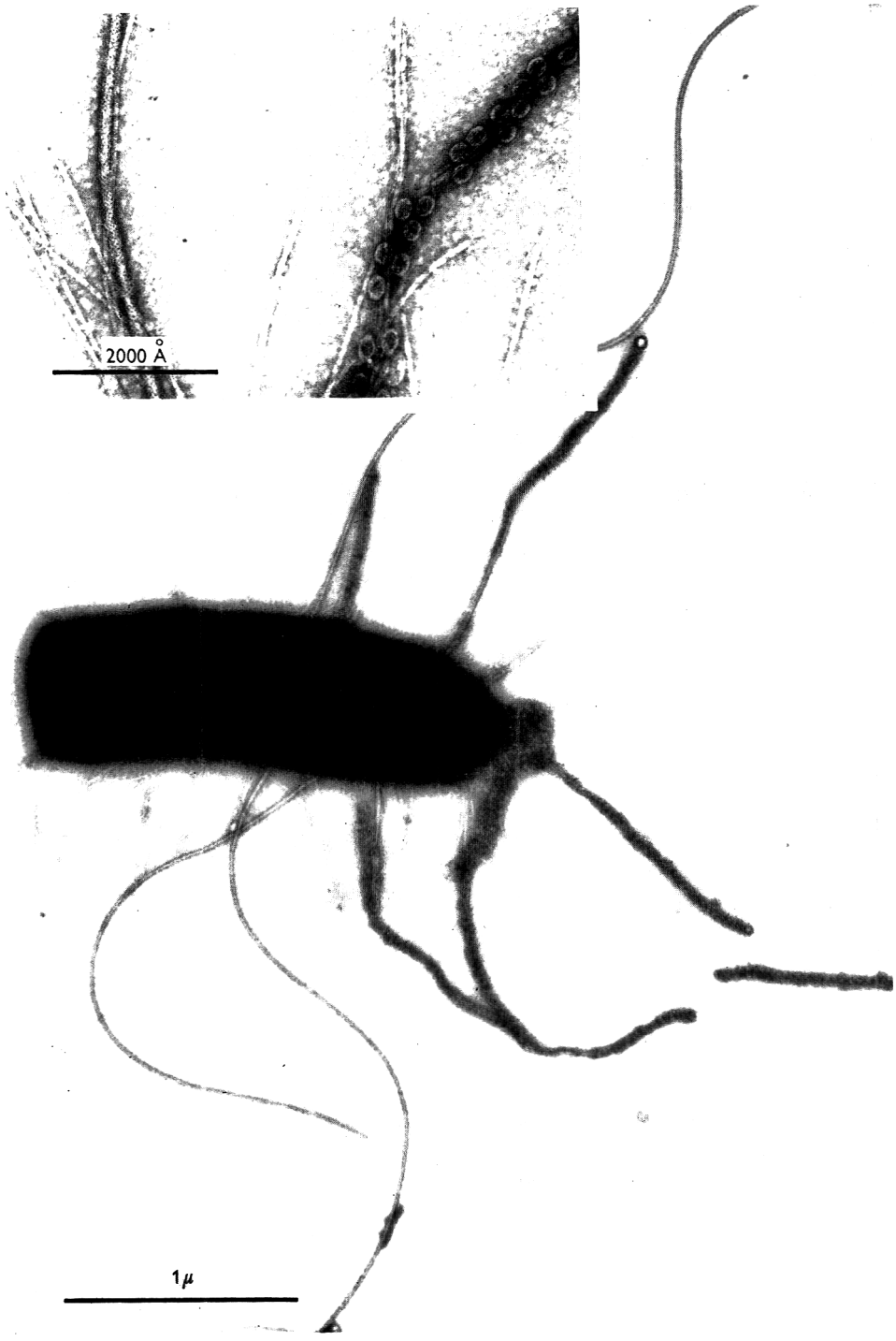

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Morphological Features of the Pili Associated with *Escherichia coli* K12 Carrying R Factors or the F Factor

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SUMMARY

Strains of *Escherichia coli* K12 carrying various R factors or the F factor were examined with the electron microscope. The F specific RNA phage MS2 was added to differentiate the specific F type pili, which adsorbed the phage, from common pili, which did not. Even when phage was not added it was possible to identify F type pili because they were wider and generally longer than common pili. F type pili, but never common pili, often possessed terminal knobs whose presence on attached pili has not been previously described. The F type pili formed by *E. coli* carrying the different R factors or HfrH could not be morphologically distinguished from each other. Only one morphological type of common pilus was observed.

INTRODUCTION

Crawford & Gesteland (1964) first reported the presence of pili on male (F^+) but not on female (F^-) bacteria which could adsorb the male-specific RNA bacteriophage R17. Brinton, Gemski & Carnahan (1964) showed that the phage receptive pili were of a special type, wider and longer than common pili (fimbriae: Duguid & Wilkinson, 1961; type I pili: Brinton, 1965) and named them F pili. Subsequently it was demonstrated that susceptibility to male-specific phages and the ability to conjugate and act as a genetic donor were both related to the presence of F pili (Valentine, Wedel & Ippen, 1965; Brinton, 1965).

Certain drug resistance factors (R factors) responsible for transmissible resistance to antibacterial agents also determine the production of pili with affinity for male-specific RNA phage (Datta, Lawn & Meynell, 1966). In a given R^+ culture, the proportion of bacteria with these F type pili was directly related to the proportion which could be infected with the phage, and to the proportion able to transmit their factor to suitable recipients.

In the present paper pili with affinity for F specific phage are referred to as 'F type pili' whether present on bacteria carrying the F factor itself or an R factor. Common pili are those which do not absorb the phage. A detailed examination has been made of the pili present on F^+ and R^+ bacteria in order to compare F type pili determined by F itself with those determined by various R factors, and to contrast these with the common pili present in many enterobacteria (Duguid & Wilkinson, 1961).

METHODS

The strains of bacteria, R factors and preparation of the samples are described in Datta *et al.* (1966). The bacteria were all derivatives of *Escherichia coli* K12 carrying either the F factor (HfrH) or one of the three R factors, R1, R124, or R237. Phage-free suspension and suspensions that had been mixed with the F-specific RNA phage MS2 at a multiplicity of about 500 plaque-forming particles/bacterium were examined.

Electron microscopy. A modification of the method of Brenner & Horne (1959) was used. A drop of the formalin-fixed culture was placed on a formvar-coated carbon-supported copper grid and either washed after it had remained for 2–3 min., or dried and then washed. The contrast agent was applied while the grid was still wet after washing. After a few seconds most of the remaining fluid was removed and the grid allowed to dry. Uranyl acetate was found to be the most satisfactory contrast agent.

The preparations were examined in a Philips EM200 microscope with 80 kV. accelerating voltage and a 25 μ objective aperture with the double condenser. The magnification of the instrument was calibrated by means of a diffraction grating replica: the calibration was reproducible to within 5%.

RESULTS

Identification of common pili

No pili absorbing the bacteriophage MS2 could be seen in F^-R^- cultures of *Escherichia coli* K12. Piliated bacteria were usually present, but all the pili were of one type, whose lengths and diameters were characteristic of common (type I) pili (see Brinton, 1965).

Identification of F type pili

In strains of *Escherichia coli* K12 carrying R1, R124 or R237 and in strain Hfr H the phage MS2 clearly distinguished common pili from F type pili, which were coated with the small spherical phage particles (Pl. 1, fig. 2). Even in cultures without MS2, F type pili were readily distinguishable, being wider and generally longer than common pili (Pl. 1, fig. 1). F type pili, but not common pili, often possessed terminal knobs. Although adsorption of F specific phage must remain the final criterion for the identification of F type pili, these morphological features are further evidence for the structural dissimilarity between F type pili and common pili.

(*a*) *Diameters of F type and common pili.* Measurements of F type pili could be made only on bacteria from cultures without phage MS2 otherwise the phage particles obscured the true diameter of the pilus. In any one area of a micrograph of a culture without phage, F type pili are clearly wider than common pili (Pl. 1, fig. 1; Pl. 2, fig. 3*b*; Pl. 4, fig. 10). However, the negative contrast technique, with its irregular distribution of stain, does not give uniform effects from one micrograph to another, nor even over different areas of a single micrograph. Measurements of pilus diameters over several micrographs therefore vary over a considerable range; common pili in some areas may be as broad as F type pili in others. The point is illustrated in Fig. 1 where the maximum and minimum diameters of individual

F type pili and neighbouring common pili are shown diagrammatically. The extreme range of diameter throughout the clearly outlined portion of an individual F type pilus (recognized either by a terminal knob or by contrast with the diameter of neighbouring common pili) on a single micrograph was measured. One or more common pili in the vicinity were similarly measured.

Although the diameters of common pili occasionally lay within the range of an F type pilus, the values for the two kinds of pili fall clearly into two groups. All pili with terminal knobs were within the F type diameter-range. There is no evidence for the existence of more than one kind of F type pilus or of one kind of common (type 1) pilus.

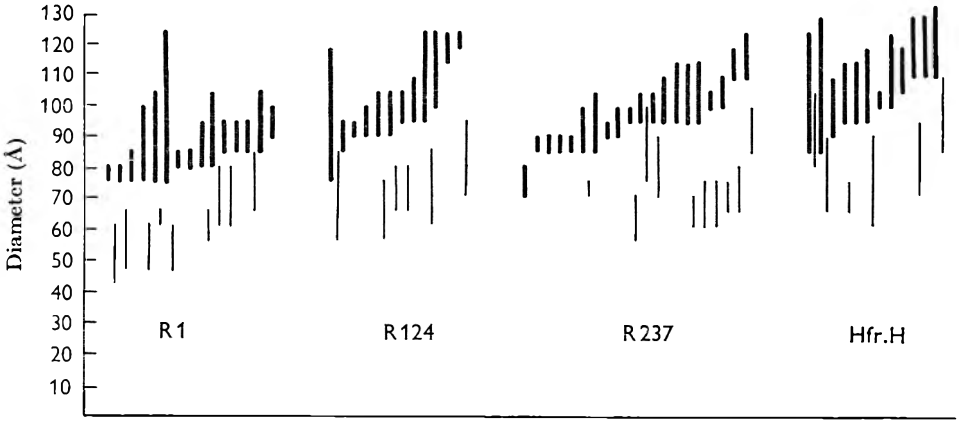

Fig. 1. The range of diameters of F type pili and common (type 1) pili formed by *Escherichia coli* K12 carrying different conjugation factors. A thick vertical line represents the diameter range of an individual F type pilus. The thin line immediately to its right represents the extreme range of diameters of one or more common pili in the vicinity of that F type pilus. The measurements are grouped according to the conjugation factor concerned.

Although (Fig. 1) the range of diameters of common (type 1) pili is large (45–100 Å), the distribution is in agreement with the figure of 70 Å deduced by Brinton (1965) from X-ray diffraction analysis and from measurements of shadowed preparations in the electron microscope. The average diameter (95 Å), and range (75–135 Å) of F type pili do not agree so well with the diameter of 85 Å given by Brinton (1965), who did not describe the method by which he arrived at this figure. Indeed, in the present sample, only 25% of F type pili had minimum diameters of 85 Å or less.

Both F type and common pili had a central dense line when prepared by the negative contrast technique. In micrographs taken near to true focus this dense line had a maximum diameter of approximately 15 Å in both types of pili. It was less prominent in F type pili, probably because of their greater thickness.

(*b*) *Terminal knobs of F type pili.* In a preparation containing phage, many of the pili which adsorbed F specific phage were enlarged at their distal extremities. Knobs were present on both short and long F type pili, but never on common pili. They did not result from the attachment of the phage, for the same knobs were seen in preparations to which no phage had been added. The shape and size of terminal

knobs was variable but they can be divided into three main types: disc-shaped, cup- or flask-shaped and spherical, The spherical knobs were vesicular, vesicular with a solid centre (sometimes laminated) or completely solid in appearance (Pl. 2, figs. 3*a–f* and fig. 4). The disc-shaped vesicular and solid knobs were respectively about 150–200, 400–800 and 250–700 Å in diameter. The spherical knobs are the most common. In some instances the F type pilus appeared to taper as it approached the knob, particularly in the case of the small solid knobs; this may be a staining artefact.

(*c*) *Lengths of F type and common pili.* F type pili associated with each of the conjugation factors varied in length from a fraction of a micron to over 20 μ; common pili seldom exceeded 1·5 μ in length.

Other features of common pili

In the strains studied, common piliation is not exclusively an all-or-none condition, as suggested in a recent review by Brinton (1965). Although the number of common pili per bacterium is most frequently zero or very large, many bacteria have only a few common pili. However, the proportion of bacteria bearing these pili varies considerably in different preparations of the same culture or even in different areas of the same grid.

Frequently, fragments of common pili broken during the preparation for electron microscopy were joined by a fine thread 15–25 Å in diameter (Pl. 4, fig. 8).

Association of common with F type pili

Some bacteria bearing F type pili without the phage MS2 label had a curious elongated structure extending from the bacterium or joining one bacterium to another (Pl. 3, fig. 5). At higher magnification this was resolved into a bundle of parallel filaments with the diameter of common pili. However, the length of these bundles exceeded that of a single common pilus. These bundles are formed by the deposition of detached common pili on the surface of an F type pilus, which is sometimes seen protruding from the end (Pl. 3, fig. 6). As the common pili are so short, they must lie in series to form a continuous sheath. A similar association of the two types of pili occurred in phage-labelled cultures, but less frequently and to a lesser degree. Where a common I pilus ran along the side of an F type pilus no phage particles were adsorbed on that side (Pl. 4, fig. 9).

In preparations of both labelled and unlabelled cultures bundles consisting solely of common I pili often occurred. Where there was more than one layer, pili in the second row often lay in the grooves between adjacent pili of the first row. This alignment gave rise to a heightening of contrast (Pl. 4, fig. 7) which appears to result from a re-inforcement of the dense central lines of the first row by superimposition of stain deposits at the edge of the second row.

Association of phage particles with F type pili

The number of phage particles attached to a 2000 Å length of F type pilus was measured in cultures of organisms carrying different conjugation factors. In most cases the average number was close to 15 (range 11–20, 7·5 particles/μ of pilus). With the ratio of phage particles to bacteria employed (about 500:1) this figure

was relatively constant, in spite of wide differences in the proportion of F type piliated bacteria. A calculation, based on the minimum distance between phage particles, suggested that 125–150 particles could lie in contact with each micron of pilus if they were closely packed and if the whole surface of the pilus was receptive. The observed average of 75 particles/μ although considerably lower than this figure, more probably results from restriction of ordered packing by random arrival of particles than of restriction of receptor sites on the pilus. In cultures to which a small number of phage particles were added it was clear that phage adsorption was initially random. With large numbers of phage particles the disposition along the pilus was not ordered.

DISCUSSION

In *Escherichia coli* K12, the F type pili associated with the R factors R1, R124 and R237 could not be distinguished morphologically from those produced by HfrH, which carries the F factor itself. There was no consistent difference either in diameter or length, and some of each kind had terminal knobs. The constancy of the average number of phage MS2 particles adsorbed per unit length of F type pilus formed by different strains is another point of similarity between them. All could readily be distinguished from common (type 1) pili.

Anderson & Strand (1965) found spherical bodies on preparations of detached F pili, and considered these to be basal structures or roots. Although basal structures can be seen on non-attached flagella, they cannot be seen on the free ends of flagella which are still attached to the bacterium (Abram, Koffler & Vatter, 1965). On the contrary, the terminal knobs of F type pili are as frequently present on the free end of attached pili as on non-attached pili. On some bacteria as many as 4 out of 5 F type pili are terminated by knobs (Pl. 2, fig. 4). For this reason, the knobs are unlikely to be basal root-like structures. There are two other possibilities. First, these structures may be present on the F type pili when they first emerge from the bacterial cell wall, possibly representing an extrusion of part of the cell wall/plasma membrane complex with the emerging tip. Secondly, the distal extremity of the F type pilus may have a special affinity for cell-wall material, associated with its supposed function in conjugation, and that stable association occurs with particles of cell wall complex present in the culture. Structures resembling terminal knobs occur from time to time in lysates of enterobacteria infected with various phages as well as in phenol-extracted cell wall preparations from enterobacteria (Lawn & Wilkinson, unpublished observations).

The bizarre structures resulting from association of common pili and F-type pili are described because they might cause difficulties in interpretation. The association is probably merely an expression of the general tendency of common pili to adhere to other structures (Duguid & Gillies, 1957, 1958) and does not necessarily imply any surface structural relationships as in the case of the single-layered and multiple-layered structures found in purified preparations of pili (Brinton, 1965). The lesser degree of association in phage-labelled cultures suggest that it is a slow process, most of it probably occurring after the addition of phage to the culture. An alternative but unlikely explanation is that the phage particles can displace the associated common pili from F type pili.

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EXPLANATION OF PLATES

All the micrographs are of *Escherichia coli* K12 carrying one of the R factors R1, R124 or R237. In some cases the F specific RNA phage MS2 was added to identify the F type pili. Preparations were made by the negative staining technique with uranyl acetate except fig. 10 for which uranyl formate was used.

PLATE 1

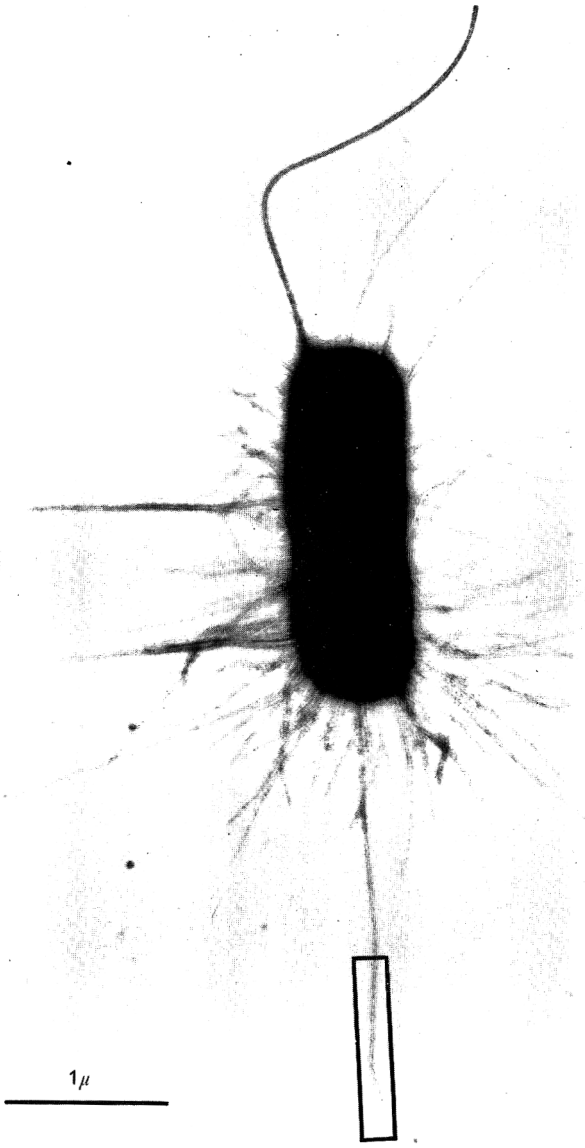
Fig. 1. *E. coli* K12, R124 without RNA phage MS2. A single flagellum and many pili are present. In this instance the F type pilus is hardly longer than neighbouring common pili, yet it can be distinguished because of its greater diameter, shown in (*b*) which is an enlargement of the area outlined in (*a*).

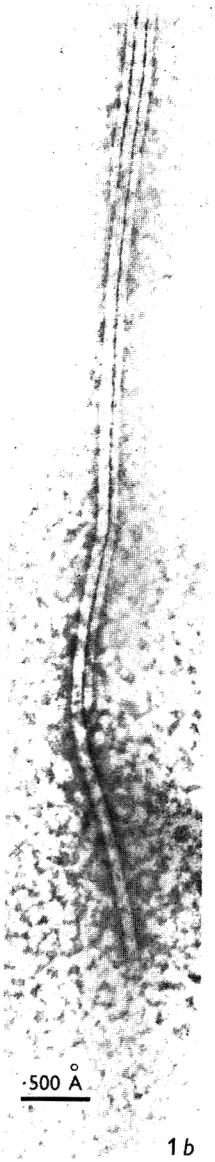
Fig. 2. *E. coli* K12 R237 mixed with the RNA phage MS2. The F type pilus, which has absorbed the round phage particles, can easily be distinguished from the common pili, which have no absorbed phage. Two flagella can be recognized by their width and distinctive surface structure.

PLATE 2.

Fig. 3. Terminal knobs of F type pili from *E. coli* K12 carrying various R factors, some with F specific phage particles. The calibration bar in (*c*) applies to all parts of the figure.

Fig. 4. A group of 5 F type pili attached to *E. coli* K12 R1. Four of the pili, which have all absorbed RNA phage MS2, are terminated by vesicular knobs. The areas outlined in (*a*) are enlarged in (*b*) and (*c*) where the terminal knobs are indicated by arrows.





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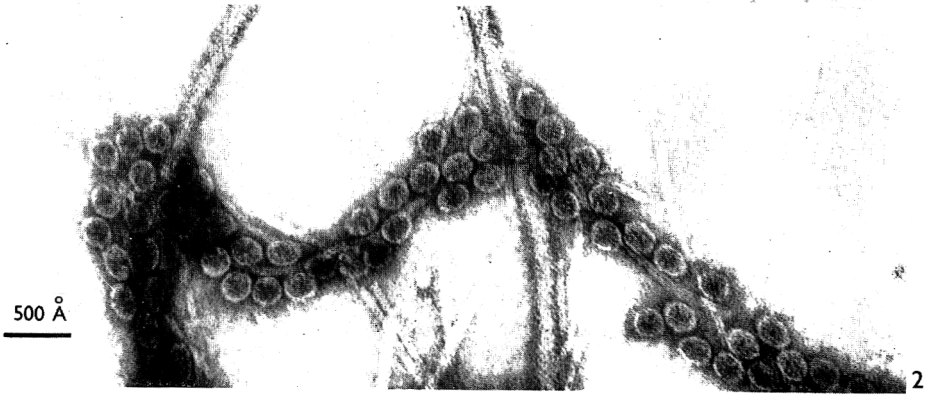

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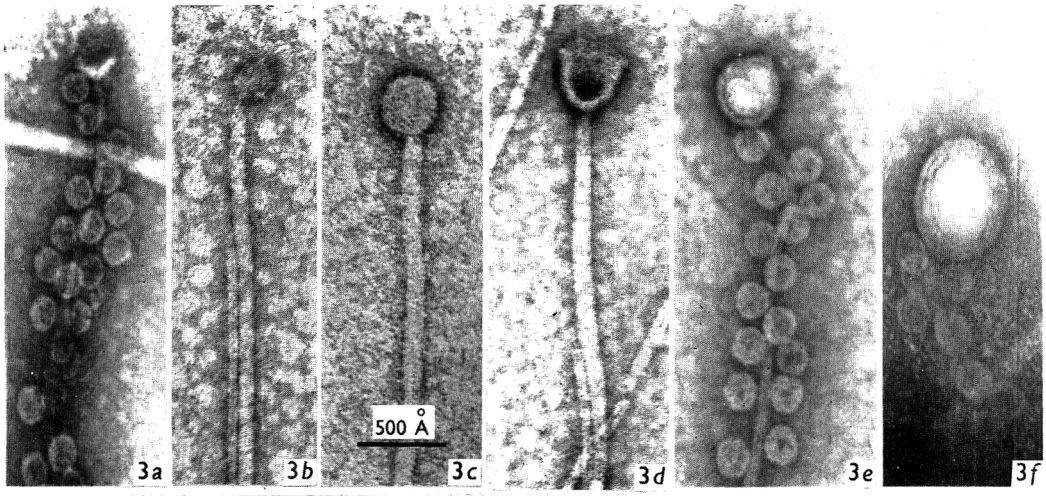

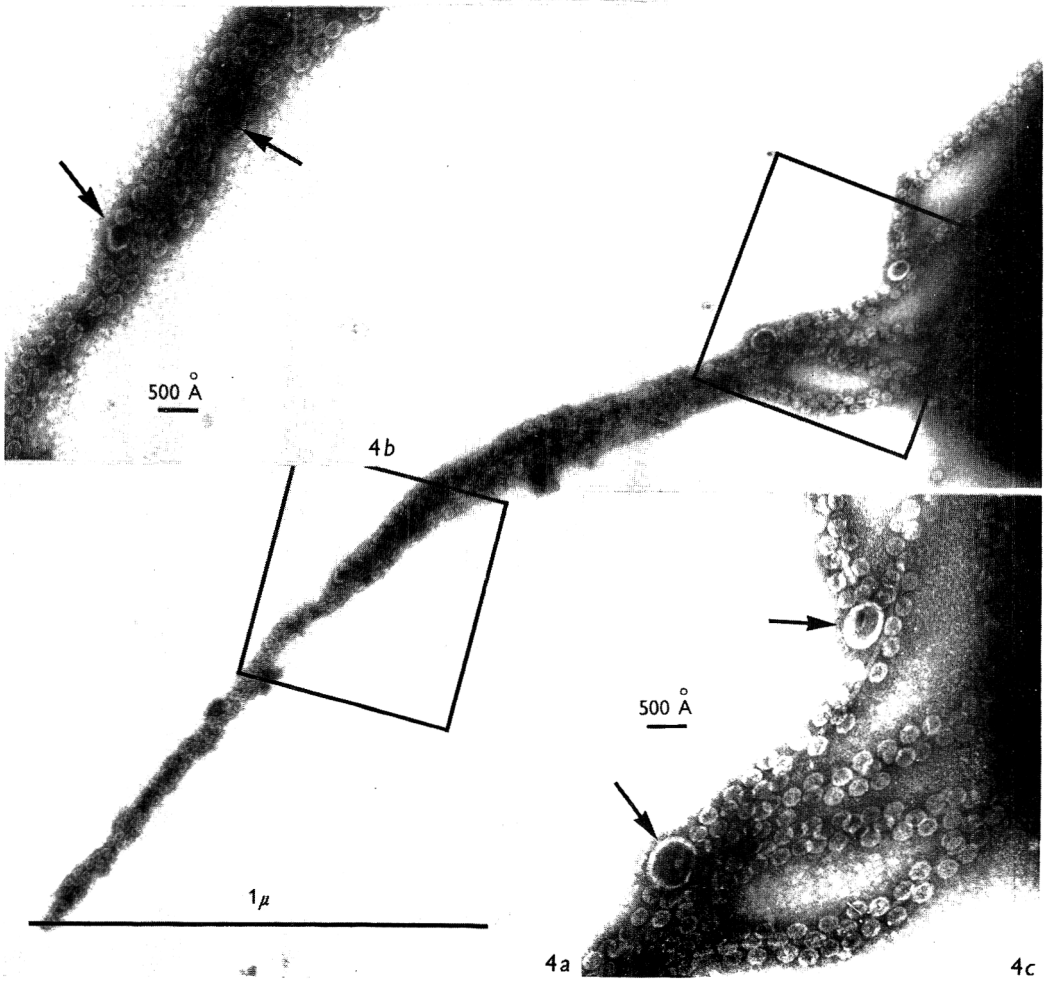

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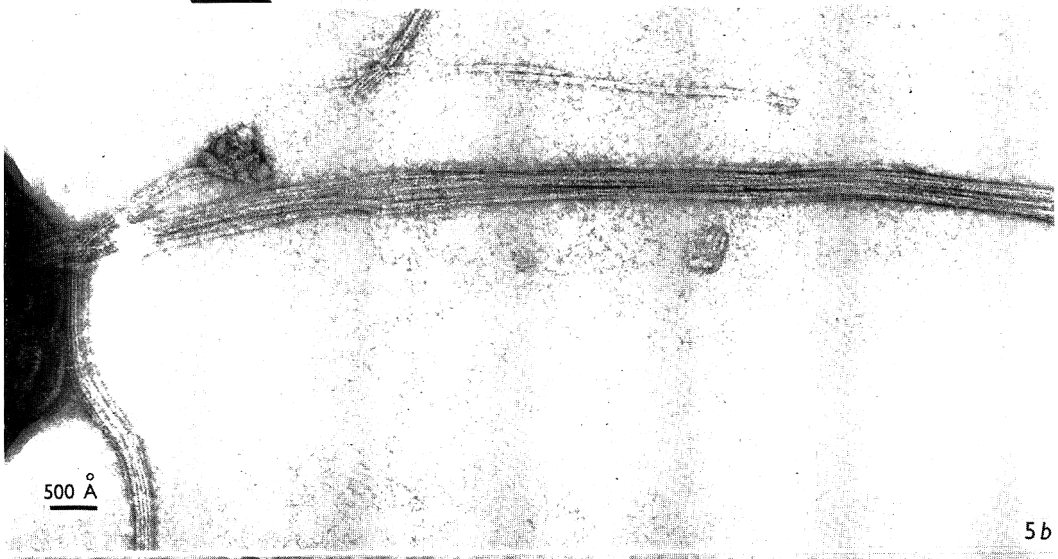

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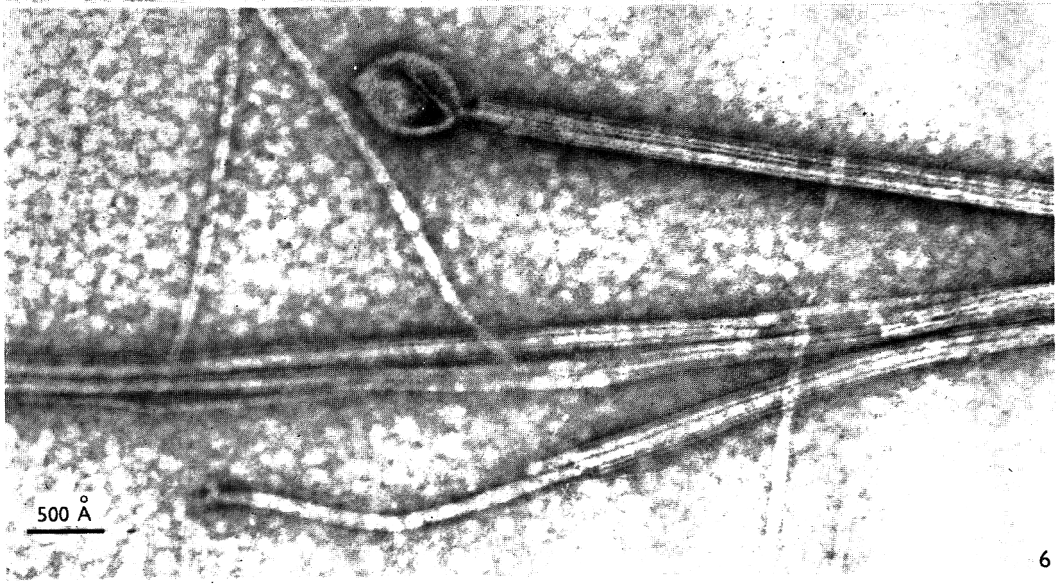

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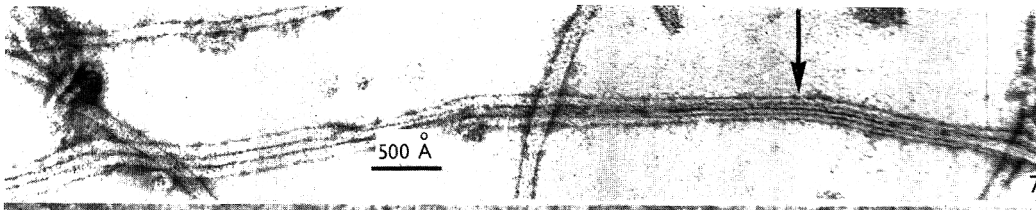

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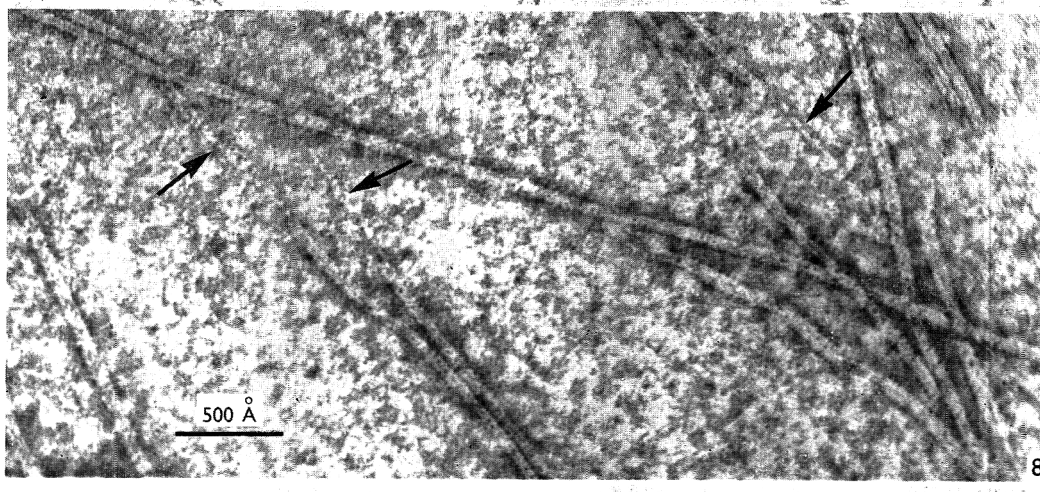

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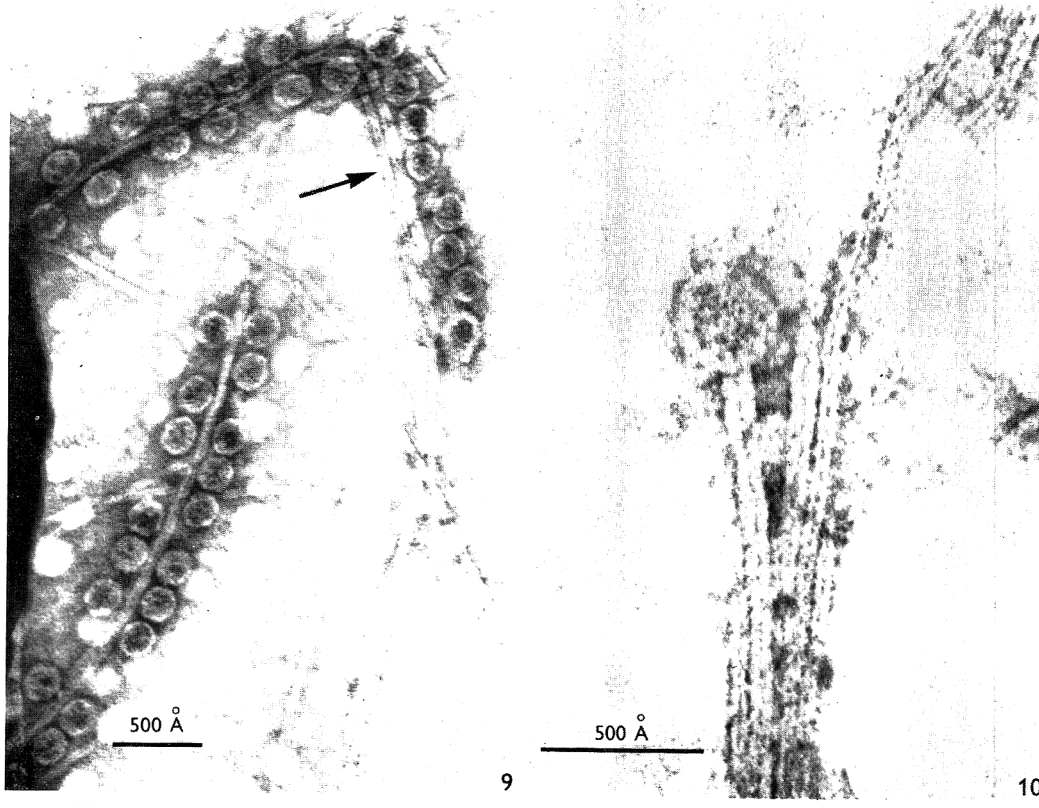

9 10

PLATE 3

Fig. 5. The bacteria (*E. coli* K12 R1) are linked by stranded threads. Part of (*a*) is enlarged in (*b*) so that the strands are defined and may be compared to detached common pili in the vicinity. No phage has been added.

Fig. 6. Two F type pili from *E. coli* K12 R237, one with a terminal knob, project from the ends of stranded bundles. The F type pili are wider than the common pili which ensheath them. No phage has been added.

PLATE 4

Fig. 7. On the left-hand side of the figure three pili lie side by side and their central dark lines are not prominent. On the right (arrow) one of the pili is superposed on the other two, giving an illusion of two pili with unusually prominent central lines.

Fig. 8. Several of the common pili in this figure have been broken. Their ends are joined by fine filaments 15–25 Å in diameter (arrows).

Fig. 9. Where a common pilus runs along a F type pilus (arrow) the phage MS2 is prevented from absorbing to the latter on the associated side. In other regions F type pili absorb phage equally on both sides. Note the flask-shaped terminal knob on one of the F type pili (*E. coli* K12 R237).

Fig. 10. Two of these pili from *E. coli* K12 R237 can be identified as F type pili because they are clearly wider than the adjacent common pili. One of the F type pili has a terminal knob. The central dark line is of similar diameter in the two types of pilus. Its apparent maximum diameter is approximately 15 Å (uranyl formate stain).