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Contents

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No. 3 *issued December* 1966

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

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

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

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

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

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

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

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

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

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

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

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

Journal of General Microbiology, Vol. 45, *No.* 1

KENNETH RUPERT BUTLIN

(*Facing p.* 1)

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Obituary Notice

KENNETH RUPERT BUTLIN, 1896–1965

Kenneth Rupert Butlin died on 1 October 1965 at his home in Teddington, Middlesex. He was 68. His wife Helen Mary and his son Martin survive him.

Butlin, known as 'Butch' to his friends, was born in Kettering, Northamptonshire, and was one of a large family. His education at Oundle School was interrupted by the First World War. He served in the Special Brigade Royal Engineers, and at the end of the War took up a scholarship at Trinity College, Cambridge. In 1921 he obtained first class honours in Chemistry and after a few months at the Royal Naval Cordite factory, he emigrated to Argentina. Here he worked on sugar and rum fermentation for Leech's Argentine Estates at Jujuy, until in 1925 he returned to England. He then worked for the H.P. Sauce and Midland Vinegar Company in Birmingham from 1926 to 1929, where his attention was directed to yeasts and to the acetic acid bacteria. In 1929 he joined a microbiology unit of the Royal Naval Cordite Factory at Holton Heath, Dorset. In 1933 this unit was transferred to the Chemical Research Laboratory, Teddington, where Butlin was to spend the rest of his research career. At that time this unit was headed by Dr A. C. Thaysen, with H. J. Bunker as second-in-command; Butlin and the late Miss M. E. Adams completed the unit.

The work of the unit encompassed cellulose decomposition and preservative treatments, bacterial sulphate reduction, sulphur-oxidizing bacteria and a variety of problems in applied microbiology. Butlin's own interest at this time was the acetic acid bacteria. His review of these organisms (Butlin, 1936*a*) was the second Special Report to be published by the then young Chemical Research Laboratory and, though long out of print, it remains an important basic work in the bibliography of the acetic acid bacteria. Butlin investigated the oxidative enzymes of *Acetobacter suboxydans* by using manometric techniques (Butlin, 1936*b*, 1938*a*, *b*) and from a knowledge of their pH sensitivity, devised a procedure for conducting the dihydroxyacetone fermentation at augmented substrate concentrations (Butlin, 1938*c*). In 1939 Butlin published with W. H. D. Wince procedures for preparing gluconic acid from glycol (Butlin & Wince, 1939*a*) and acetol from propyleneglycol (Butlin & Wince, 1939*b*) on a production scale.

With the declaration of the Second World War, Thaysen's unit turned to the problem of producing food yeast (*Torula* (now *Candida*) *utilis*) from molasses on a large scale. The intention was to use such yeast as a food supplement, for men or animals, and the Teddington unit supplied material for experiments made by the Medical Research Council and the medical services of the armed forces. Butlin was in charge of the pilot plant to produce this material; the plant was semi-continuous and his experience with it probably laid the foundation of his later interest in continuous cultivation. Butlin's research was not formally published; accounts of it may be found in a report of the food yeast project (Report, 1944) and briefly in an entertaining contribution by Butlin to the first Czechoslovakian Symposium on Continuous Culture (Butlin, 1958).

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In 1944 H. J. Bunker left the Teddington laboratories to enter the brewing industry and in 1945–46 A. C. Thaysen departed to take charge of the Colonial Microbiological Institute in Trinidad, British West Indies. Butlin was then the senior microbiologist, but the unit lost its administrative independence. Butlin and Miss Adams, with Miss Margaret Thomas who joined them about this time, formed the microbiology section of W. H. Vernon's Corrosion Group, charged with studying the role of sulphate-reducing bacteria in the anaerobic corrosion of buried iron pipes. As a result of the classic paper of von Wolzogen Kuhr & van der Vlugt (1934) sulphate-reducing bacteria had been incriminated as the major causative agents of underground corrosion; Starkey & Wight (1945) had published an exhaustive study of the mechanism of such corrosion and since some pre-war work by Bunker (1939*b*) had opened the field at Teddington, co-operation in research on corrosion might have seemed a logical niche for the residue of Thaysen's group of microbiologists.

Butlin did not wholly share this view. Almost nothing, for example, had been learned about the biochemistry of sulphate-reducing bacteria since Baars's (1930) thesis published 15 years before; authentic pure cultures of these bacteria were not available and doubt existed whether many workers in the field had in fact used pure cultures at all. Butlin, Miss Adams and Miss Thomas therefore embarked on microbiological studies designed to facilitate the isolation and cultivation of sulphate-reducing bacteria; to re-investigate with pure cultures the claims of Kluyver & Baars (1932) and of Starkey (1938) to have converted thermophilic into mesophilic sulphate-reducing bacteria (and vice-versa) by 'training'; to discover with pure cultures whether the hydrogenase present in these bacteria enabled them to grow autotrophically: to reduce CO_2 at the expense of the oxidation of hydrogen by sulphate. In a basic publication in this field (Butlin, Adams & Thomas, 1949*a*) they described their method, by using 3% (w/v) sodium sulphite to inhibit contaminants, of regularly obtaining pure cultures. Though exposure to such a high salt concentration is now known to exclude salt-sensitive types, it provided reproducible working material for fundamental and applied studies. They came to the conclusion that these bacteria were faculative autotrophs (Butlin & Adams, 1947; Adams, Butlin, Hollands & Postgate, 1951) and that Starkey's claim of adaptive interconversion, accompanied by drastic morphological change, was correct (Butlin, Adams & Thomas, 1949*b*). By hindsight we now know that both demonstrations were probably incorrect: a peculiar assimilatory reaction could simulate autotrophy in their test conditions (Mechalas & Rittenberg, 1960; Postgate, 1960) and the apparent adaptations to unfamiliar temperatures must have been unreal (see Campbell & Postgate, 1965). But, for the period before the ready availability of radio-isotopes, and before Campbell, Frank & Hall's (1957) identification of the thermophile as *Clostridium* (now *Desulfotomaculum*) *nigrificans*, Butlin's group's experiments were as rigidly designed as seemed possible. Their adoption of Bunker's (1939*a*) suggestion that growth could be stimulated by organic materials (Bunker & Butlin, 1947) made reasonable concentrations of bacteria available in pure culture for the first time. By 1948 it was obvious that further fundamental research in the biochemistry of sulphate-reducing bacteria was needed. R. P. Linstead, the then Director of the Chemical Research Laboratory, had considerable sympathy with Butlin's desire to expand the microbiological activities of the laboratory, and agreed to the appointment of J. R. Postgate as a 'Senior Research Investigator' (on a

temporary basis) to Butlin's section. This appointment was intended to strengthen the effort available for basic research and it led to a succession of publications from Teddington on the physiology of the sulphate-reducing bacteria, many of which were apparently far removed from practical application. It gave Butlin great satisfaction that, while on the one hand his unit laid the groundwork for a rigid demonstration of the role of cathodic depolarization in underground corrosion, it also overthrew the concepts of 25 years on the topic of respiratory mechanisms with the discovery of cytochrome c_3. As a perusal of the laboratory reports for those years will show (*Chemistry Research* 1947, 1948, 1949, 1950, 1951), basic demonstrations of the role of sulphate-reducing bacteria in corrosion were progressing steadily, their presence in tubercles was established (Butlin, Adams & Thomas, 1949*c*), and important field trials were initiated (Butlin & Vernon, 1949*a*, *b*; Butlin, Vernon & Whiskin, 1951*a*, *b*). Yet Butlin's enthusiasm for corrosion of iron was limited and his publications on concrete corrosion (Butlin, 1948), water pollution (Butlin, 1949) and microbiological sulphur production (Butlin, 1950) illustrated his search for new worlds to conquer. Official support for these interests came in 1951 when Butlin's section became independent of the Corrosion Group, and received the status of the Microbiology Group of the Chemical Research Laboratory.

In 1950 Dr S. T. Cowan, curator of the National Collection of Type Cultures (NCTC), announced to the microbiological world that he proposed to discard some 300 type cultures which were of negligible medical interest. Butlin seized upon this prospect, arranged house-room for the cultures at Teddington and obtained official sanction for the foundation of the National Collection of Industrial Bacteria (NCIB). Within months the culture collection at Teddington expanded to more than 1000 isolates and it included some strangely exacting organisms. On the advice of the late Professor A. J. Kluyver, a continental type of beer was thought to be the best medium for subculturing acetobacter species, and a modest but regular supply was duly arranged. The bacteria required but a limited proportion of the beer available; I recall several pleasant afternoon visits with Butlin to the Collection's laboratory because the Curator, W. S. Greaves, had signified his intention of 'subculturing the acetobacters'. Certain residues therefore required disposal. The present-day importance of the NCIB, now amalgamated with the National Collection of Marine Bacteria at the Torry Research Station, Aberdeen, needs no stressing for microbiologists.

In 1950 also, Butlin accompanied by J. R. Postgate, made a trip to the then separate countries of Tripolitania and Cyrenaica in North Africa, to visit certain sulphur-producing lakes which were reported to exist in that area. At that time Britain was suffering from the world sulphur shortage; because of the war, the rate of exhaustion of known sulphur deposits had exceeded the rate of discovery of new ones, and Britain, whose industry relied almost entirely on imported sulphur, was faced with small quotas and mounting costs, the latter mainly in hard currencies. Any natural source of sulphur was worth investigating, and as the lakes near El Agheila seemed, from geologists' descriptions, to be contemporary examples of sulphur formation by microbes, the investigation seemed justified on economic grounds as well as for its scientific value. The expedition has been written up (*Chemistry Research*, 1950, 1951; Butlin & Postgate, 1954*a*), but even Butlin's ability to infuse an element of humanity into a cold official report failed to convey

the hilarity, absurdity, yet fundamental success of that expedition. Stranded in Benghazi because the local authorities had to impress their transport shortage on a visiting Military Official (as soon as the Official had passed on to Egypt, transport miraculously appeared); driving through an un-cleared mine field with a fatalistic Arab at the wheel of a Land Rover; taking *leben* (a fluid sort of yoghourt) and exchanging belches with the local Bedouin; joining in an Arab sing-song after an Arab wedding party in which the expedition somehow became involved; the loss for 18 hr, and happy re-appearance absolutely unharmed, of a 15 cwt. truck containing all the expedition's equipment (the non-European part of the expedition had decided to visit relatives on the way home). These and a thousand other incidents make one regret that Butlin never wrote the book 'Frolics of a microbiologist' which he sometimes contemplated.

From a scientific point of view the expedition achieved all it set out to do: the lake Ain-ez-Zauia, which yielded about 100 tons of sulphur a year, proved to be an almost text-book example of the sulphur cycle in action; sulphate-reducing bacteria and sulphide-oxidising bacteria jointly converting sulphate via sulphide to sulphur, powered, as it were, by the North African sun. The process was copied in the laboratory at Teddington and research was started on how best to adapt the process industrially. Because of the sulphur shortage, the expedition received excessive press publicity: reports of the expedition, usually somewhat garbled, were published in newspapers all over the world, the B.B.C. broadcast accounts of it, the Teddington group was inundated with telephone calls from reporters for several weeks and the Press Office of the Department of Scientific and Industrial Research collected several hundred cuttings on the subject during 1950. The situation was in many ways highly amusing (a press report starting with the words: 'The market in African mud holes isn't exactly bullish these days . . .' was adopted by Butlin as a Christmas card) but it had political repercussions. Shortly after Butlin's return the late Right Hon. Mr Richard Stokes, then Minister of Materials, was negotiating an increase in Britain's sulphur quota from Washington; he found it difficult to convince the U.S. of our real needs in view of widespread press reports that African bacteria at Teddington were about to solve the world sulphur problem. A stiff instruction to play down the story of the sulphur bacteria reached the laboratory's headquarters from Mr Stokes's delegation.

The experiments on African bacteria did not solve the sulphur shortage; no one in his senses had thought they would. But there arose from them the project for the microbiological production of sulphide, which work Butlin directed personally and which engaged most of his attention until his retirement. The energy-consuming step of natural sulphur formation is the reduction of sulphate to sulphide and, by using an industrial waste product to replace the carbon fixed by photosynthetic bacteria in the natural process, an industrial method for the preparation of reduced sulphur should be feasible (Butlin, 1950; Butlin & Thomas, 1954). Sewage sludge proved to be the only economically suitable raw material, and though there was insufficient sewage available to meet the whole sulphur needs of Great Britain, an appreciable proportion could be obtained this way. Butlin, with Miss Sylvia Selwyn and D. S. Wakerley, developed a process for the sulphide fermentation of sewage sludge and the extraction of the sulphide which, with the co-operation of Dr S. G. Burgess of the London County Council, was brought to the pilot plant scale (Butlin,

Selwyn & Wakerley, 1956, 1960; Burgess, Butlin & Postgate, 1958; Burgess & Wood, 1961). Sulphide fermentation of sewage brought with it the bonus, to sewage technologists, of improved settling of the digested sludge. But by 1959, when the feasibility of the project had been well established, the sulphur crisis was long over and official support of the project was withdrawn. Such processes have, however, been used in Eastern Europe and the work of Butlin's group on this topic would provide the basis for a resumption of the work when the world's sulphur resources become seriously short again. World demand for native sulphur began to exceed world supply once more in 1964.

Though primarily interested in the microbiological production of sulphide, Butlin also initiated other applied studies in his unit. He was responsible for the bacteriological side of Knolles's (1952) lagoon procedure for recovering waterlogged pits for building without gross nuisance from H_2S production from sulphate reduction; his group's use of chromate as a practical inhibitor of these bacteria (see Drummond & Postgate, 1955) arose because a disconcerted Local Authority consulted him over the discovery that some fly-by-night had quietly dumped great quantities of chromate in a clay-pit in the middle of the town. A most happy malfeasance, as it happened, because chromate is still one of the most effective inhibitors of sulphate-reduction for use in such circumstances. Contamination of stored petroleum spirit grounded British military aircraft twice at critical times during this period; the problem was referred to Butlin but his recommendations, to his annoyance, were not adopted in time (*Chemistry Research*, 1952, 1956). 'Fossil disease' , a spontaneous crumbling of pyritic fossils that can occur in museums, was traced tentatively to *Thiobacillus ferro-oxidans* (a finding that was never published). Consultant work on biological effluent disposal was started and later, with Drs S. L. S. Thomas and V. A. Knivett, some biochemical work on microbial phenol oxidation was initiated (*Chemistry Research*, 1953). Butlin's wide interest in the economic importance of microbes is illustrated by the general review (Butlin & Postgate, 1954*b*) and a later article (Butlin, 1962). He sought to convert his group into a national centre for research into Economic Microbiology, with the NCIB as its pivot. In 1956–58, V. A. Knivett had started studies on the methane fermentation, using continuous culture techniques (*Chemistry Research*, 1957, 1958); considerable progress had been made on the biochemistry of bacterial oxidation of phthalates and aryl sulphates (*Chemistry Research*, 1954 to 1958); F. W. Ochynski's investigation of multi-stage continuous culture of phenol bacteria was in progress; Miss M. E. Adams had added concrete corrosion and leaching of minerals by thiobacilli to her programme (*Chemistry Research*, 1957, 1958); the initiation of research into coal microbiology was under discussion. Moreover, the arrival of Dr G. Booth in the Corrosion Group had initiated a new and more productive period of collaboration, studying the mechanism of bacterial corrosion of metals. Visiting scientists and vacation students contributed to the group's work regularly (a particularly memorable visitor was Dr J. C. Senez, now Director of the French C.N.I.R.S. Laboratory of Bacterial Chemistry in Marseilles, who spent 2 months at Teddington in the early '50's and became a life-long friend of Butlin's). The group was advised by a special Committee of microbiologists chaired first by the late Professor D. D. Woods, later by Professor S. R. Elsden; general advice to industry on problems of a microbiological character was part of the group's routine. Nevertheless, practical problems did not distract

Butlin entirely from fundamental matters and he contributed valuably to reviews of a basically academic nature (Butlin & Postgate, 1953, 1956) as well as reviewing applied subjects (Butlin, 1953, 1956). By 1958, Butlin's dream of a British centre for research in Economic Microbiology seemed to be approaching realization, and a strong bid for expansion of the group's programme and resources was made by Butlin himself and by his Advisory Committee.

At this point one is obliged to say something of the disagreeable events that led to the disbandment of Butlin's group on 1 May, 1959. This act, which shocked microbiologists both here and abroad, was a most unfortunate example of administrative ineptitude; a decision which benefited no-one and which caused remarkable ill-feeling (*Chemical Age*, 29 Nov. 1958; *The Times*, 29 Nov. 1958). The Teddington laboratory, by then called the National Chemical Laboratory, has since been disbanded itself, and the Department of Scientific and Industrial Research (D.S.I.R.) has also been abolished; no purpose will be served by raking over the old embers. Nevertheless, it is proper to record the elements of the situation here. The D.S.I.R.'s Water Pollution Research Laboratory had been offered a substantial grant by the Federation of British Industry provided the greater part of it was devoted to the microbiology of water pollution. The D.S.I.R. proposed to transfer the research side of Butlin's group, but not Butlin, to the Water Pollution Research Laboratory and to modify the group's programme accordingly; the NCIB was to go to Aberdeen. Great objections were raised by Butlin and his colleagues, by the Advisory Committee and by the Institute of Professional Civil Servants but, in the event, the D.S.I.R. decided to enforce the disbandment and transfer upon Butlin's group. Only three of the Teddington Group actually made the physical move to the Water Pollution Research Laboratory and within a year two of these found employment elsewhere; Butlin, who had resisted the break-up of his group most strenuously, was compulsorily retired by the D.S.I.R. two years before he need have been.

After his retirement Butlin became a private consultant to industry, advising on microbiological problems such as effluent disposal and, at one time, visiting the U.S.A. on behalf of a major oil company to survey the position about research on nitrogen fixation. These activities led to no formal publications.

During his scientific career he was an active member of the Society for Applied Bacteriology, the Microbiology Group of the Society of Chemical Industry and the Society for General Microbiology. In July 1962, our Society made him an Honorary Member, a gesture which gave Butlin intense pleasure for many reasons, not least of which was the fact that, in a list of honorands with many distinguished letters both before and after their names, Butlin's was the only one with nothing but a modest 'M.A.'.

It would be wrong to conclude this obituary without a word about Butlin the man. His exuberant friendly personality made a lasting impression on all who met him, and inspired great affection and loyalty. He was a lover of classical music, a connoisseur of wine, and he derived great enjoyment from travelling, paintings, good food and convivial company. As another obituarist has written (*The Times*, 2 Oct. 1965), he 'had no time for the two cultures'; he refused to divorce science from everyday culture, and this refusal is exemplified by the uncommonly high standard of writing that characterized his scientific exposition. But, though an intellectual, he was in no way austere: in the right company a pint of draught beer

was as delightful as a vintage *Château Cheval Blanc*, impromptu music-hall songs as enjoyable as the grand opera that he loved. Despite a lifelong impediment in his speech, which disappeared completely when he sang, his company was always the gayer, the more enriched, for his presence. No event could remain wholly formal when Butlin was around. If this obituary has shown moments of unseemly gaiety, it is surely as Butch would have wished.

J. R. POSTGATE

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With 1 *plate*
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Study of Osmophilic Yeasts producing Invertase

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SUMMARY

Samples of sugar cane (*Saccharum officinarum*), cane juice, raw sugar and refinery samples were screened for osmophilic yeasts able to ferment sucrose. One of the isolates showed properties bordering on the original definition of an obligate osmophil. Therefore, diagnostic tests were adapted to fit ecological considerations. These modifications in technique were adopted for all isolates when it was found that more consistent results could thus be obtained. As a result of these tests relevant organisms were identified as *Torulopsis apicola*, *T. globosa*, *T. lactis-condensi*, *T. bacillaris*, *Candida guilliermondii*, *Saccharomyces florentinus* and a new species, *T. kestoni*.

INTRODUCTION

It has been known for some years (Scarr, 1951) that *Saccharomyces rouxii* and *S. mellis*, growing in impure concentrated sucrose solutions, will ferment any invert sugar present to form organic acids when the subsequent decrease in pH value allows a slow chemical inversion to follow. There was no detectable invertase production by normal diagnostic tests. Torulopsis species, growing more slowly, were also noted, but, under the conditions of test, did not appear to be biochemically active. Recently, however, an incident was reported of fermentation in a saturated sucrose solution containing no invert sugar and from it only torulopsis species were isolated. The following account describes the isolation and identification of osmophilic yeasts from this and other sources.

METHODS

Definition of concentration. Osmophilic yeasts grow in a highly selective medium. At very high concentrations of dissolved solids, the exact concentration is important and unless otherwise stated is defined here as ° Brix. This is the weight of dissolved solids as % of weight of solution determined at 20°.

Bacteria will grow, albeit slowly, in solutions up to 40° Brix and yeasts and moulds up to 75–79° Brix. For convenience, osmophilic organisms are defined here as those which can grow at concentrations over 65° Brix, which in pure sucrose solutions are conditions near to saturation (saturated sucrose solution at 20° = 66·7 °Brix).

Isolation, screening and storage. Plate cultures were made on osmophilic agar (Scarr, 1959) and colonies picked off into filter-sterilized 20 % (w/v) sucrose yeast–water solution containing a trace of bromocresol green, in McCartney bottles with a Durham tube. These were incubated for a maximum of 3 weeks at 27°. The contents of bottles showing acid and gas formation were then transferred to 65° Brix sucrose solutions containing 0·5 % Difco Bacto Peptone, 0·1 % $MgSO_4.7H_2O$ and 0·2 % KH_2PO_4 (sterilized at 5 lb./in.^{2} for 10 min.) and incubated at 27° for a further 3

weeks. Cultures still showing a positive result were then streaked on osmophilic agar and stored on slopes of this medium.

Isolates from cane, cane juice, air (entrapped on a membrane filter), raw sugar and intermediate refinery products were screened in this manner.

Diagnostic tests. Preliminary tests were made by the standard methods of Lodder & Kreger van-Rij (1952) but using liquid auxanogram tests and growth on vitamin-free medium according to Wickerham (1951). The fermentation tests were incubated for 3 weeks at 27°.

Subsequently modifications were made in the fermentation and carbon assimilation techniques by using 10% (w/v) of the appropriate sugar or carbon source, except for ethanol, inulin, succinic, lactic and citric acids which were tested at normal strength and salicin at 1% and in saturated solution. Assimilation of soluble starch was tested by the plate auxanogram.

Liquid assimilation media were prepared with Difco Yeast Nitrogen Base and carbon source at final strength, sterilized by filtration and dispensed aseptically as 5 ml. volumes into empty plugged sterile tubes. In the nitrogen assimilation test the Difco Yeast Carbon Base was re-inforced with extra glucose where necessary.

Inocula were taken from 72-hr slopes on osmophilic agar (incubated at 30°), washed and centrifuged in sterile Ringer's solution; one drop of washed suspension was used per tube. We noticed no loss of viability after this treatment.

RESULTS

One hundred isolates were examined. After screening tests and the elimination of obvious duplicates, the number of cultures was ten. For comparative purposes, a culture of *Saccharomyces rouxii*, Boutroux (code Sa 24) isolated from a Mauritius raw sugar, was included in the examination. The ten species isolated fell into three genera which are considered separately.

Cultures T7A, T8, T9, T10, T11 and T12

Data for this series of isolates are summarized in Tables 1 and 2.

In Culture T7A (isolated from a refinery sample) 10% sugar broths only gave a fermentation pattern corresponding to *Torulopsis apicola* Hajsig (1958). From this aspect and its very poor growth on wort agar, it resembles the 'obligate osmophil' of Kroemer & Krumbholtz (1931); but many osmophilic yeasts can be gradually accustomed to more standard conditions, so we do not consider this a difference of taxonomic significance. Assimilation of L-sorbose, xylose and adonitol were also positive at 10% concentration only.

Culture T8 (isolated from raw, unrefined syrup) was a typical isolate of *Torulopsis globosa* (Olson & Hammer); but here again, fermentation tests gave more consistent results at the higher concentration.

The biochemical reactions of Culture T9 (isolated from the air) at first indicated a connexion with *Torulopsis dattila*; but this was discounted as our isolate was a very much smaller organism than the authentic strain from the Centraal Bureau voor Schimmelcultures, Delft, Holland (CBS) and differed in growing without added vitamins, and growing strongly with ethanol as carbon source. It is smaller than *T. bacillaris* and utilizes more carbon compounds in the full auxanogram. Any con-

nexion with *T. apicola* can also be eliminated, since Culture T9 ferments glucose and sucrose more quickly, with the additional fermentation of raffinose; and again, it assimilates more compounds. We confirm the results of Dr W. Slooff (private communication), that the assimilation pattern resembles that of *Candida guilliermondii*, although more significant differences in fermentation become apparent when tested at 10% concentrations. Culture T9 may be related to *C. guilliermondii*, but it must be ascribed to *Torulopsis* as it is asporogenous, forms no pseudomycelium on potato or corn-meal agar slide-cultures, and does not split arbutin. It is also a point of difference that we found the assimilation tests on salicin and cellobiose were only

Table 1. *Morphological characteristics of osmophilic yeasts*

Code	Growth in wort broth	Growth on wort agar	Pseudo-mycelium	Ascospores
T7	(1–1·5) × (2–2·5) μ* sediment only	(1–1·5) × (1·5–2·5) μ growth poor, browns	Negative	Not formed
T8	(4–6·5) × (4·5–6·5) μ sediment only	(4·5–7·5) × (5–7·5) μ growth poor, cream	Negative	Not formed
T9	(1·5–2·5) × (2–3·5) μ sediment only	(0·75–1·5) × (1·5–3) μ browns, growth poor	Negative	Not formed
T10	(1·5–3) × (2–5) μ sediment only	(1·5–3) × (2·5–5) μ dark brown, growth poor	Negative	Not formed
T11	(3·5–5) × (2·5–5·5) μ sediment, thin ring and faint islets	(2·7–4·5) × (3·5–6·5) μ white, creamy, margin entire	Negative	Not formed
T12	(2·5–5) × (3·5–5) μ sediment only	(2·5–4) × (2·5–5) μ pale brown, growth poor	Negative	Not formed
P4A	(1·5–4) × (2–5) μ sediment, ring and islets	(1–4) × (1·5–4) μ smooth, glistening, creamy-white	Blastodendrion type	Not detected
P4B	(1·5–4) × (2–5) μ sediment, ring and islets	(1–4) × (1·5–4) μ smooth, glistening, creamy-white	Blastodendrion type	Not detected
Sa24	(2–4) × (3–7·5) μ sediment only	(3–6) × (3·5–7·5) μ white—dirty-white, viscous	Negative	In newly isolated cultures
Sa26	(3–5·5) × (5·5–9) μ sediment, thin ring and islets	(2·5–6) × (4·5–8·5) μ creamy-white, margin crenated	Long, slender cells, sparse	Not detected

* Cell sizes of 3-day cultures, macroscopic appearance after 1 month at ambient temperature.

positive with inocula prepared by Wickerham's (1951) starvation method. With washed organisms as inocula (a necessity here, particularly with some of the 'obligate osmophils') the results were negative; β-glucosidase production is probably therefore very slight. With this combination of characteristics, we consider this to be a new species which we have named *T. kestoni*. A culture has been deposited at the Centraal Bureau voor Schimmelcultures. The Latin diagnosis is included at the end of this account.

Culture T10 (isolated from unrefined liquor) can be directly typed as *Torulopsis lactis-condensi* (Hammer). Culture T11 (also isolated from unrefined liquor) shows

Table 2. *Biochemical characteristics of Torulopsis spp.*

	Cultures											
	T7		T8		T9		T10		T11		T12	
	10%	2%	10%	2%	10%	2%	10%	2%	10%	2%	10%	2%
Fermentation												
Glucose	+	−	+	V*	+	+	+	+	+	+	+	+
Sucrose	+	−	+	VW	+	VW	+	+	+	+	+	+
Raffinose	−	−	+	VW	+	−	+	+	+	−	+	+
Maltose	−	−	−	−	−	−	−	−	−	−	−	−
Galactose	−	−	−	−	−	−	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	−	−	−	−	−	−
Melibiose	−	−	−	−	−	−	−	−	−	−	−	−
Assimilation of carbon compounds												
	10%	1%	10%	1%	10%	1%	10%	1%	10%†		10%	1%
Glucose	+	+	+	+	+	+	+	+	+		+	+
Galactose	W	−	−	−	+	−	−	−	+		W	−
Sucrose	+	+	+	+	+	+	+	+	+		+	+
Maltose	−	−	+	+	+	−	−	−	+		W	−
Lactose	−	−	−	−	−	−	−	−	−		−	−
L-sorbose	+	−	+	+	+	+	−	−	+		+	−
Cellobiose	−	−	−	−	+	−	−	−	−		+	−
Trehalose	−	−	+	+	−	−	−	−	nt		+	+
Melibiose	−	−	−	−	+	−	−	−	+		+	−
Raffinose	+	+	+	+	+	+	+	+	+		+	+
Melizitose	−	−	−	−	W	W	−	−	nt		−	−
Inulin	.	−	.	W	.	−	.	−	+		.	+
Starch	.	−	.	−	.	−	.	−	−		.	−
Xylose	+	−	−	−	+	−	−	−	nt		−	−
L-arabinose	−	−	−	−	−	−	−	−	−		−	−
D-arabinose	−	−	−	−	−	−	−	−	−		−	−
D-ribose	+	W	−	−	+	+	−	−	nt		−	−
L-rhamnose	−	−	−	−	−	−	−	−	−		−	−
Ethanol	.	+	.	+	.	+	.	−	−		.	W
Glycerol	+	+	+	+	+	+	−	−	+		+	−
Erythritol	−	−	−	−	W	−	−	−	−		−	−
Adonitol	+	−	+	+	+	−	−	−	nt		−	−
Dulcitol	−	−	−	−	−	−	−	−	nt		−	−
D-mannitol	+	+	+	+	+	+	−	−	+		+	+
D-sorbitol	+	+	+	+	+	+	−	−	+		+	W
α-methyl glucoside	−	−	+	+	+	+	−	−	+		+	+
Salicin	−	−	−	−	W	−	−	−	−		−	−
Lactic acid	.	−	.	−	.	W	.	−	−		.	VW
Succinic acid	.	−	.	−	.	+	.	−	nt		.	+
Citric acid	.	−	.	−	.	+	.	−	+		.	W
Inositol	−	−	−	−	−	−	−	−	−		−	−
Arbutin	−		−		−		−		−		−	
KNO_3	−		+		−		+		+		−	
Growth in vitamin-free medium	W		+		+		−		+		+	
Growth at 37°	−		−		+‡		−		−		−	

* V = variable; W = weak.

† In T11, 10% solutions only were tested, except where the lower concentrations were used as in ethanol, starch, etc. nt = not tested.

‡ Growth at 37° positive on osmophilic agar only.

Table 3. *Biochemical characteristics of osmophilic yeasts*

	Cultures							
	P4A		P4B		Sa24		Sa26	
	10 %	2 %	10 %	2 %	10 %	2 %	10 %	2 %
Fermentation								
Glucose	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	−	−	+	+
Raffinose	+	W	+	W	−	−	+	+
Maltose	−	−	W	−	+	−	+	+
Galactose	+	+	+	+	−	−	+	+
Lactose	−	−	−	−	−	−	−	−
Melibiose	−	−	−	−	−	−	+	+
Assimilation of carbon compounds								
	10 %	1 %	10 %	1 %	1 %	10 %	10 %	1 %
Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	W	−	+	+
Sucrose	+	+	+	+	−	−	+	+
Maltose	+	+	+	+	+	VW	+	+
Lactose	−	−	−	−	−	−	−	−
L-sorbose	+	+	+	+	−	−	−	−
Cellobiose	+	+	+	+	−	−	−	−
Trehalose	+	+	+	+	−	−	+	+
Melibiose	+	+	−	−	−	−	+	+
Raffinose	+	+	+	+	−	−	+	+
Melizitose	+	+	+	+	−	−	+	+
Inulin	.	+	.	+	.	−	.	−
Starch	.	−	.	−	.	−	.	−
Xylose	+	+	+	+	−	−	−	−
L-arabinose	+	+	+	+	−	−	+	W
D-arabinose	−	−	+	+	−	−	−	−
D-ribose	−	−	+	+	−	−	−	−
L-rhamnose	+	+	+	+	−	−	−	−
Ethanol	.	+	.	+	.	−	.	−
Glycerol	+	+	+	+	W	−	−	−
Erythritol	+	+	+	+	−	−	−	−
Adonitol	−	−	+	+	−	−	−	−
Dulcitol	+	+	+	+	−	−	−	−
D-mannitol	+	+	+	+	−	−	−	−
D-sorbitol	+	+	+	+	−	−	−	−
α-methyl glucoside	+	+	+	+	W	W	+	+
Salicin	+	+	+	+	−	−	−	−
Lactic acid	.	+	.	+	.	−	.	+
Citric acid	.	+	.	+	.	W	.	−
Succinic acid	.	+	.	+	.	−	.	−
Inositol	−	−	−	−	−	−	−	−
Arbutin	+		+		−		−	
KNO_3	−		−		−		−	
Growth in vitamin-free medium	+		+		−		+	
Growth at 37°	+		+		+*		+	

* Growth at 37° on osmophilic agar only.

typical morphological characteristics of *T. globosa* (Olson & Hammer) together with a strong reaction in the vitamin-free medium. It is unusual, however, in that it assimilates galactose and on the potato-agar slide cultures produces occasional organisms which show an abortive elongation similar to those in the authentic strain of *T. dattila*. It may represent an intermediate form with species of the genus *Candida*, but these differences are not considered sufficiently decisive to justify the creation of a new variety.

Culture T12 (from a South American cane juice sample) was typed as *Torulopsis bacillaris* (Kroemer & Krumbholtz). The negative reaction with KNO_3 eliminated any relationship with *T. lactis-condensi*; the configuration of the organisms (singly and in pairs) in wort broth also eliminated the possibility of its being *T. stellata* which produces star-like clusters, or the variety *T. stellata* var. *cambresieri* which splits arbutin.

Cultures P4A and P4B

Culture P4A was isolated from a West Indian sugar cane and Culture P4B from a South American cane juice. These two isolates were obviously identical and were identified as *Candida guilliermondii* (Cast.) Langeron et Guerra (Tables 1, 3). Formation of pseudomycelium on potato agar was observed after 7 days at 27°, but sporulation was not seen on V8 and Gorodkowa agar slants or carrot plugs even when mixed with a possible mating strain (Kreger van Rij, 1964). Culture P4B is similar to a strain described by Kreger van Rij which assimilated melibiose. Of interest is the very high osmotic tolerance of these strains, a further indication of the cosmopolitan distribution of this species.

Cultures Sa24 and Sa26

Culture Sa24 is a typical strain of *Saccharomyces rouxii*, but the fermentation of maltose was only definite in 10% solutions. This observation is similar to that of Santa Maria (1964) with Spanish cane molasses.

Culture Sa26 was isolated from the air in a sugar factory and was identified as *Saccharomyces florentinus*, Castelli. Small amounts of mycelium only were seen on potato agar slide cultures after 7 days incubation. Sporulation was not observed on V8 or Gorodkowa agar or carrot plugs after 12 weeks at 27°. To our knowledge, this is the first time that this organism has been found in an osmophilic habitat.

DISCUSSION

Barnett (1957) advocated the use of special biochemical tests for specific groups of yeasts, but the fat-splitting enzyme test used for the identification of *Candida lipolytica* shows that such an approach has been used for some time. The limitations of such tests are that often the media used are not sufficiently standard for biochemical use and merely form a medium for morphological observation, for example, Koji extract (Onishi, 1957) and honey agar (Lochead & Heron, 1929); malt extract can be included in media of this type. On the other hand, increased sugar concentrations can be used in more chemically defined media.

In identifying these highly osmophilic yeasts either by the standard methods or by using an increased sugar concentration as here, consideration must be made of the nature of the original microhabitat; simply to record growth on an increased con-

centration of sugar in an agar medium would appear to be insufficient (Scarr & Rose, 1965). The practical assessment of the industrial importance of this group of osmophilic yeasts; either alone or with *Saccharomyces rouxii* and *S. mellis* would be in relation to the storage of raw sugar and other sucrose products for any length of time. This has yet to be determined.

Latin diagnosis

Torulopsis kestoni sp.nov.

In musto maltato cellulae subovoidae $(1{\cdot}5\text{–}2{\cdot}5) \times (2{\cdot}0\text{–}3{\cdot}5)\mu$, singulae, binae et glomerae. Post unem mensem, sedimentum.

In agara maltato cultura (post unem mensem 17°) fusca, tempore breve moritar. Pseudomycelium nullum.

Fermentatio ambis concentrationibus 2 et 10% glucosi et sacchari. Fermentatio raffinosi concentratione sola 10%. In media minerale cum 1 et 10% glucoso, saccharoso, L-sorboso, raffinoso, D-riboso, glycerolo, D-mannitolo, D-sorbitolo et α-methyl glucosido crescit. Cum 10% sola galactoso, maltoso, cellobioso, xyloso, adonitolo et salicino (exiguum) crescit. Cum 1% sola acido succinico, acido citrico et acido lactico (exiguum) crescit.

Arbutin non finditur. Formatio esterium nulla. Kalium nitricum non assimilantur. Non necessariae ad crescentium sunt vitaminae externae.

The authors wish to thank the Directors of Tate and Lyle Ltd., for permission to publish this paper and Miss W. Slooff of the Centraal-Bureau voor Schimmelcultures for her criticism and very valuable suggestions.

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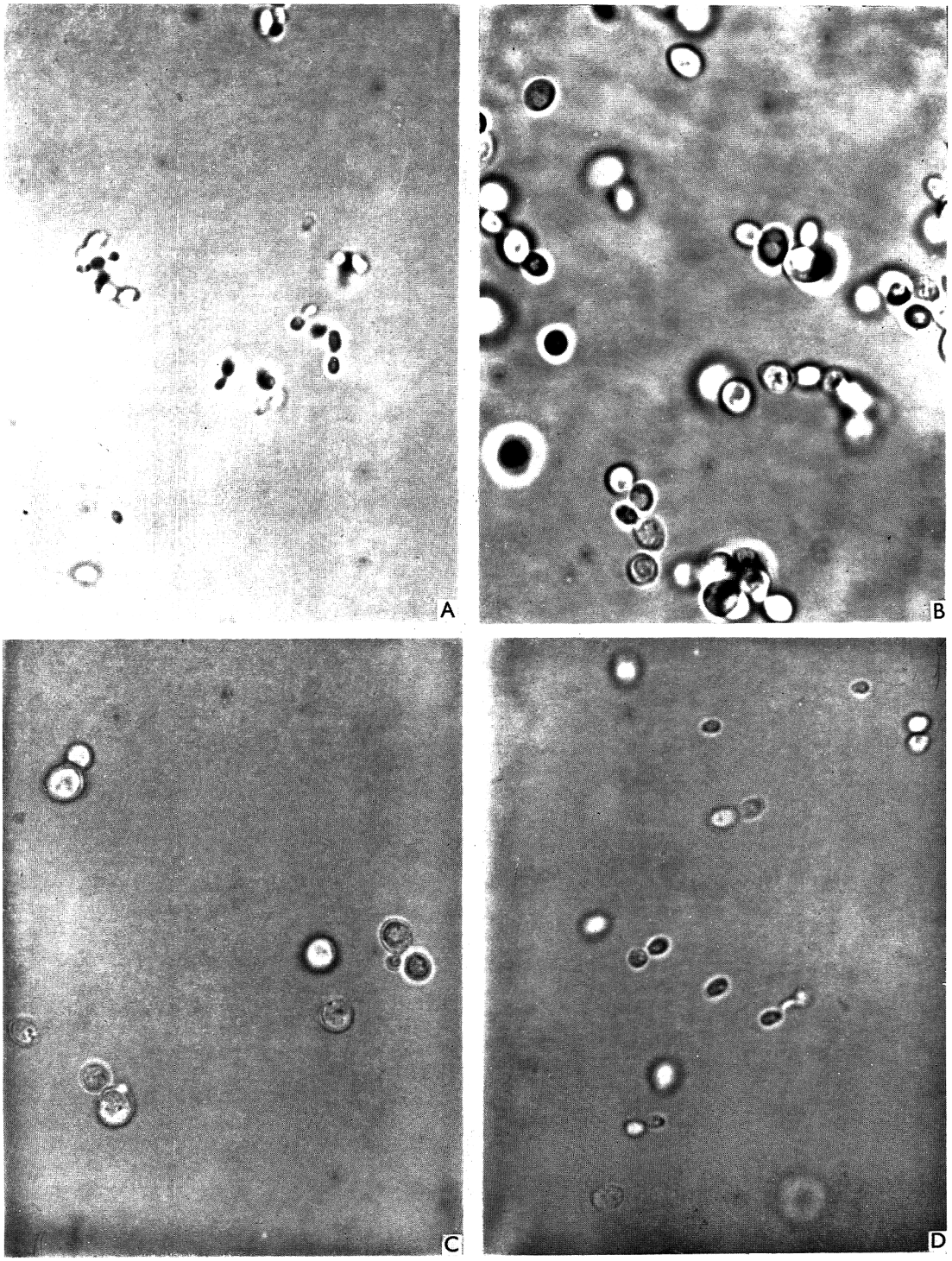
EXPLANATION OF PLATE

Cultures mounted in glycerine jelly from a slope of osmophilic agar.

(*a*) *Torulopsis kestoni* (T9). (*b*) *T. bacillaris* (T12). (*c*) *Saccharomyces rouxii* (Sa24). (*d*) *Candida guilliermondii* (P4B).



M. P. SCARR AND D. ROSE

(*Facing p.* 16)

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Reactions of Fungi to Exposure to 10 Atmospheres Pressure of Oxygen

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SUMMARY

Of 103 species of fungi exposed to 10 atmospheres (atm.) pressure of oxygen at 25° for 7 days 52 resumed growth after treatment. Of these, 22 recovered from a similar treatment for 14 days. On resumption of growth, growth rates were the same as those of untreated colonies, after a lag period between decompression and recovery. The lag period varied with species, length of exposure, inoculum source, and in some cases between replicates of a species. More detailed investigation of the reaction of *Fusarium solani*, *Rhizopus arrhizus*, *Mucor racemosus* and *M. plumbeus* showed that generally the lag periods increased with increasing exposure times, that their extinction points (i.e. the exposure which killed all replicates) varied, and that at exposures approaching the extinction point there was selective survival either of spores or of strains in the latter three fungi.

INTRODUCTION

The effects of high oxygen pressures on living organisms have been under investigation for many years. Studies have extended over a wide range of test organisms, including man (Bean, 1945). Deep-sea fish are known to live at a depth of 1365 m. where the partial pressure of oxygen is equivalent to about 115 atm. of pure oxygen. Frogs and an alligator have survived, apparently with no ill effects, after exposure to 3·5 atm. of oxygen for 65 hr and 4·3 atm. of oxygen for 75 min., respectively. A guinea pig and a dog subjected to the latter treatment did not survive, and this and other experiments suggested that cold-blooded animals are more tolerant of high oxygen pressures than are warm-blooded. Cleveland (1925) succeeded in killing the protozoan parasites in insects, earthworms, frogs, goldfish and salamanders by subjecting them to oxygen pressures of up to 3·5 atm. for periods well below those which killed the hosts. In similar experiments to free rats from protozoa, he found that the host was killed before the parasite. These effects are of great significance in the medical field, where high oxygen pressures are cautiously administered to human beings, e.g. in the resuscitation of new-born babies (Hutchison, 1963) and in the alleviation or cure of anaerobic infection (Boerema, Brummelkamp & Miejne, 1964). Little is known, however, about the direct effect of high oxygen pressures on micro-organisms. Bert (1878) reported the inhibitory or destructive action of hyperoxygenated air at 10 atm. on putrefactive organisms in wine and meat, on moulds, yeasts and *Bacillus anthracis*, although hyperoxygenated air at 20 atm. did not kill the glanders organism. Of 20 micro-organisms studied by Berghaus (see Bean, 1945) 15 remained viable after 24 hr at 35 atm. of oxygen.

Thaysen (1934) recorded that the growth of 4 micro-organisms was retarded by exposure to 10 atm. pressure, and McAllister, Stark, Norman & Ross (1963) reported that exposure of eight bacteria and two fungi to 2 atm. of oxygen for 42 hr temporarily inhibited growth and permanently altered three of the bacteria (*Pseudomonas pyocyanea* (*aeruginosa*), *Staphylococcus aureus* and *Escherichia coli*). Caldwell (1963, 1964, 1965) described the effect of high oxygen pressures on a variety of organisms, including fungi, bacteria, mites and dormant plant seeds. Bacteria appeared to be remarkably resistant to up to 10 atm. of oxygen for up to 14 days, and *E. coli*, *S. citreus*, *S. albus* and a chromobacterium species recovered from a 28-day exposure (Caldwell, 1965). Fungi subjected to 10 atm. pressure of oxygen varied in the length of exposure which they could tolerate. *Aspergillus niger*, *Sordaria fimicola* and *Penicillium cyclopium* recovered from exposure for 18 days, 14 days and 10 days, respectively. Caldwell noted that, whereas bacteria recover almost immediately after treatment, with fungi there was a lag period between decompression and resumption of growth, and that the lag period was longer with longer exposure at any one pressure, or with the same time of exposure at higher pressures. To investigate various aspects of the tolerance of a wider range of fungi the present work was undertaken.

METHODS

The species of fungi used in this investigation numbered 103 and comprised 26 Phycomycetes, 16 Ascomycetes, 2 Basidiomycetes and 59 Fungi Imperfecti. Four replicates of each were grown on malt agar (25 g. malt extract and 15 g. agar/l.) in Petri dishes. Two plates were retained as controls in an incubator at 25° and the diameter of the colonies was measured as an indication of growth rate. Two plates, after growing for 2 days at 25° to ensure establishment, were placed in a pressure vessel, flushed out with oxygen, and then kept at 10 atm. of oxygen in a constant temperature room at 25° for various periods. At the end of the experimental period, the pressure was released slowly, the treated plates removed to an incubator at 25°, observed for 14 days, and any increases in colony diameter measured.

The objection might be raised that inhibitory effects on growth in pressure vessels first flushed out with oxygen (thus removing air) might be the result of lack of CO_2. This possibility was tested by introducing pure oxygen into unflushed pressure vessels, which therefore retained CO_2 at atmospheric concentrations. Growth was inhibited in both flushed and unflushed vessels; therefore it was assumed that the absence of atmospheric concentrations of CO_2 in flushed vessels was not the cause of inhibition, and that the latter was due to the high partial pressure of oxygen.

At first all cultures were subjected to pressure for 14 days. Subsequently, those fungi which did not recover after this treatment were subjected to pressure for 7 days. Finally, a more detailed study was made with *Mucor plumbeus*, *M. racemosus*, *Fusarium solani* and *Rhizopus arrhizus* to elucidate points which arose during the previous survey.

RESULTS

Twenty-two fungi recovered from exposure to 10 atm. of oxygen for 14 days (Table 1). They were 6 Phycomycetes (*Achlya bisexualis*, *Circinella mucoroides*, *Mucor pusillus*, *M. ramannianus*, *Syncephalastrum cinereum*, *Thamnidium elegans*);

2 Ascomycetes (*Gymnoascus subumbrinus, Sordaria fimicola*); and 14 Fungi Imperfecti (*Aspergillus amstelodami, A. chevalieri, A. flavus, A. fumigatus, A. niger, A. quercinus, A. tamarii, A. terreus, Penicillium chrysogenum, P. cyclopium, P. cylindrosporium, P. decumbens, P. varioti, P. velutinum*).

Thirty fungi were resistant to exposure for 7 days, but not to 14 days, to 10 atm. of oxygen. They were 7 Phycomycetes (*Absidia glauca, Mucor plumbeus, M. racemosus, M. rouxii, M. hiemalis* (minus strain), *Syncephalis cornu, Zygorhynchus*

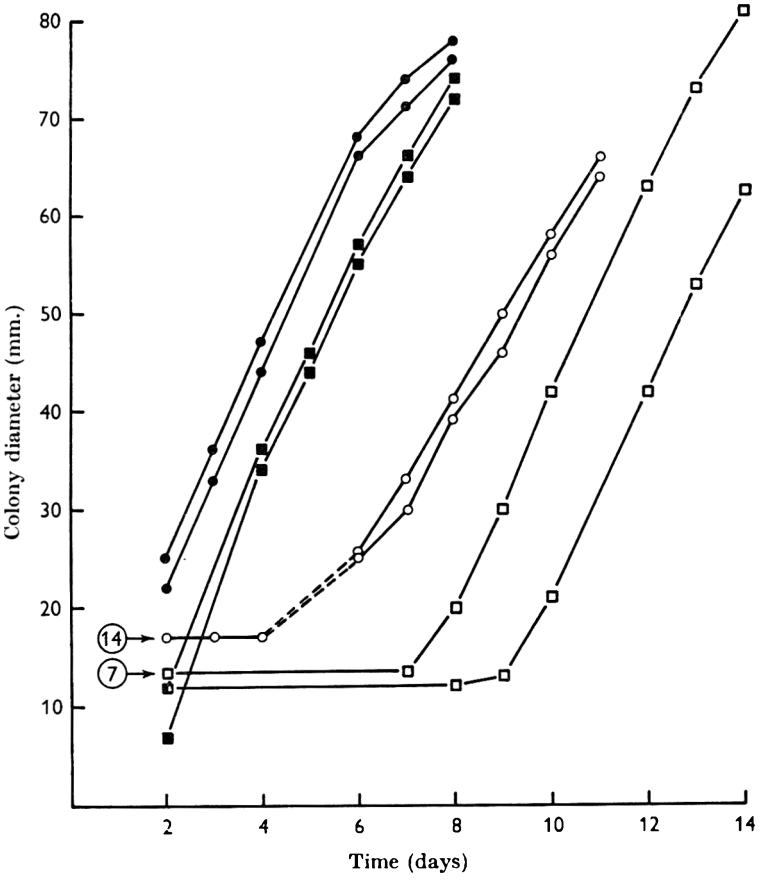

Fig. 1. Growth of *Penicillium stipitatum* and *Circinella mucoroides* after exposure to 10 atm. of oxygen for 7 to 14 days respectively. ■, *Penicillium stipitatum* control; □, *P. stipitatum* treated for 7 days; ●, *Circinella mucoroides* control; ○, *C. mucoroides* treated for 14 days.

exponens); 5 Ascomycetes (*Chaetomium indicum, Endomycopsis capsularis, Eremascus albus, Preussia* (*Perisporium*) *funiculata, Saccharomyces cerevisiae*); 1 Basidiomycete (*Fomes pomaceus*); and 16 Fungi Imperfecti (*Ascochyta pisi, Aspergillus nidulans, A. ochraceus, A. wentii, Candida albicans, Cladosporium herbarum, Colletotrichum dematium, Penicillium camembertii, P. claviforme, P. janczewskii, P. notatum, P. spinulosum, P. stipitatum, P. terrestre, Trichoderma viride, Verticillium lateritium*).

Several points of interest arose from this preliminary survey.

Table 1. *Reaction of fungi to 10 atm. pressure of oxygen at 25° C*

Figures indicate days after removal from pressure when cultures resumed growth. Brackets indicate that only one replicate of two recovered. Points in blank spaces indicate that replicates did not resume growth.

Classification	Reference	Fungus	No. of days under pressure: 7	14
PHYCOMYCETES				
Saprolegniales	E 30	*Achyla bisexualis*	.	(13)
Peronosporales	E 21	*Phytophthora cactorum*	.	.
	IMI 82514	*Pythium ultimum*	.	.
Mucorales	IMI 15406	*Absidia glauca*	3	.
	IMI 77986	*Circinella mucoroides*	.	3
	IMI 45954	*Mortierella hygrophilla*	.	.
	IMI 21216	*Mucor hiemalis* (+)	.	.
	IMI 21217	*M. hiemalis* (−)	(6)	.
		M. hiemalis (−)	3	.
	IMI 78407	*M. mucedo*	.	.
	E 65	*M. plumbeus*	2	.
	IMI 71629	*M. pusillus*	.	4
	IMI 35716	*M. racemosus*	(7)	.
	IMI 35044a	*M. ramannianus*	.	4–5
	IMI 68072	*M. rouxii*	(6)	.
	E 104	*Phycomyces blakesleeanus*	.	.
	IMI 63218	*P. nitens* (+)	.	.
	E 29	*P. nitens* (−)	.	.
	IMI 71487	*Rhizopus arrhizus*	.	.
	E 37	*R. cohnii*	.	.
	IMI 40564	*R. oryzae*	.	.
	E 22	*R. stolonifer*	.	.
	E 1	*Syncephalastrum cinereum*	.	2
	IMI 77606	*Syncephalis cornu*	(3)	.
	IMI 43624	*Thamnidium elegans*	.	(8)
	IMI 29216	*Zygorhynchus exponens*	13	.
Entomophthorales	E 27	*Entomophthora* sp.	.	.
ASCOMYCETES				
Endomycetales	NCYC 127	*Endomycopsis capsularis*	6	.
	E 3	*Eremascus albus*	6	.
	E 8	*Saccharomyces cerevisiae*	7	.
	IMI 23657	*Schizosaccharomyces octosporus*	(*c.* 25)	.
	NCYC 580	*Zygosaccharomyces bailii*	.	.
Eurotiales	IMI 41112	*Gymnoascus subumbrinus*	.	(11)
Erysiphales	E 80	*Preussia* (*Perisporium*) *funiculata*	2	.
Sphaeriales	E 95	*Chaetomium indicum*	4–5	.
	E 92	*Glomerella cingulata*	.	.
	E 96	*G. tucumanensis*	.	.
	E 100	*Mycosphaerella macrospora*	.	.
	E 24	*Sordaria fimicola*	.	1–2
	E 99	*Venturia inacqualis*	.	.
Helotiales	E 110	*Diplocarpon rosae*	.	.
	IMI 61849	*Sclerotinia fructigena*	.	.
Pezizales	IMI 57472	*Pyronema domesticum*	.	.

Table 1 (*cont.*)

Classification	Reference	Fungus	No. of days under pressure: 7	14
BASIDIOMYCETES				
Agaricales	E 20	*Fomes pomaceus*	(9)	.
	IMI 79125	*Merulius lacrymans*	.	.
FUNGI IMPERFECTI				
Sphaeropsidales	E 14	*Ascochyta pisi*	2	.
	IMI 17361	*Phoma alternariaceum*	.	.
	IMI 45944	*P. eupyrena*	.	.
	IMI 46259	*P. glomerata*	.	.
	IMI 49948	*P. violacea* (*P. pigmentivora*)	.	.
	E 91	*Phyllosticta sorghina*	.	.
	E 84	*Pyrenochaeta terrestris*	.	.
	E 86	*Selenophoma donacis*	.	.
Melanconiales	E 93	*Colletotrichum dematium*	(7)	.
	E 94	*C. gossypii*	.	.
Moniliales	E 44	*Alternaria brassicae*	.	.
	E 28	*A. brassicicola*	.	.
	E 73	*A. tenuis*	.	.
	IMI 17455	*Aspergillus amstelodami*	.	3
	E 48	*A. chevalieri*	.	3
	E 47	*A. flavus*	.	(5)
	E 46	*A. fumigatus*	.	4–5
	E 51	*A. nidulans*	3	.
	E 10	*A. niger*	.	(3) variable
	E 50	*A. ochraceus*	2	.
	E 67	*A. quercinus*	.	5–7
	E 53	*A. tamarii*	.	(10)
	E 52	*A. terreus*	.	3
	E 45	*A. wentii*	(4)	.
	E 32	*Botrytis allii*	.	.
	E 33	*B. cinerea*	.	.
	IMI 89362	*Caldariomyces fumago*	.	.
	E 25	*Candida albicans*	2–3	.
	E 23	*Cladosporium herbarum*	(6)	.
	E 83	*Epicoccum purpurascens*	.	.
	E 81	*Fusarium avenaceum*	.	.
	E 82	*F. merismoides*	.	.
	IMI 68412	*F. solani*	.	.
	IMI 53994	*Helminthosporium australiense*	.	.
	IMI 45769	*H. cynodontis*	.	.
	IMI 53993	*H. hawaiiense*	.	.
	IMI 61868	*H. rostratum*	.	.
	IMI 53252	*H. sorghicola*	.	.
	IMI 102237	*H. torulosum*	.	.
	E 60	*Penicillium brevi-compactum*	.	.
	IMI 27831	*P. camembertii*	(3)	.
	E 57	*P. chrysogenum*	.	9
	E 64	*P. claviforme*	(6)	.
	E 55	*P. cyclopium*	.	9–11
	E 61	*P. cylindrosporium*	.	5
	E 61	*P. cylindrosporium*	.	8
	E 59	*P. decumbens*	.	(11)
	E 26	*P. janczewskii*	3	.

Table 1 (*cont.*)

Classification	Reference	Fungus	No. of days under pressure: 7	14
	IMI 15378	*P. notatum*	8	.
	E 54	*P. spinulosum*	3	.
	IMI 69250	*P. stipitatum*	5–6	.
	E 56	*P. terrestre*	(4)	.
	E 58	*P. verrioti*	.	(9)
	E 62	*P. velutinum*	.	5–6
	E 62	*P. velutinum*	.	7–9
	E 101	*Aureobasidium pullulans* (*Pullularia pullulans*)	.	.
	E 98	*Stilbum* sp.	.	.
	E 97	*Trichoderma koningii*	.	.
	IMI 45553	*T. viride*	2	.
	E 38	*Trichothecium roseum*	.	.
	IMI 54377	*Verticillium lateritium*	4–5	.

IMI = Commonwealth Mycological Institute; NCYC = National Collection of Yeast Cultures; E = Fungus collection, Department of Botany, University of Exeter.

1. *Relationship between reaction of a fungus to treatment and its taxonomic position.* There appears to be no correlation between the taxonomic position of a fungus and its reaction to treatment. Thus two mucor species withstood 10 atm. of oxygen for 14 days, two withstood pressure for 7 but not 14 days, and two did not withstand 7 days (Table 1). At least one member of each of the Classes Phycomycetes, Ascomycetes and Fungi Imperfecti recovered from exposure for 14 days to 10 atm. of oxygen, and all Classes had members which recovered after 7-day treatments. A relatively large number of species of the genera *Aspergillus* and *Penicillium* were investigated, and here a high proportion (14/25) were resistant to exposure for 14 days. A smaller proportion (9/25) withstood exposure for 7 days, and only two (*P. brevicompactum* and *P. camembertii*) did not recover after either treatment.

2. *Growth rates of control and treated fungi.* The criterion of growth rate was increase in colony diameter; measurement of two control colonies gave the normal growth rate for each fungus. While in the pressure vessel, the growth of all colonies ceased, but when treated colonies resumed growth after removal from pressure the growth rate after recovery was the same as in control colonies. Typical graphs are presented in Fig. 1 for *Penicillium stipitatum* after a 7-day treatment and for *Circinella mucoroides* after a 14-day treatment. As soon as growth was resumed, the rate was the same as if there had been no intermediate exposure to high oxygen pressure.

3. *Recovery of colonies more than 14 days after treatment.* For the most part, cultures were kept under observation for 14 days after treatment and then discarded. Over this period, the lag time between removal from pressure and resumption of growth varied with different fungi, and was as much as 13 days with *Achlya bisexualis* and *Zygorhynchus exponens* (Table 1). On one occasion cultures of *Schizosaccharomyces octosporus*, *Rhizopus arrhizus*, *R. stolonifer*, *Phytophthora cactorum*, *Alternaria brassicicola*, *Botrytis allii* and *B. cinerea*, which had been exposed to 10 atm. of oxygen for 7 days, were retained for 32 days after treatment. Although

none of the other cultures resumed growth, one replicate of *Schizosaccharomyces octosporus* recovered between 14 and 32 days after removal from pressure (estimated at 25 days by extrapolation from the growth curve). It must be stressed, therefore, that the results in Table 1, other than that for *S. octosporus*, refer to observations up to 14 days after treatment only. Perhaps some fungi would have recovered in a longer period.

4. *Variability in recovery of replicates.* In Table 1 there are some instances in which only one replicate of two recovered after treatment (figures in brackets) and, in a few instances where the treatments were repeated, it was found that behaviour of the treated fungi differed at different times. As an example of the latter, the first time two replicates of the minus strain of *Mucor hiemalis* were exposed for 7 days to 10 atm. of oxygen, one only resumed growth 6 days after removal from pressure; on repeating the experiment, both replicates recovered 3 days after removal from pressure. *Aspergillus niger* was very variable in this respect. The first result of a 14-day exposure was that neither replicate recovered; in a second experiment, both resumed growth after 3 days; in a third, neither recovered; in a fourth, both recovered in 3 days. In a larger experiment with 6 replicates, 2 recovered in 10 days, 1 in 16 days, 1 in 19 days after treatment, and 1 did not recover within 34 days of treatment. Furthermore, there was some variation between replicates even when both did resume growth after treatment. Usually they did so together, but in some cases there was a lag of a day, or at most 2 days, between recovery times (see e.g. *Mucor ramannianus* and *Penicillium cyclopium* in Table 1).

It appeared possible that the position of a culture in the pressure vessel might be related to its subsequent behaviour. Consequently, 10 replicates of *Rhizopus arrhizus* were placed in a known order in a pressure vessel, no. 1 at the top, no. 10 at the bottom. They were then subjected to 10 atm. pressure of oxygen for 4 days. Five of the ten resumed growth after treatment, namely nos. 1, 2, 3, 7, 9 and 10. Such a random distribution of recoveries would appear to argue against position in the stack as a factor which influenced recovery. In subsequent treatments, where placement was recorded, there was no evidence that this was a significant factor.

Investigations of variability of response to oxygen pressure

The several aspects of variability of response were investigated in more detail. Ten replicates of *Mucor plumbeus* (both replicates had recovered previously), 12 replicates of *Aspergillus wentii* (1 of 2 replicates had recovered previously) and 10 replicates of *Fusarium solani* (neither replicate had recovered previously) were exposed to 10 atm. of oxygen for 7 days. All the replicates of *Mucor plumbeus* resumed growth 2–3 days after removal from pressure. Only 10 of the 12 replicates of *A. wentii* did so; the remaining two had not recovered after 40 days. All the replicates of *F. solani* resumed growth 4–5 days after removal from pressure. In each experiment, all replicates had been subcultured from the same inoculum of the appropriate fungus on to the same medium, and had been treated identically.

Since the sources of the inocula were not of known age, an examination was made on the reaction of subculture originating from young and from old cultures of *Mucor racemosus*. Three-day and 42-day colonies were used as sources of inocula, having grown for these periods on malt agar at 25°. Five subcultures were made from each, grown for 2 days, then placed under oxygen at 10 atm. for 7 days. All

Table 2. *Times of recovery growth of fungi after exposure to 10 atm. of oxygen for various periods*

Fungus		No. days exposure to 10 atmospheres of oxygen												
		2	3	4	5	6	7	8	9	10	11	12	13	14
Fusarium solani	Recoveries	.	.	6/6*	10/10	10/10	9/10 10/10	0	.	.	.	.	.	.
	Time lag (days)	.	.	3·8	3·0	4·6	6·1 4·6	.	.	.	.	.	.	.
Rhizopus arrhizus	Recoveries	.	10/10	5/10	8/10	5(+3)† 10	.	3/10	4/10	0	.	.	.	.
	Time-lag (days)	.	1·4	1·8	2·2	3·0	.	1–2 L	1	.	.	.	.	.
Mucor racemosus	Recoveries	5/5	.	5/5	.	.	5/5 10/10	.	5/5	1/10	0	.	.	.
	Time-lag (days)	1·2	.	2·8	.	.	4·1 4·1	.	6·5	3 L	.	.	.	.
Mucor plumbeus	Recoveries	5/5	.	5/5	.	.	10/10 5/5 12/12	.	5/5	.	9/10	4/10	2/10	0
	Time-lag (days)	1·2	.	2·4	.	.	3·1 3·4 2·6	.	3·8	.	3 L	4–6 L	3–5 L	.

* Numerator = no. of colonies which recovered, denominator = no. of replicates. † Figure in brackets refers to abnormal growth on recovery (see text). . Expt. not carried out. L, recovery from localized points in colonies.

the replicates recovered, but those from the young inocula did so in an average of $5{\cdot}4 \pm 0{\cdot}6$ days while those from the old inocula took $4{\cdot}4 \pm 0{\cdot}5$ days. This result is significant at the 5% level. In a similar experiment with 5 replicates each of inocula from a 7-day and from a 171-day culture of *M. plumbeus*, all resumed growth at the same time 4 days after removal from pressure. It would appear therefore that although inocula taken from old colonies of *M. racemosus* were slightly more resistant to high oxygen pressure than those of young colonies, there was no significant difference in this respect with *M. plumbeus*.

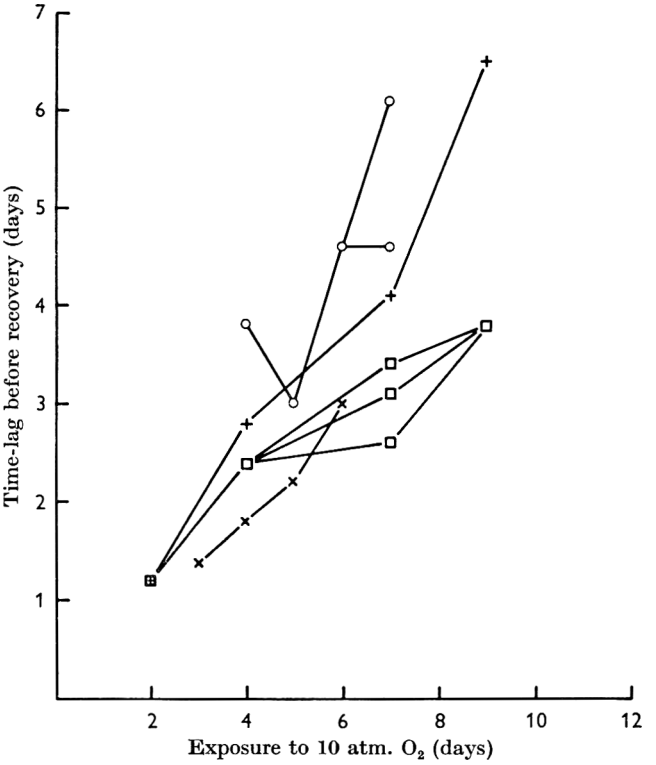

Fig. 2. Relationship between length of exposure to 10 atm. oxygen and time-lag before recovery. ●, *Fusarium solani*; ×, *Rhizopus arrhizus*; +, *Mucor racemosus*; □, *Mucor plumbeus*.

More detailed investigations were made on *Mucor racemosus*, *M. plumbeus*, *Rhizopus arrhizus* and *Fusarium solani*, with a procedure modified in the light of previous experience. The modifications were: (*a*) to use at least 5 replicates of each fungus; (*b*) to extend observations over at least 21 days after treatment in cases of non-recovery; (*c*) to standardize the inocula by always taking them from 3-day colonies. The object of these experiments was to relate time of exposure to oxygen under pressure to the lag time before recovery, and to find the extinction point at which the fungus did not recover from the oxygen pressure treatment. The results of these experiments are summarized in Table 2.

It is clear from Table 2 that longer exposures to oxygen at 10 atm. pressure were in general correlated with longer lag periods before growth under normal conditions was resumed. This is more clearly shown in Fig. 2.

The extinction point (i.e. the number of days of exposure to oxygen at 10 atm. pressure which killed all replicates) was 8 days for *Fusarium solani*, 10 days for *Rhizopus arrhizus*, 11 days for *Mucor racemosus* and 14 days for *M. plumbeus*. With *F. solani* this was very clear-cut. All replicates recovered from exposure to O_2 pressure for 7 days whereas none recovered from exposure for 8 days. On repeating the experiments, 9 of 10 replicates recovered from the 7-day treatment while none recovered from the 8-day treatment. The behaviour of the other three fungi was not so clear-cut. All replicates of *M. racemosus* recovered from a 9-day treatment, 1 of 10 from a 10-day treatment and none from an 11-day treatment. The one culture which recovered from the 10-day treatment appeared to resume growth at one localized spot within the colony; from this spot, which was located in the sporing centre of the original colony, a new colony developed which spread over the original, apparently dead mycelium. It looked very much as if a spore or a group of spores had survived the treatment and had later resumed growth.

There was some evidence of a similar selective process in colonies of *Mucor plumbeus* after exposure to 10 atm. of oxygen for 11, 12 and 13 days. This treatment was very near the extinction point for this fungus, which is 14 days. After the 11-day treatment, it was observed on the third day after removal from pressure that centres of growth appeared in the sporing region of some colonies; only 1 colony did not recover at all, and the remaining 9 replicates resumed growth at 1–6 or more distinct loci. After the 12-day treatment, 4 out of 10 replicates showed a similar pattern of growth, but here growth was resumed 4–6 days after removal from pressure; in 2 colonies there was one locus, and in the other two there were 2 loci. After the 13-day treatment, 2 of 10 replicates resumed growth, each at one locus, 3–5 days after treatment. This new growth spread over the non-growing mycelial rim of the original colony, and where there was more than one centre of growth, remained distinctly lobed for several days. Eleven 'strains' were isolated from 5 plates which had received treatment for 11 days, and two replicates of each 'strain' subjected to treatment for 12 days. In two instances both replicates resumed growth 3–4 days after treatment at a single locus in the sporing centre of each colony; the colony as a whole did not resume growth.

Rhizopus arrhizus showed very variable behaviour. Five replicates of 10 recovered from a 4-day exposure and 8 of 10 from a 5-day exposure. After a 6-day exposure, 5 of 10 replicates recovered normally (i.e. apparently the whole colony resumed growth at the same time) 3 days after removal from pressure. Two of the remaining 5 replicates did not resume growth within 35 days after O_2 pressure treatment. However, localized centres of recovery appeared in the other replicates 9–14 days after removal from pressure, but the new growth was atypical; instead of being rapid and producing a fluffy aerial mycelium it developed as a slow-growing, semi-submerged or superficial mycelium. On examination, the hyphae were found to be disorganized internally, and to have a stunted growth form. After developing for a few days in this manner, all growth ceased in the plates, although subcultures from these colonies grew in tubes in a similar manner for a few days before also ceasing to develop. Of 10 replicates from the normal stock colony of *R. arrhizus* subjected to pressure for 8 days, 3 resumed growth from localized recovery points 2 days after exposure. Here growth was normal and rapid. After a 9-day exposure, 4 of 10 replicates resumed growth 5 days after treatment. After a 10-day exposure there was no recovery.

The presence of localized recovery points observed in replicates of *Mucor racemosus*, *M. plumbeus* and *Rhizopus arrhizus*, all of which were sporing at the time of exposure to oxygen pressure, seems to indicate that growth was resumed by resistant cells or spores. If this were so, colonies arising from such selected hyphae or spores would be expected to give subcultures which were also resistant. This was investigated by using *R. arrhizus* as the test organism. Five replicates subcultured from a 3-day stock culture were grown for one day. Two further subcultures were then taken from each replicate before it was subjected to oxygen pressure for 6 days. These secondary subcultures (A 1, A 2, B 1, B 2, etc.) after growing for one day, were then subjected to the same treatment. After removal from pressure, three of the original cultures, (A, B, C) and one of the subcultures (B 2) resumed growth. The fact that only 1 subculture resumed growth of 6 from the original cultures A, B and C does not strongly support the idea that ability to recover is a 'strain' characteristic. However, since each of the cultures A–E may have consisted of a mixture of strains, the result cannot be taken as disproving the existence of strains with greater powers of recovery than others. If such strains do exist, it is more likely that they would be detected by taking subcultures only from cultures which recovered after treatment. Therefore a further set of subcultures (A 3–6, B 3–6, C 3–6) from colonies A, B and C which had recovered after treatment, were grown for one day, then subjected to the same treatment. After removal from oxygen pressure, 3 of these subcultures (C 3, C 5, C 6) resumed growth. Although this result is also inconclusive, the fact that 3 of 4 subcultures from one source did recover suggests that the treatment may have some selective effect on the colonies.

DISCUSSION

Of 103 fungi tested in the present study, 52 recovered from exposure to 10 atm. oxygen for 7 days, and of these, 22 withstood exposure for 14 days. Resistant species were not confined to any one Class nor to a limited number of genera. It would appear therefore that fungi are substantially more resistant to high oxygen pressures than are plant (Caldwell, 1956, 1964) and animal (Bean, 1945) tissues, although not so resistant as some bacteria (Caldwell, 1965).

When the fungal colonies resumed growth after treatment, it was found that the subsequent growth rates, measured as increase in colony diameter, were the same as that of control colonies not subjected to high oxygen pressure. Since there was a lag period between treatment and resumption of growth, it would appear that the suspension of certain metabolic systems, presumably by oxidation induced by high oxygen pressure, requires a period of adjustment after removal from pressure before the complete metabolic cycle and hence growth can be resumed. One such system, which appears to be of variable sensitivity in different fungi, has been found by Dr G. G. Pritchard of this Department to be the pyruvate oxidase system (private communication). The period of adjustment may be a lengthy one (21–28 days in *Schizosaccharomyces octosporus* after a 7-day treatment), the time generally being correlated with the time of exposure to high oxygen pressure, longer exposures resulting in longer lag periods. This agrees with Caldwell's observations on fungal recovery (Caldwell, 1963).

Caldwell (1965), working with bacteria, noted that after 28-day exposure it was

possible that not all the bacteria, but only some individuals of the colony, perhaps strains, survived. McAllister *et al.* (1963) refer to 'hyperbars', which may be individuals selected or selectively altered by the treatment. Such selective survival was noted in the present work, with the three fungi *Rhizopus arrhizus*, *Mucor racemosus* and *M. plumbeus*, when exposure times approached the extinction point, which was 10 days, 11 days and 14 days, respectively; and selective survival was first apparent after exposures of 8 days, 10 days and 11 days, respectively. Exposure of *R. arrhizus* for 6 days did result in a degree of selectivity in that 3 cultures out of 10 replicates resumed growth 9–14 days after treatment. The new growth, however, was then very abnormal, with internally disorganized hyphae; this is regarded as selective damage rather than selective survival.

At first it appeared likely that selection occurred from spores or from strains in a mixture either by chance from the stock colony during subculture, or as a result of exposure of replicates to high oxygen pressure. Both possibilities were examined with *Rhizopus arrhizus*, but with inconclusive results. Nevertheless, variability of response of replicates to high oxygen pressure is puzzling. When only two replicates were used, as was the case for most of the fungi investigated here, there were several occasions when only one resumed growth after treatment, or, when both recovered, there was a difference in the lag period of 1 or 2 days. Furthermore, in the initial survey (Table 1) both replicates of *R. arrhizus* and of *Fusarium solani* did not recover from a 7-day treatment, whereas in later experiments with a larger number of replicates the extinction points were 10 days and 8 days, respectively. *Aspergillus niger* was perhaps the most variable fungus, the replicates either not resuming growth at all or recovering at times varying from 3 to 19 days after a 14-day exposure to high oxygen pressure. This suggests that some variability also existed in the initial subcultures.

Fairly consistent results were obtained with *Fusarium solani*, *Mucor racemosus* and *M. plumbeus* (Table 2) using the modified procedure described on p. 25, except when exposure time approached their extinction points. *Rhizopus arrhizus* on the other hand was much less reliable even with the modified procedure. It is likely that there are many strains in the stock cultures of *R. arrhizus* and that this causes the wide variation of response to treatment.

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A Numerical Taxonomical Study of Some Corynebacteria and Related Organisms

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SUMMARY

A numerical Adansonian analysis has been performed on the results of a wide range of biochemical and physiological characters of organisms at present classified in the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Arthrobacter* and *Jensenia*. The results show that some of the organisms need to be reclassified. *Corynebacterium ulcerans* could be considered as another type of *C. diphtheriae*, and *C. pyogenes* and *C. haemolyticum* have little similarity to any of the other corynebacteria. *Corynebacterium equi* and *Jensenia canicruria* appear to be closely related to the mycobacteria examined. The plant pathogens *C. rathayi*, *C. betae* and *C. michiganense* seem to be distinct from most of the animal parasites in this genus. The mycobacteria, nocardia and most of the corynebacteria from animal sources form one large group at the 51 % similarity level. Acid-fastness does not seem to be a good criterion for separating the genus *Corynebacterium* from the genus *Mycobacterium*, some of the corynebacteria showed varying degrees of acid-fastness.

INTRODUCTION

In the genus *Corynebacterium*, *Bergey's Manual* (1957) lists thirty-three species. In addition to these there are others which have been described, and some which have been transferred from this genus to other genera in *Bergey's Manual* or by other authors, but which are still retained as corynebacteria in culture collections and by some authors. Several workers have suggested that the genus *Corynebacterium* contains some species, particularly those from plant and soil sources, which should not be there (Conn, 1947; Conn & Dimmick, 1947; Clark, 1952; Cummins, 1962*a*; da Silva & Holt, 1965). Cummins (1962*b*) and Perkins & Cummins (1964) found that most of the plant pathogens they examined had markedly different cell-wall compositions from those of the animal parasites in the genus. Harrington (unpublished) has found that *C. michiganense* has a cell-wall amino acid composition similar to those of most of the other plant pathogens, while the soil strain *C. fimi* has the amino acid pattern of the animal parasites.

Not only is there confusion within the genus *Corynebacterium*, but also disagreement as to its taxonomic position in relation to other genera, notably *Mycobacterium* and *Nocardia*. Mainly on the basis of acid-fastness, *Bergey's Manual* (1948) transferred the genus *Corynebacterium* to a new family, Corynebacteriaceae, in the *Eubacteriales*, the genera *Mycobacterium* and *Nocardia* remaining in the Actinomycetales. The seventh edition of *Bergey's Manual* (1957) continued this division, although Bisset & Moore (1949), Jensen (1953) and others had pointed out that

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morphologically these three genera are closely related and not always distinct from each other. Cummins & Harris (1958) found that these genera have almost identical patterns of cell-wall components, and Kwapinski (1956, 1964) and Cummins (1962*b*) have shown serological relationships between members of these genera.

This present work includes an Adansonian numerical study of the taxonomy of some species currently classified as corynebacteria, and of the relationships of these organisms to others in some related genera.

METHODS

Strains examined. Table 1 shows the origins of the strains used in this study. Those received from culture collections have the names they bore when they were received, those from the Department's collection have the names under which they are maintained.

Table 1. *The strains used and their sources*

From the National Collection of Type Cultures

Corynebacterium equi	1621	*C. diphtheriae gravis*	7283
C. diphtheriae P.W. 8	380	*C. hofmannii*	231
C. renale	7448	*C. bovis*	3224
C. flavidum	746	*C. xerosis*	7243
C. ulcerans	8640	*C. ovis*	4681
C. segmentosum	934	*C. murium*	949
C. fimi	7547	*C. viscosum*	2416
C. haemolyticum	8452	*C. pyogenes*	9825

From the National Collection of Plant Pathogenic Bacteria

C. fascians	764	*C. betae*	372
C. rathayi	797	*C. michiganense*	382

From the National Collection of Dairy Organisms

C. helvolum	1192	*Arthrobacter* sp.	1233
Arthrobacter sp.	1234		

From the Department's collection

C. diphtheriae intermedius	*C. diphtheriae mitis*
C. hofmannii	*Mycobacterium phlei*
M. lacticola	*M. smegmatis*
M. butyricum	*Jensenia canicruria*

Isolated by the author

Nocardia sp. from human saliva
Nocardia sp. from soil

Strain maintenance. The strains were maintained in a medium of the following composition, percentages are w/v. Tryptone (Oxoid L42) 0·5 %, proteose peptone (Oxoid L46) 0·25 %, casein hydrolysate (Allen and Hanbury Ltd., acid hydrolysed, vitamin-free) 0·25 %, NaCl 0·1 %, KH_2PO_4 0·02 %, Na_2HPO_4 0·06 %, $MgSO_4$ 0·001 %, $MnCl_2$ 0·001 %. The pH was adjusted to 7·2. This medium supported good growth of all the strains.

Characters examined. Table 2 shows the characters examined. With the exception of the Voges-Proskauer test, the methyl red test, reactions in litmus milk, growth in Koser citrate, growth on Hoyle medium, and liquefaction of heat-coagulated serum, all the tests were performed using the maintenance medium as the basal nutrient

Table 2. *Characters examined*

Acid from: *
  Arabinose Xylose
  Rhamnose Glucose
  Fructose Mannose
  Galactose Maltose
  Lactose Sucrose
  Raffinose Starch
  Inulin Dextrin
  Glycogen Glycerol
  Erythritol Adonitol
  Mannitol Dulcitol
  Sorbitol Salicin
  Aesculin Inositol
DHA from glycerol
Gluconate oxidation
Lipolysis on:
  Butter Castor oil
  Tweens 20, 40, 60 and 80
  Glycerol tributyrate
Lecithinase production
On egg yolk medium:
  Lipolysis Proteolysis
Gelatin hydrolysis
Coagulated serum hydrolysis
Phosphatase production
pH in basal medium after 7 days
Nitrate reduction
Catalase production
Sodium hippurate hydrolysis
Peroxide production
H_2S from basal medium
H_2S from NaS_2O_3
H_2S from cysteine
Resistance to antibiotics:
  Polymyxin B Mycostatin
  Tetracycline Penicillin
  Oleandomycin Novobiocin
  Chlortetracycline Terramycin
  Chloramphenicol Kanamycin
  Erythromycin Neomycin
  Streptomycin Bacitracin
Growth on Hoyles medium
Growth at initial pH values:
  5·0 6·0 7·0 8·0 9·0
Pigmentation
Morphology: presence of:
  Long rods > 5 μ
  Medium rods 3 μ–5 μ
  Short rods < 3 μ
  Coccal forms
  Oval forms
  Branching
Methyl red test
Voges-Proskauer test
Salicin hydrolysis
Aesculin hydrolysis
Starch hydrolysis
Reactions in litmus milk
Oxid. or ferm. of glucose
Polysaccharide on medium +
  Glucose Sucrose
Intracellular starch on medium +
  Glucose Sucrose
Growth in Koser citrate
Acetic acid from ethanol
Calcium lactate oxidation
Tyrosine breakdown
Xanthine breakdown
Lysine decarboxylation
Ornithine decarboxylation
Phenylalanine deamination
Sodium malonate utilisation
Ammonia from arginine
Serum hydrolysis
Casein hydrolysis
Ammonia from basal medium
Urease production
Nitrite reduction
Oxidase production
Methylene blue reduction
Benzidine/H_2O_2 test
Haemolysis of horse blood
Motility
Anaerobic growth
Reaction to the Gram stain
Tolerance of NaCl:
  2·5 % 5·0 % 10·0 %
Tolerance of potassium tellurite:
  0·0175 % 0·035 %
Tolerance of sodium selenite:
  0·0175 % 0·035 %
Tolerance of triphenyltetrazolium chloride:
  0·001 % 0·0025 % 0·01 % 0·025 %
Tolerance of sodium taurocholate:
  2·0 % 5·0 %
Growth at:
  5° 25° 30° 37° 45°
On basal medium, acid-fast to:
  H_2SO_4 1 % 2 % 5 % 10 % 20 %
On basal medium + 2 % glycerol, acid-fast to:
  H_2SO_4 1 % 2 % 5 % 10 % 20 %

* No strain produced gas from any carbohydrate.

medium. The strains from animal sources were incubated at 37°, all the others at 30°. For complete details of the test procedures, see Harrington (1964).

For the numerical analysis, all the characters, with the exceptions listed below, were recorded as positive or negative. Pigmentation was scored as white, creamy

yellow, pink or orange; haemolysis as α, β, or negative; pH of the basal medium after 7 days as no change, raised or lowered; reactions in litmus milk as acid, acid with clot, no change, or alkaline; digestion and reduction of the indicator were scored as positive or negative; pathway of glucose breakdown, where applicable, as oxidation or fermentation. To make the processing of the results, which was done by hand, easier, degrees of positivity were not taken into account. All the characters were given equal weight and the equation proposed by Sneath (1957) was used to obtain the similarity values. This equation is $S = a/(b+c+a)$, where a = the number of tests in which both members of a pair are positive, $b+c$ = the number of tests in which one member is positive and the other negative, and negative matches are not included.

Acid-fastness. Two days' growth on the maintenance medium and on the maintenance medium + 2 % glycerol was used. Smears were made on microscope slides and stained for 5 min. with hot strong carbol fuchsin, then washed with water and treated with four changes of sulphuric acid, each application lasting 1·5 min. The acid was washed off the slide with water and the smear blotted dry between each acid change. After the last washing the smears were counter-stained with dilute malachite green. The first concentration of acid used was 1 %. Smears were then made of those strains which showed more than approximately 25 % of the cells retaining the carbol fuchsin, and these were stained as before, with 2 % acid being used to effect decolorization. This process of elimination was repeated with 5 % acid, 10 % acid and 20 % acid until complete or almost complete decolorization was achieved.

RESULTS

Numerical analysis. After the similarity values had been calculated as percentages, a cluster analysis was performed as described by Sneath (1962), a strain being admitted to a group at the highest similarity level it had with any member of that group. Figure 1 is the similarity matrix after the cluster analysis had been performed, and Figure 2 is the dendrogram derived from the similarity matrix.

The main points of interest are:

(1) The relatively wide range of S values among the strains of *Corynebacterium diphtheriae.*

(2) The high S values of *Corynebacterium ulcerans* with the 'gravis' and 'mitis' strains of *C. diphtheriae.*

(3) *Corynebacterium pyogenes* and *C. haemolyticum* have very low S values with all the other strains examined here.

(4) *Jensenia canicruria* and *Corynebacterium equi* come within the mycobacterium group.

(5) The plant pathogens with the atypical cell wall patterns, *Corynebacterium rathayi*, *C. betae* and *C. michiganense*, have low S values with most of the corynebacteria from animal sources, and these values are lower than those of the other plant pathogen, *C. fascians*, particularly with the strains of *C. diphtheriae.*

(6) The mycobacteria, the nocardia and the majority of the animal parasitic corynebacteria are in one group at the 51 % S level.

Acid-fastness. Table 3 shows the results of the acid-fast staining of those strains which showed any degree of acid-fastness on either medium. Most of those that

C. diphtheriae gravis	1	100																																						
C. diphtheriae mitis	2	87	100																																					
C. diphtheriae P.W.8	3	77	76	100																																				
C. diphtheriae intermedius	4	67	58	51	100																																			
C. ulcerans	5	60	62	50	40	100																																		
C. flavidum	6	53	46	48	53	41	100																																	
C. renale	7	46	40	38	51	37	57	100																																
C. xerosis	8	49	45	45	45	46	56	46	100																															
Nocardia sp. (oral)	9	35	44	50	40	47	37	32	34	100																														
C. hofmannii (231)	10	45	40	40	36	38	44	41	55	35	100																													
C. hofmannii	11	36	33	34	33	31	45	40	49	31	86	100																												
C. equi	12	30	29	31	39	41	39	36	51	33	54	50	100																											
Nocardia sp. (soil)	13	39	34	40	37	49	37	37	42	41	48	39	61	100																										
Jensenia canicruria	14	48	38	42	42	45	44	36	48	39	55	48	59	59	100																									
Mycobacterium smegmatis	15	47	39	48	37	41	41	34	38	36	45	48	53	61	57	100																								
M. butyricum	16	43	36	45	36	43	42	36	41	39	46	39	51	65	61	66	100																							
M. phlei	17	41	33	38	36	39	39	43	35	35	46	40	41	55	54	54	65	100																						
M. lacticola	18	47	38	46	39	44	47	40	44	38	40	35	43	49	62	58	60	63	100																					
C. ovis	19	37	33	30	42	30	46	46	41	30	34	39	27	23	27	22	26	30	36	100																				
C. murium	20	31	27	29	31	29	36	36	35	28	34	35	32	22	24	24	31	27	28	41	100																			
C. helvolum	21	36	32	32	29	40	37	41	35	26	42	36	35	40	49	36	43	36	41	19	26	100																		
C. viscosum	22	28	26	23	33	36	38	42	38	26	41	37	35	36	41	35	39	38	42	26	38	54	100																	
C. fascians	23	33	30	25	42	38	37	38	37	40	32	25	40	40	42	42	40	39	40	25	33	38	40	100																
C. betae	24	16	20	13	23	30	33	33	38	29	26	21	35	48	32	38	34	30	30	24	26	30	36	46	100														
C. rathayi	25	13	14	14	16	26	28	21	30	25	33	29	37	39	35	26	35	24	22	20	25	48	33	36	48	100													
C. michiganense	26	14	17	15	19	25	32	24	34	27	32	28	43	40	36	28	30	26	23	21	21	31	32	40	61	68	100												
C. segmentosum	27	25	26	20	27	31	28	25	34	38	41	41	35	37	35	23	28	29	27	30	40	37	38	28	26	39	30	100											
Arthrobacter sp. (1233)	28	31	33	31	35	34	40	28	43	31	43	40	44	43	50	31	36	41	38	39	32	50	48	33	36	46	43	62	100										
Arthrobacter sp. (1234)	29	33	27	25	34	34	30	38	31	27	35	28	37	35	48	29	36	34	36	28	28	48	48	36	30	41	35	52	57	100									
C. bovis	30	25	27	26	30	31	32	35	38	26	42	38	35	37	33	41	28	32	29	34	23	32	25	22	25	33	32	46	51	41	100								
C. fimi	31	31	31	27	29	33	32	21	33	33	18	16	26	29	26	29	30	23	32	19	13	26	26	28	31	26	29	16	27	20	22	100							
C. pyogenes	32	10	13	13	13	20	19	25	19	13	13	13	17	8	16	9	9	8	17	20	14	17	25	13	19	24	18	16	18	16	25	15	100						
C. haemolyticum	33	14	9	11	10	15	18	17	20	14	12	14	7	8	12	12	11	7	11	16	13	15	13	10	7	20	15	14	15	16	20	4	48	100					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33					

Fig. 1. The similarity matrix of all the strains examined. The similarity values are expressed as percentages.

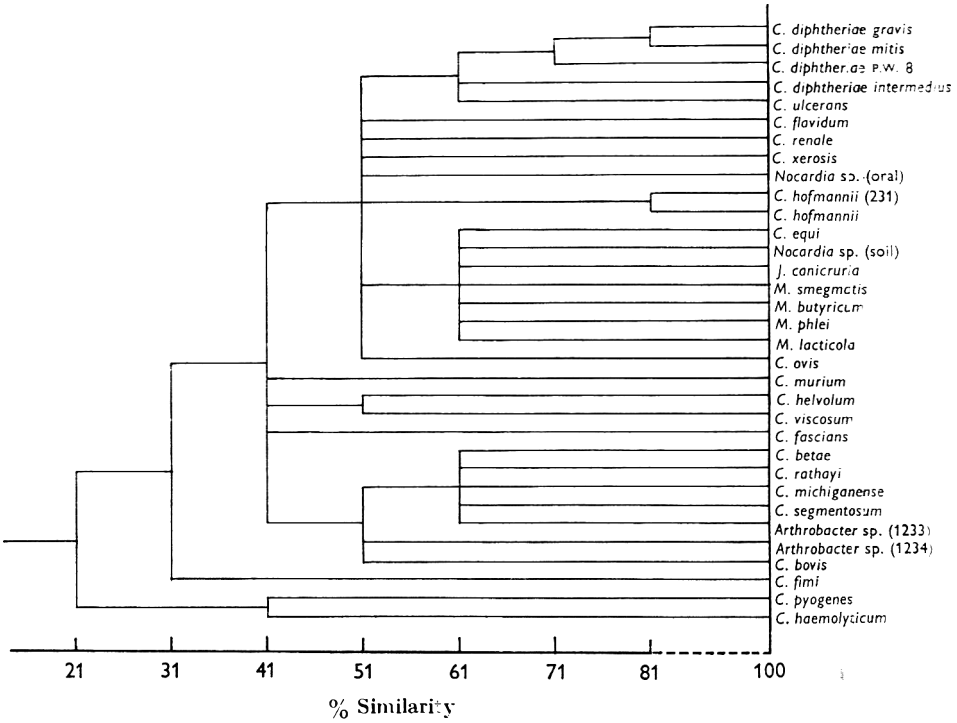

Fig. 2. Dendrogram obtained from the similarity matrix.

Table 3. *Acid-fastness of strains grown on different media*

	Maintenance medium					Maintenance medium +2 % glycerol				
Concentration of H_2SO_4	1 %	2 %	5 %	10 %	20 %	1 %	2 %	5 %	10 %	20 %
C. diphtheriae gravis	+	+	−	−	−	+	+	+	−	−
C. diphtheriae mitis	+	−	−	−	−	+	+	+	−	−
C. diphtheriae P.W. 8	+	+	+	−	−	+	+	+	−	−
C. ulcerans	+	−	−	−	−	+	+	+	−	−
C. flavidum	−	−	−	−	−	+	−	−	−	−
C. renale	−	−	−	−	−	+	−	−	−	−
Nocardia sp. (oral)	+	+	−	−	−	+	+	+	+	−
C. hofmannii (231)	+	−	−	−	−	+	−	−	−	−
Nocardia sp. (soil)	+	−	−	−	−	+	−	−	−	−
Jensenia canicruria	+	+	−	−	−	+	+	−	−	−
M. smegmatis	+	+	+	−	−	+	+	+	+	−
M. phlei	+	+	+	−	−	+	+	+	+	−
M. butyricum	+	+	−	−	−	+	+	+	+	−
M. lacticola	+	+	+	−	−	+	+	+	+	−
C. helvolum	−	−	−	−	−	+	−	−	−	−

showed some resistance to decolorisation when grown on the maintenance medium showed an increased resistance when grown on this medium + 2 % glycerol. Even on the glycerol-containing medium all the mycobacteria examined were decolorized by 20 % sulphuric acid, while on this medium *C. ulcerans* and the '*gravis*', '*mitis*' and P.W. 8 strains of *C. diphtheriae* were resistant to 5 % acid.

DISCUSSION

Among the four strains of *Corynebacterium diphtheriae* there is a range of similarity values, from 87 % for the '*gravis* + *mitis*' pairing down to 51 % for the *intermedius* + P.W.8 pairing, the lowest values being those of pairings which include the '*intermedius*' strain. In view of this wide range of S values, the position of *C. ulcerans*, which has been found to cause diphtheria-like lesions and produce a similar toxin to that of *C. diphtheriae* (see Wilson & Miles, 1964), is of interest. It was found here that this organism has an S value of 62 % with the '*mitis*' strain and 60 % with the '*gravis*' strain. The taxonomic rank of *C. ulcerans* is still a matter of some dispute. Saxholm (1951) and Henricksen & Grelland (1952) thought the organisms of the *C. ulcerans* type, the so-called 'diphtheria-like corynebacteria', could either be regarded as a distinct species or as a variety of *C. diphtheriae*. Howard & Jann (1954) felt that their results with bacteriophages lytic for the diphtheria-like corynebacteria showed these organisms to be distinct from *C. diphtheriae*, although two of their diphtheria-like corynebacteria resembled *C. diphtheriae* in that they were resistant to all the phages used. Wilson & Miles (1964) believed that *C. ulcerans* is sufficiently well defined to qualify for species rank, basing their decision on the results of Jebb (1948) who found it to differ from *C. diphtheriae* in only four characters. If the four strains of *C. diphtheriae* examined here are to be regarded as representatives of one species, then on the S values of the '*gravis*' and '*mitis*' strains with *C. ulcerans* found here, this organism should be reclassified as a variety of *C. diphtheriae*.

Corynebacterium pyogenes and *C. haemolyticum* were found by Cummins & Harris (1956) to have cell-wall amino acid and sugar patterns characteristic of the streptococci, not the corynebacteria, and they suggested that these organisms should be reclassified as streptococci. The similarity values of these two strains with all the other strains are low, which supports the hypothesis that they are not corynebacteria. Sneath & Cowan (1958), in their numerical taxonomical study of a wide range of bacteria, found that *C. pyogenes* fell in the group which contained the streptococci.

There is a distinct group at the 61 % level made up of the mycobacteria, *Corynebacterium equi*, *Jensenia canicruria*, and the soil strain of *Nocardia*. The results for the mycobacteria are in general agreement with those of Cerbon & Bojalil (1961), who found that *Mycobacterium smegmatis* and *M. phlei* were on the same branch of their dendrogram, the branch dividing at the 60 % level into two groups. It is of interest here that *C. equi* and *J. canicruria* appear in the mycobacteria group. Jensen (1934) placed *C. equi* in the genus *Mycobacterium* because of its acid-fastness, and Jensen (1952) considered that its existence showed that there was a close relationship between the genera *Corynebacterium* and *Mycobacterium*. The strain examined here showed no trace of acid-fastness. The genus *Jensenia* was proposed by Bisset & Moore (1949) to include the unicellular soil diphtheroids, but *J. canicruria*, described by these authors in 1950, has never gained much support as a specific entity, being identified as a nocardia by some authors and as a mycobacterium by others (see Adams & McClung, 1960; Gordon & Mihm, 1961; Kwapinski, 1964; Jones & Bradley, 1964). Sneath & Cowan (1958) found it to be in the mycobacterium group of their numerical taxonomy.

Corynebacterium rathayi, *C. betae* and *C. michigancnse* appear to be more distinct from most of the animal parasitic corynebacteria, particularly those of the *C. diphtheriae* group, than do *C. fascians* and *C. fimi*, and this distinction is similar to that found with the cell-wall composition patterns of these organisms. Da Silva & Holt (1965) have compared some plant pathogens with *C. diphtheriae* and they concluded that the plant pathogens were sufficiently distinct from *C. diphtheriae* to warrant their exclusion from the genus *Corynebacterium*. They suggested that *C. fascians*, being so different from all the other coryneforms they examined, should be excluded from the family Corynebacteriaceae, a proposal the present author cannot agree with. Of the plant pathogens, *C. fascians* appears from the numerical taxonomy presented here, to be the one most closely related to the animal strains, a relationship that is also suggested by the cell-wall studies of Cummins (1962*b*).

The position of two of the strains from animal sources cannot be readily explained. *Corynebacterium segmentosum*, a species no longer recognised by *Bergey's Manual* (1957), is from the human respiratory tract, and *C. bovis* is commonly found in cow's milk, yet both fall into the Arthrobacter-plant pathogens group at the 51 % level of the dendrogram.

The large group at the 51 % level contains almost all the strains with the cell-wall composition which Cummins & Harris (1958) and Cummins (1962*b*) found to be typical of the true corynebacteria, the mycobacteria and the nocardias, and their presence in this group could further indicate a close relationship between these three genera.

In the distinction between and the separation of the genera *Corynebacterium* and *Mycobacterium*, the character of acid-fastness has always been a major factor, the corynebacteria being described as non-acid-fast and the mycobacteria as acid-fast. However, 'acid-fast' does not seem to be a well-defined term, and although its meaning is clear in medical laboratory procedure referring to *M. tuberculosis*, it is not always certain that the same exact meaning is used for other species of this genus and other genera. *Bergey's Manual* (1957) uses such descriptions as 'weakly acid-fast', 'acid-fast when grown under the proper conditions' and 'not as strongly acid-fast as . . .', which confirm the idea that the criterion of acid-fastness is not a sound taxonomic one. This is further supported by the results of the acid-fast staining reported here, in which the '*gravis*', '*mitis*' and P.W.8 strains of *C. diphtheriae*, and *C. ulcerans* were resistant to decolorization by 5 % sulphuric acid when grown in the presence of 2 % glycerol, and on this medium the oral nocardia was as acid-fast as the four mycobacteria.

The results of this present work, taken with the results of Kwapinski (1956, 1964), Cummins & Harris (1958) and Cummins (1962*b*), and the morphology of these organisms, lead the present author to support the suggestion, made by Cummins (1962*a*) on the basis of cell wall composition, that the genera *Corynebacterium*, *Mycobacterium* and *Nocardia* could be merged to form one genus. Even if this is not generally accepted, there is no reason to continue the separation of the genus *Corynebacterium* from the genera *Mycobacterium* and *Nocardia* into different orders mainly on the basis of acid-fastness.

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Some Chemical Changes in the Mycelium of *Aspergillus flavus* during Autolysis

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SUMMARY

Some chemical changes which took place in the mycelium of *Aspergillus flavus* during autolysis were studied. *A. flavus* underwent a 'neutral autolysis' in which a decrease of 73·9% in mycelial dry weight occurred. Glucose, xylose, maltose and mannitol were found in autolysing mycelium of *A. flavus*. The loss of free glucose amounted to 10 mg./flask during the whole period of autolysis. Xylose was decreased to half its initial content by the third day of autolysis and then disappeared by the ninth day, whereas maltose decreased at the beginning and then remained constant from the eighty-first to the hundred and eleventh day of incubation. Eighty-nine per cent of the initial amount of mannitol was lost during the first 9 days of autolysis, whereas fat seemed to be uninvolved in the process. Eighteen different amino acids were found in the autolysing mycelium of *A. flavus*. The general picture was of decreasing concentrations during autolysis, and amino acids decreased their content by about 95% with respect to the concentration present at the beginning. This decrease partially accounted for the loss of mycelial-nitrogen observed during autolysis.

INTRODUCTION

Low molecular weight compounds such as amino acids and simple sugars contained in mycelium of filamentous fungi have been the subject of extensive investigation. The nature and variation in amount of these compounds and especially of amino acids has attracted the attention of many workers. A large amount of information has been obtained about the nature, and amount of changes in the amino acid pool in mycelium of filamentous fungi at different stages of their life-cycle (Ritter, 1955; Pillai & Srinivasan, 1956; Meyers & Knight, 1961; Chattaway, Toothill & Barlow, 1962).

Bent & Morton (1964) and Bent (1964) have made a detailed study on the changes of free and combined amino acids during the successive phases of spore germination, exponential growth and nitrogen starvation with several filamentous fungi. Nevertheless, very little information exists about the free sugars and of the free amino acids pools and their changes in the mycelium during the autolytic phase of growth. This terminal phase has been relatively neglected and especially as concern simple carbohydrates. We have not found any report dealing with the behaviour and quantitative changes of free sugars in mycelium during autolysis. Reports describing the presence or absence of free amino acids and free sugars

in autolysates from mycelium of *Aspergillus phoenicis* are those by Emiliani & Parera (1958) and by Emiliani & Ucha de Davie (1962). Tandon & Chandra (1962) described the nature and qualitative changes observed in the amino acids pool of *Colletotrichum gloeosporioides* during autolysis. In the present work the qualitative and quantitative changes of the free sugars, mannitol and free amino acids during the autolytic phase of growth in *Aspergillus flavus* have been studied.

METHODS

Organism. Aspergillus flavus Link was bought from the Centraal Bureau voor Schimmelcultures, Baarn, Holland. This organism was used throughout the present studies of autolysis in a static liquid cultures.

Culture medium. Modified Raulin-Thom medium of the following composition was: glucose (anhydrous), 50 g.; tartaric acid, 2·7 g.; ammonium tartrate, 2·7 g.; $(NH_4)_2SO_4$, 5 g.; $(NH_4)_2HPO_4$, 0·4 g.; K_2CO_3, 0·4 g.; $MgCO_3$, 0·27 g.; distilled water, 1000 ml. To avoid excessive sporulation $FeSO_4$ and $ZnSO_4$ were omitted.

Twenty litres of this medium were prepared and distributed in 100 ml. amounts in Jena conical flasks of 300 ml. capacity. These filled flasks were plugged with cotton-wool and sterilized by steaming for 30 min. on three successive days. When at room temperature they were inoculated with 1 or 2 ml. of a spore suspension obtained by gently rubbing the surfaces of thirty slopes of *Aspergillus flavus* growing on malt agar for 20 days.

The inoculated flasks were incubated in the dark at 24–25°. At regular intervals flasks were randomized and the sample flasks taken for analysis. The number of flasks taken at any given time depended upon the amount of growth of the mould; thus at the beginning of the autolytic phase, samples of five flasks were sufficient, while as autolysis proceeded the number of flasks taken was progressively increased. Mycelium was separated from the cultures by filtration. The pads of mycelium were washed with distilled water on a Buchner funnel, and these washings discarded. The culture filtrate was kept frozen until needed.

Treatment of mycelium. Mycelium was cut into small pieces and dried in an oven at 80–100° then in a desiccator over P_2O_5 to constant weight, and finally powdered in a small coffee mill ('Turmix' model moka-mix).

In a typical experiment a weighed portion of the finely powdered mycelium was exhaustively extracted in a Soxhlet apparatus with light petroleum (b.p. 50–70°). The solid residue from this solvent was Fraction I. The defatted mycelium was next extracted with diethyl ether and the material after distilling off the ether constituted Fraction II. The lipid-free mycelium was carefully removed from the extraction thimble, dried at 80–100° and weighed. This mycelium was placed in a beaker and continuously extracted by cold (22°) distilled water (100 ml./g. lipid-free mycelium) for 1 hr with mechanical stirring. This extraction was repeated twice, using 50 ml. water each time. A fourth extraction gave an extract which did not produce any spot when a portion of it was chromatographed after concentration for both sugars and amino acids. These aqueous extracts were combined (Fraction III) and the following analyses made: (*a*) in a portion of this extract (100 ml.) free amino acids were estimated; (*b*) in 1 ml. of this extract total free reducing substances were determined; (*c*) a portion of this extract (100 ml.) was concentrated at 70° under

reduced pressure to a final volume of 10 ml. and individual free reducing sugars and mannitol separated, identified and estimated.

Fat. The content of crude fat was estimated by direct gravimetric determination.

Reducing substances. Total free reducing substances were determined by the method of Somogyi (1945) in conjunction with that of Nelson (1944).

Determination of sugars: Glucose, xylose and maltose. Individual sugars were separated and identified on paper chromatograms by the general method of Partridge (1948) with modifications for quantitative determination (Jermyn & Isherwood, 1949). For each sample, three sheets of Whatman no. 1 filter paper (36 × 48 cm.) were used. On each six spots, four standards and two unknown, each of 0·10 ml. were placed by means of a micropipette. The papers were developed by descending chromatography for 72 hr. with the butan-1-ol + acetic acid + water (40 + 10 + 22, by vol.) solvent. The chromatograms were dried and the four strips with standards on each sheet cut out and sprayed with silver nitrate in acetone-ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950). Areas corresponding to the spots for each sugar and a corresponding paper blank were cut out from the chromatogram and extracted with water at 70° for 45 min. (Flood, Hirst & Jones, 1947). The Somogyi–Nelson method was used for the quantitative determination of these separated sugars.

Determination of mannitol. The areas corresponding to mannitol in the chromatograms were cut out and eluted with water at 70° for 45 min. In this eluate mannitol was estimated by a modification of the colorimetric method of West & Rapaport (1949) using sodium arsenite instead of stannous chloride.

Estimation of amino acids. One hundred ml. of Fraction III were concentrated to dryness at 60–70° under reduced pressure and extracted with 50 ml. of the ethanol + water + conc. HCl (95 + 4·5 + 0·5, by vol.) mixture described by Baliga *et al.* (1955). This extract was concentrated and the amino acids separated and identified by descending paper chromatography, using as solvent the butan-1-ol + acetic acid + 96% (v/v) ethanol + water (40 + 10 + 10 + 20, by vol.) mixture on Whatman no. 1 filter paper. Elution of the spots and estimations of the total amount of amino acids have been carried out by the method of Giri, Radhakrishnan & Vaidyanathan (1953) as modified by Kay, Harris & Entenman (1956). Readings were done in a Beckman spectrophotometer, model B at 575 mμ. Standards were prepared using leucine of analytical purity obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Results are expressed as mg./flask.

Estimation of kojic acid. In exploratory experiments we have observed that kojic acid could be quantitatively removed by diethyl ether from mycelium which had been previously extracted by light petroleum. In accordance with this observation fraction II was treated by distilled water (25 ml.) and the mixture warmed to 60° for a few min. on a water bath, filtered under suction, and the solid residue washed on the filter repeatedly with warm distilled water. The filtrate and washings were made up to volume (50 ml.) and to a portion of this solution the method used by Arnstein & Bentley (1953), modified by Bentley (1957) was applied for the estimation of kojic acid. Readings were done at 540 mμ. Standards were prepared with kojic acid of analytical purity obtained from The British Drug Houses Ltd. (England).

Total nitrogen. Was determined by the micro-Kjeldahl method.

RESULTS

The criterion of autolysis we have used through this work was the loss in weight of dry mycelium. Between the ninth and the twelfth day of incubation autolysis set in. The tenth day of incubation was therefore taken as the first day of autolysis and the word 'day' in the following text refers to 'day of autolysis'.

By the 117th day the mycelium lost 73·9% of its maximum initial dry weight (Fig. 1).

The culture filtrate (Table 1) stayed at pH 4·6 at the initiation of the autolytic phase of growth. It shifted to 7·5 at ninth day and then remained in the neighbourhood of 7 through the whole period of incubation.

During the first 87 days of the autolytic cycle mycelium lost 70% of its initial nitrogen content. The content of fat remained more or less constant, being uninvolved in the process of autolysis. *Aspergillus flavus* grown in the medium used in this work produced a relatively small amount of kojic acid. This amount, 0·8 mg./flask (see Table 1) at the 0 day, disappeared from mycelium coinciding with the initiation of the autolytic phase. No kojic acid could be detected at the third day.

Table 1. *Weight of dry mycelium, pH of the culture filtrate and nitrogen, fat and kojic acid of Aspergillus flavus at various stages of autolysis*

Period of incubation (days)	Period of autolysis (days)	pH culture fluid	Mycelium dry weight (g./flask)	Total N in dry mycelium (mg./flask)	Fat (mg./flask)	Kojic acid in mycelium (mg./flask)
9	0	4·2	1·0066	49·3	19·8	0·8
12	3	4·6	0·8493	39·9	.	.
18	9	7·5	0·6336	29·1	20·9	.
30	21	7·5	0·4789	20·1	20·5	.
66	57	7·8	0·3725	15·2	19·7	.
81	72	7·4	0·3092	14·8	20·0	.
96	87	7·4	0·2981	14·6	19·6	.
111	102	7·1	0·2899	15·6	17·6	.
126	117	.	0·2594	.	15·8	.
251	242	7·0	0·2618	19·3	15·7	.

Behaviour of the free reducing substances and of the free sugars. The total amount of free reducing substances (Fig. 2) decreased as autolysis set in. Its initial concentration, nearly 20 mg./flask at the beginning of autolysis, diminished to 3·7 mg./flask at the 102nd day. There was then a slight increase to 4·5 mg./flask, dropping finally to 0·8 mg./flask.

Glucose, xylose and maltose were found in the autolysing mycelium of *Aspergillus flavus*. The general picture for free glucose was of decreasing (Fig. 2) concentration from the beginning of the autolytic phase of growth. It presents a similar pattern to that of total free reducing substances. The diminution of this sugar between the 0 and the 202nd day amounted to 10 mg./flask. The content of free glucose accounts for an average of 54% of the content of free reducing substances through the whole period of incubation. At the end of the period of autolysis here studied the total reducing activity of this aqueous extract was practically due to the presence of glucose.

Xylose which was present in mycelium of *Aspergillus flavus* at the beginning of autolysis in a concentration of nearly 4·0 mg./flask reduced its content to half (2·0 mg./flask) at the third day and then disappeared between the third and the ninth day (Fig. 2).

The amount of maltose present in autolysing mycelium of *Aspergillus flavus*, 2·2 mg./flask at the beginning of autolysis continuously decreased during the process. From the 72nd to the 102nd day the content of maltose remained constant. At the end of this cycle only traces could be detected (Fig. 2).

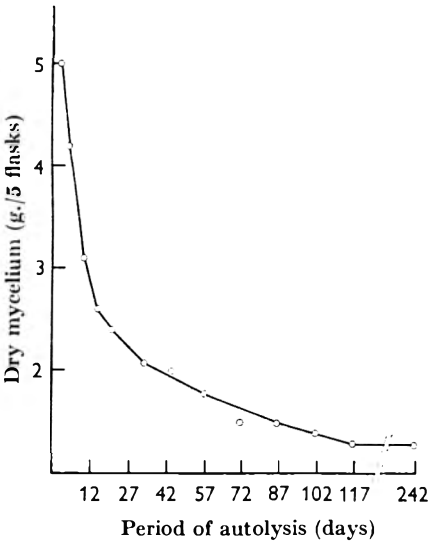

Fig. 1

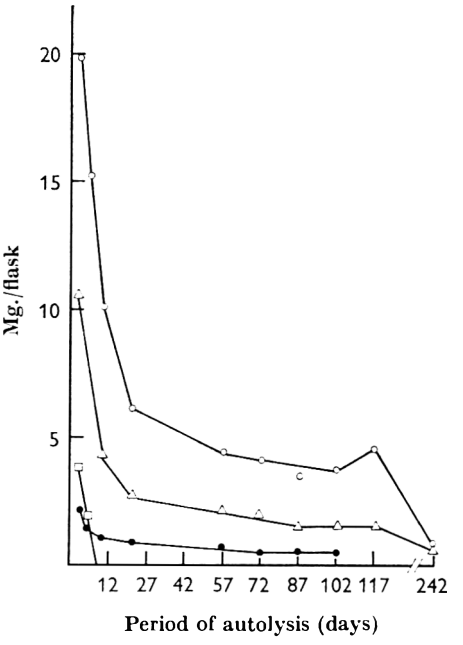

Fig. 2

Fig. 1. Variation in the weight of dry mycelium (g./5 flasks) of *Aspergillus flavus* during the autolytic phase of growth.

Fig. 2. Changes in the concentration of total free reducing substances, ○; glucose, △; maltose, ●; xylose, □; in the aqueous extracts from mycelium of *Aspergillus flavus* during the cycle of autolysis.

Behaviour of mannitol. The amount of mannitol present in mycelium was relatively high at the beginning of autolysis (Fig. 3). The diminution of its content was very sharp. Eighty-nine per cent of its initial content was lost within the first 9 days, since its concentration dropped from 75·49 mg./flask, at the beginning of autolysis, to 7·6 mg./flask at the ninth day. The rate of diminution slows between the ninth and the 57th day. Then it remained constant, the final amount being 1·5 mg./flask.

Behaviour of the free amino acids. Sixteen different amino acids were separated and identified, two other present in substantial amounts, were not identified (unknown I and II). The general picture was of decreasing concentration of free amino acids during autolysis. Glycine, threonine, proline and tryptophan disappeared by the 117th day. The initial concentration of the constituents of the pool,

24·2 mg./flask (Fig.4), decreased to nearly an eighth (3·3 mg./flask) during the first 20 days. At the end of the period of autolysis the content of amino acids present was 0·3 mg./flask.

The concentration of each amino acid, as estimated by visual comparison are recorded in Table 2. The total amount of leucine, alanine, glutamic acid, serine, cysteine, unknown I and II accounts for the gross weight in the bulk of amino acids. Neither arginine or γ-aminobutyric acid could be traced in the whole cycle of autolysis.

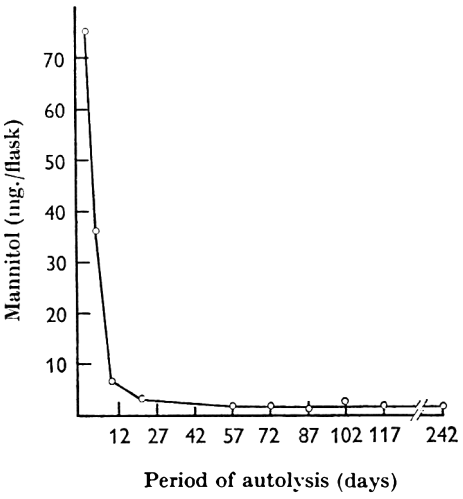

Fig. 3

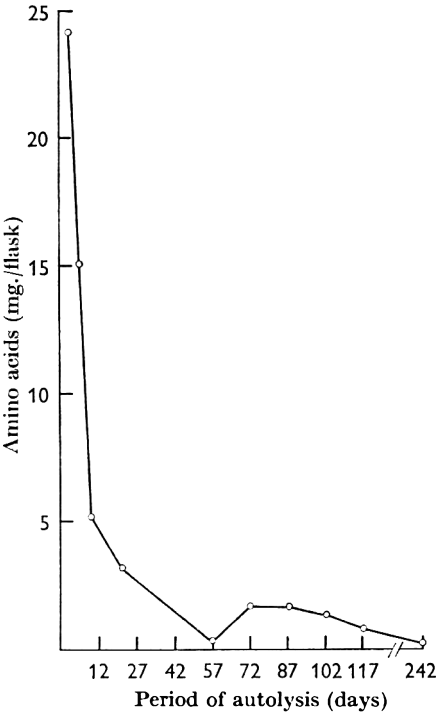

Fig. 4

Fig. 3. Variation of the concentration of mannitol in the aqueous extracts from mycelium of *Aspergillus flavus* during autolysis.

Fig. 4. Changes in the amount of the free amino acids present in autolysing mycelium of *Aspergillus flavus*.

DISCUSSION

In the present work we found a reduction of 73·9 % in mycelium weight of *Aspergillus flavus* during autolysis. As far as our information goes this was the highest loss recorded in mycelial weight for the genera *Aspergillus* and *Penicillium* during the autolytic phase of growth.

The sharp diminution in the content of mannitol in the cells (98 %) of autolysing mycelium of *Aspergillus flavus* recalls the behaviour of this sugar alcohol in *A. terreus*, grown in a medium containing NO_3^- as N source, the disappearance of this substance during autolysis amounting to 95 % of its initial content (unpublished work).

Table 2. *Free amino acids present in autolysing mycelium of Aspergillus flavus*

Amino acid	Period of autolysis (days)										
	0	3	9	21	39	57	72	87	102	117	242
Glycine	+	T	T	T	T	+	+	T	T	–	–
Proline	+	T	+	T	T	T	T	T	T	?	–
Threonine	T	T	T	T	T	T	T	T	T	–	–
Tryptophan	T	T	T	T	T	T	T	T	T	–	–
Lysine	T	T	T	T	T	T	T	T	T	T	T
Valine	T	T	T	T	T	T	T	T	T	T	T
Leucine	+	+	+	+	+	+	+	+	+	+	T
Alanine	+	+	+	+	+	+	+	+	+	+	T
Glutamic acid	+	+	+	+	+	+	+	+	+	+	T
Serine	+	+	+	+	+	+	+	+	+	+	T
Cysteine	+	+	+	+	+	+	+	+	+	+	T
Unknown I	+	+	+	+	+	+	+	+	+	+	T
Unknown II	+	+	+	+	+	+	+	+	+	+	T
Phenilalanine	T	T	+	T	T	T	T	T	T	T	T
Methionine	+	+	+	T	T	+	+	T	T	+	+
Tyrosine	+	+	+	T	T	T	T	T	T	+	+
Aspartic acid	+	+	+	+	+	+	+	+	T	+	T
Asparagine	+	+	+	T	T	+	+	+	+	T	T

+, Present in substantial amount; –, absent; T, traces.

Amino acids reduced their content in 86 % with respect to the concentration present at the beginning of autolysis. The reduction in the amount of these nitrogenous compounds partially accounted for the loss of nitrogen suffered by the mycelium during the first 96 days of incubation.

Ritter (1955) found an increase in the content of asparagine, aspartic acid and glutamic acid contained in mycelium of *Aspergillus niger* during autolysis, whereas the concentration of alanine, arginine, glycine, valine and proline continuously decreased. Tandon & Chandra (1962) reported that excepting glutamic acid and alanine the components of the pool obtained from *Colletotrichum gloeosporioides* disappeared at the thirteenth day of autolysis (28 days of incubation). This relatively early disappearance of these components in the pool could be ascribed to the accelerated rate in which changes take place, since in Tandon & Chandra's experiments the fungus was grown in shake cultures.

Pillai & Srinivasan (1956) grew *Aspergillus flavus* in a medium physiologically alkaline (Czapek-Dox medium, $NaNO_3$ as N source) and they observed a decrease in the mycelial dry weight from the 15th day up to the 30th day of incubation thus indicating that autolysis, as measured by the loss in mycelial dry weight, had begun at the 15th day of incubation. Concentration of free amino acids at the 15th day of incubation, were, according to Pillai & Srinivasan (1956) 1335 mg./100 g. dry mycelium. Since at the 30th day of incubation this concentration amounted to 765 mg./100 g. dry mycelium, this indicated that there had been a reduction in the content of free amino acids of 42 % in these fifteen first days of autolysis. In our experiments using Raulin–Thom medium this reduction amounted to 71 % in the first 15 days of autolysis, therefore indicating that autolysis in a medium physiologically acid (Raulin–Thom medium, NH^{4+} as N source) affected free amino acids to

a greater extent than it did in the case of autolysis in a medium physiologically alkaline.

Since pH of our cultures was above 4·0 throughout the whole period of autolysis no γ-aminobutyric acid present in the pool could be expected, confirming the observation of Simonart & Chow (1954) that *Aspergillis oryzae* only formed γ-aminobutyric acid when pH of the culture was below 4·0. Later, working with *Penicillium griseofulvum*, Bent & Morton (1964) confirmed this finding.

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The Genetic Constitution of Certain Penicillinase Micro-mutants in *Staphylococcus aureus*

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SUMMARY

Treatment of the naturally occurring magno-inducible *Staphylococcus aureus* strain 8325–18 with ethyl methane sulphonate allows the isolation of mutants which synthesize much less penicillinase than the parent. Both inducible and constitutive mutants can be isolated and their activities range from 0·1 to 50% of the basal activity of the wild type. All the mutants examined here synthesize a penicillinase indistinguishable from the wild type in terms of substrate profile and reaction with specific anti-exo-penicillinase serum. Examination of the genetic make-up of the mutants shows that they all possess an intact inducibility (i^+) gene. It is theoretically possible that the lesion in the micro-inducible strains could lie in the penicillinase structural gene, but such a location is extremely unlikely for the micro-constitutives. The most likely location for these mutants is a region analogous to the operator of the lactose segment of *Escherichia coli*; however, their properties are incompatible with the recent suggestion that the operator region should be divided into two distinct parts.

INTRODUCTION

Novick (1963) has shown that penicillinase micro-mutants may be obtained from cultures of *Staphylococcus aureus* after treatment with mutagens. Two classes were observed: micro-inducible strains, in which the uninduced enzyme level was less than that found in the wild type but in which the induction ratio was normal ($\sim$ 40-fold); and micro-constitutive strains, in which the uninduced level was less than the wild type but in which the induction ratio was approximately 1·0. Within this classification, strains have been isolated with basal penicillinase activities ranging from about 0·1 to 50% of normal. Some micro-inducible strains synthesize normal amounts of penicillinase protein with low specific enzyme activity, but all the micro-strains examined here synthesize penicillinase indistinguishable from the wild type.

Penicillinase micro-mutants have also been obtained from wild-type *Bacillus licheniformis* by treatment with ethyl methane sulphonate or nitroso-guanidine (Dubnau & Pollock, 1965). In these mutants the enzyme level varied from 0·25 to 5% of normal and some of these appear to make a 'mutein'—or mutant protein (Collins *et al.* 1965).

The genes responsible for penicillinase synthesis are normally carried on an extra-chromosomal particle—or plasmid (Novick, 1963; Richmond, 1965*a*). So far a number of different types of plasmid have been identified on the basis of the genetic markers accompanying the penicillinase genes on the plasmid and the immunological properties of the penicillinase molecule itself (Novick & Richmond, 1965;

Richmond, 1965*a*). Of the ten different plasmid types that can be distinguished on these grounds, only three (α, β and γ) are involved in the work described here. All the micro-mutants which are studied in this account are carried on the α-plasmid since they are obtained by treatment of strain ($\alpha.i^+p^+$) with mutagens. The only difference between the α- and γ-plasmids is that the latter carries genes responsible for erythromycin resistance (em^R). Transduction of the γ-plasmid to cells already containing α leads to recombination between α and γ and such recombined plasmids ($\alpha \ldots \gamma$) have been used in certain experiments described below which require selection of transductants with erythromycin.

The β-plasmid differs from γ in that recombination with α after transduction is rare: instead plasmid diploids are established (Richmond, 1965*b*). Such diploids have been used in these experiments to test some of the control properties of micro-mutants in much the same way that Jacob & Monod (1961) have used merodiploids of the lactose system in *Escherichia coli* to investigate the control properties of the *lac* genes.

So far two plasmid-borne genetic regions determining penicillinase synthesis have been identified in *Staphylococcus aureus*. One (i^+) confers inducible properties on enzyme synthesis, and the other (p) is the structural gene for the penicillinase molecule itself (Novick, 1963; Richmond, 1965*a*). Since all the mutants examined here seem to synthesize wild-type penicillinase, one question to be answered is whether their micro-properties are caused by a mutation in the *i*-gene, or elsewhere. The genetic experiments reported later suggest that both micro-inducible and micro-constitutive mutants carry an intact i^+ gene and it is necessary, therefore, to invoke the presence of a second control region in the penicillinase system to explain the behaviour of these micro-mutants. Mapping experiments show that in the case of strains μ 1, μ 15, μ 56 and μ 88 at least, this region probably lies between the i_1^- and p_2^- loci, and this location suggests—by analogy with mutants in the β-galactosidase system of *Escherichia coli* (Jacob & Monod, 1961)—the presence of a genetic region of the 'operator' type. In the latest version of the 'operator-hypothesis' for the control of enzyme synthesis, Jacob & Monod (1965) suggest that the operator as originally envisaged must be divided into two parts, one responsible for the recognition of the *i*-gene product (still called the operator) and the other controlling the rate of expression of the operon (the promoter). The properties of the penicillinase micro-inducible mutants could be caused by a mutation in a promoter, but the micro-constitutives cannot readily be interpreted on the basis of a divided operator/promoter since a single mutational change in these strains affects both the recognition of the *i*-gene product and the rate of expression of the structural gene. It appears therefore, that an operator region may be involved in the penicillinase system of *S. aureus*, but its subdivision into two parts, as in the β-galactosidase system, does not need to be postulated, A preliminary account of some of this work has appeared previously (Richmond, 1965*a*, 1966).

METHODS

Organisms. All the micro-mutants examined in this paper (see Table 1) were obtained from strain 8325($\alpha.i^+p^+.Hg^R.em^s$) (Novick & Richmond, 1965) by treatment with ethylmethane sulphonate (Novick, 1963). They were subcultured

routinely on blood agar and the presence of the α-plasmid checked at each sub-culture by showing the presence of the Hg^R marker with a mercuric chloride disc (Green, 1962). Other stock strains, e.g. 8325($\alpha.i^+p^+.Hg^R.em^S$), 8325(N), 147 ($\beta.i^-_{223}p^+.Hg^R.em^3$), and 147($\beta.i^+p^+.Hg^R.em^S$) have already been described (Novick & Richmond, 1965). The recombinant strain 8325($\alpha.i^-_1p^+.Hg^R...\gamma.em^R$) was obtained by transducing strain 8325($\alpha.i^-_1p^+.Hg^R.em^S$) with phage 80 α (Novick, 1963) propagated on strain 258($\gamma.i^+p^+.Hg^R.em^R$) (Hashimoto, Kono & Mitsuhashi, 1964; Richmond, 1965*a*) and selecting on erythromycin agar. All the micro-strains were then converted to the erythromycin-resistant state (em^R) by transduction with phage obtained by irradiating the constitutive strain 8325($\alpha.i^-p^+.Hg^R...\gamma.em^R$). The transductants were selected on medium containing 10 μg. erythromycin/ml. and the micro-strains picked after staining the colonies to show the low amount of penicillinase synthesised.

Media and conditions for induction of penicillinase. The organisms were grown routinely in CY medium (Novick & Richmond, 1965) and induced by addition of 0·5 μg. methicillin/ml. The specific enzyme activity (enzyme units/mg. dry wt. bacteria) was measured 3·75 hr after induction (Richmond *et al.* 1964). The amount of penicillinase synthesized by strains was determined by staining colonies growing on CY medium agar containing 0·2% (w/v) starch with penicillin + iodine solution as described by Novick & Richmond (1965).

Transduction and selection of transductants. The phages used for transduction were carried as prophage by lysogenic donor strains. Exponentially growing cultures of the donor strains were centrifuged and the bacteria resuspended to a density of 5×10^8 organisms/ml. in physiological saline. The suspension of organisms (2 ml.) was irradiated in a Petri dish (Hanovia lamp), and 1·5 ml. immediately transferred to 1·5 ml. pre-warmed CY medium containing 0·3% (w/v) glucose. The culture was incubated at 35° for 1 hr, then centrifuged, and the phage freed from bacteria by membrane filtration. This method of preparation usually gave phage titres of about 10^9 p.f.u./ml. for both strains 8325 and 147.

Transduction experiments were carried out as described by Novick (1963; Novick & Richmond, 1965). The transductants were usually selected with penicillin as described by Richmond (1965*b*). Where selection of the *Hg* marker was required, the transductants were plated at a density of 5×10^7 recipient organisms/plate on mercuric chloride agar of the following composition: 'Oxoid' peptone, 2% (w/v); NaCl, 0·5% (w/v); Davis agar, 1% (w/v); adjusted to pH 7·4 (Moore, 1960) Mercuric chloride was added to the molten agar to a final concentration of 65 μg./ml. immediately before pouring. The plates were used within 24 hr of mixing the peptone and the $HgCl_2$ solution. Selection with erythomycin was carried out as described above.

Immunological titration of A- and C-type penicillinase. The presence of A- or C-type penicillinase was detected with specific anti-exo-penicillinase serum as described by Richmond (1965*b*, *c*).

RESULTS

Characteristics of mutants

Table 1 shows the specific activity of exponentially growing cultures of the micro-mutants considered below. Among the micro-inducible strains, the uninduced

enzyme level varied from 0·1 to 10 % of normal, whereas among the micro-constitutives the variation is from 2–10 %. The induction ratio is close to normal (40-fold) in all the micro-inducible strains except one (strain μ 89), where the ratio is about 15.

All the mutants were examined to see whether there is any evidence for their being double mutants or deletions. Ultraviolet light-induced revertants have been obtained from the micro-inducible strains μ 1, μ 15 and μ 33 and from the micro-constitutive strain μ 56. In all cases the revertants were indistinguishable from the wild-type strain 8325($\alpha.i^+p^+$). These observations suggest that each of these mutants carry point mutations.

Table 1. *Specific enzyme activity of micro-mutants*

Strain No. 8325($\alpha.i^+p^-$)	Phenotype	Specific enzyme activity		Induction ratio
		Uninduced	Induced	
	Magno-inducible	10	300	30
μ 1	Micro-inducible	0·002	0·08	36
μ 15		0·006	0·02	33
μ 33		0·02	0·6	30
μ 88		0·13	5	38
μ 89		0·02	0·3	15
μ 55	Micro-constitutive	0·03	0·035	1·2
μ 56		1·1	1·6	1·2
μ 102		0·27	0·3	1·0

Specific enzyme activity = enzyme units/mg. dry wt. bacteria.

$$\text{Induction ratio} = \frac{\text{Specific enzyme activity of induced culture}}{\text{Specific enzyme activity of uninduced culture}}$$

Crude preparations of the enzyme synthesized by each mutant strain were indistinguishable from normal in their reaction with specific anti-exo-penicillinase serum (Richmond, 1963, 1965*c*). Examination of the relative rates of hydrolysis of benzyl penicillin, phenoxymethyl penicillin, ampicillin, 6-amino penicillanic acid and quinacillin by the enzymes synthesized by the mutant strains showed no significant difference in behaviour from the wild-type enzyme. The extracellular enzymes synthesized by fully induced cultures of strain μ 33 (micro-inducible) and μ 56 (micro-constitutive) were purified. The specific enzyme activity of both preparations was close to that of the purified wild-type enzyme (Richmond, 1963) and there was no difference in immunological behaviour between the mutant and the wild type. This is, therefore, *prima facie* evidence that micro-strains are not caused by mutations in the penicillinase structural gene. However, a mutation in a structural gene can theoretically lead to a mutant protein which is indistinguishable from the wild type in substrate profile and reaction with specific antiserum, and the possibility of this being the case here is discussed later.

Genetic constitution of the micro-mutants

One type of mutation that could give rise to micro-strains is one in the controlling (i^+)-gene. In the case of the micro-inducible strains, the mutation would have to alter the properties of the *i*-gene so that its product became an exceptionally effective repressor of the structural gene, yet retained its ability to recognize the inducer. To give a micro-constitutive phenotype, the mutant would have to modify the

i-gene product so that recognition of the inducer was lost in addition to its increased effectiveness as a repressor.

In practice, any alteration in the properties of the *i*-gene should be detected by examining the effect of the micro-mutation carried on one penicillinase plasmid on a known constitutive (i^-) genotype carried on a second plasmid in the same cell (Richmond, 1965*b*; Novick & Richmond, 1965). If the micro-mutation is in the *i*-gene, it should express its effect when 'trans' to the (i^-) constitutive gene; that is, a plasmid bearing a micro-mutation in the *i*-gene should repress the uninduced level of penicillinase formation by a constitutive plasmid to the micro-level.

Table 2. *Physiological characteristics of parent strains, diploids and segregants in an experiment in which phage from strain 147($\beta.i^-_{223}p^+$) was used to construct a diploid with the micro-inducible strain, μ 33.*

	Specific enzyme activity		
	Uninduced	Induced	Induction ratio
Parent strain			
147($\beta.i^-_{223}p^+$)	158	317	2
Micro-inducible strain, μ 33	0·02	0·65	32
Diploid*	5·8	296	51
Segregants			
8325($\beta.i^-_{223}p^+$)	161	288	1·8
Micro-strain (μ 33?)	0·017	0·8	46
Stock strains			
147($\beta.i^+p^+$)	5·2	271	52
8325($\beta.i^-_{223}p^+$)	150	274	1·8

* The activities quoted for the diploid cultures are the average of measurements on six diploid clones.

Specific enzyme activity = enzyme units/mg. dry wt. bacteria.

$$\text{Induction ratio} = \frac{\text{Specific enzyme activity of induced culture}}{\text{Specific enzyme activity of uninduced culture.}}$$

Appropriate diploid cultures were constructed by using the micro-strains as recipients in a transduction experiment in which the transducing phage was obtained by irradiating the magno-constitutive strain 147($\beta.i^-_{223}p^+$). The recipient cultures were infected at a multiplicity of 1·1 p.f.u./recipient organism, and the transductants selected on agar containing penicillin (see Methods). Since recombination between components of an α/β plasmid diploid are uncommon (Richmond, 1965*b*), only two types of segregant are expected from this type of diploid. One of them (carrying the $\beta.i^-_{223}p^+$ genome) is constitutive (see Table 2 for enzyme levels) whereas the other (carrying the α.micro.p^+ genome) has the phenotype characteristic of the particular micro-strain involved (Table 1). These two segregants may readily be distinguished from the diploid (see Table 2 for typical enyzme levels), and from one another, by comparing the relative size of the penicillinase haloes produced by uninduced colonies growing on starch/CY agar after staining with penicillin+iodine (Novick & Richmond, 1965). Table 2 gives the physiological characteristics of the parent, diploid and segregant strains obtained when the recipient was the micro-inducible strain μ 33. The presence of the μ 33 genome in the uninduced diploid did not lower the specific activity (enzyme units/mg. dry wt.

bacteria) to the micro-level but only to the values characteristic of the wild-type parent. These results, therefore, suggest that the mutation in the micro-inducible strain μ 33 does not lie in the *i*-gene, and comparable results have been obtained with all the other micro-inducible strains tested (μ 1, μ 15, μ 88 and μ 89). Similarly, when the micro-constitutive strains were used as recipients (e.g. μ 55, μ 56 and μ 102, Table 3), the diploid containing both the micro- and the magno-constitutive genomes was fully inducible with a normal basal level, so the micro-constitutive strains also behave as though they have a normal *i*-gene (i^+).

Table 3. *Physiological characters of parent strains, diploids and segregants in an experiment in which phage from strain 147($\beta.i^-_{223}p^+$) was used to construct a diploid with the micro-constitutive strains μ 55, μ 56 and μ 102.*

	Specific enzyme activity		Induction ratio
	Uninduced	Induced	
Parent strains			
147($\beta.i^-_{223}p^+$)	161	298	1·8
μ 55	0·02	0·035	1·7
μ 102	0·32	0·30	1·0
μ 56	1·3	2·0	1·5
Diploid			
In μ 55*	4·2	281	69
In μ 102*	6·1	268	44
In μ 56*	8·3	249	31
Segregants			
8325($\beta.i^-_{223}p^+$) from diploid in μ 55	142	307	2·1
8325($\beta.i^-_{223}p^+$) from diploid in μ 102	168	301	1·9
8325($\beta.i^-_{223}p^+$) from diploid in μ 56	171	308	1·8
Micro-strain (μ 55?)	0·02	0·035	1·7
(μ 102?)	0·32	0·21	0·7
(μ 56?)	1·8	3·0	1·7
Stock strains			
147($\beta.i^+p^+$)	4·6	238	51
8325($\beta.i^-_{223}p^+$)	171	317	1·8

* The activities quoted for the diploid cultures are the average values obtained with six diploid clones of each strain.

Specific enzyme activity = enzyme units/mg. dry wt. bacteria.

$$\text{Induction ratio} = \frac{\text{Specific enzyme activity of induced culture}}{\text{Specific enzyme activity of uninduced culture}}$$

Examination of the diploid comprising the ($\beta.i^-_{223}p^+$. magno-constitutive) and ($\alpha.i^+p^+$.micro-constitutive) plasmids can also show whether the micro-mutation on the α-plasmid can be counteracted by a product of the β-plasmid acting 'trans' in the diploid. If the wild type can correct a micro-mutation in the penicillinase system, then the diploid between the ($\beta.i^-_{223}p^+_O$) and ($\alpha.i^+p^+_A$.micro-constitutive) plasmids should synthesize both A- and C-type penicillinase (characteristic of the α- and β-plasmids respectively—Richmond, 1965*a*) to the full wild-type level in the presence of inducer. However, if the micro-mutation cannot be corrected by the wild-type plasmid acting 'trans', then the diploid should form C-type enzyme at the full induced rate (i.e. about 300 units/mg. dry wt. bacteria), whereas the A-type enzyme should only be present at the level found in the micro-state (e.g. 1·6 units/

mg. dry weight bacteria in the case of the μ 56; see Table 1). Measurement of the amount of A- and C-type enzyme synthesized by the diploid constructed by transducing the plasmid from strain 147($\beta.i^{-}{}_{223}p^{+}$) into strain μ 56, showed that, at most, the A-type enzyme amounted to 3 % of the C-type, and micro-constitutive mutations in the penicillinase system cannot, therefore, be counteracted in a diploid by the wild-type allele.

Genetic location of some micro-mutations

The positions of the micro-mutations μ 1, μ 15, μ 56 and μ 88 were mapped in relation to a known mutation in the structural gene, $p^{-}{}_{2}$; (Richmond, 1963, 1965*a*, *b*) and to the *Hg* and *em* loci, by taking advantage of the fact that the micro-strains

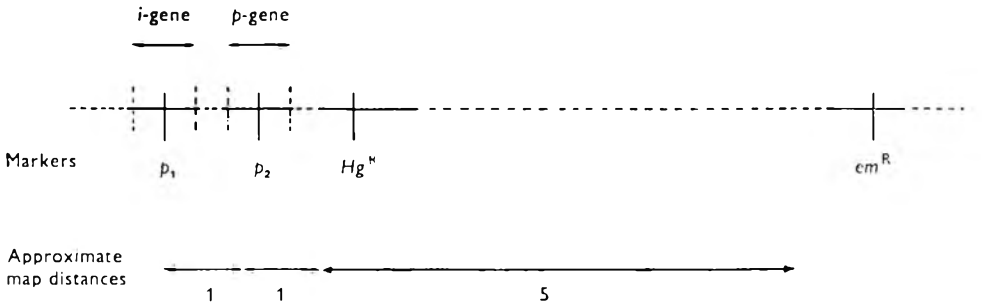

Fig. 1. Order and approximate distances between markers on the recombined $\alpha \ldots \gamma$ plasmid. Markers: *i*, penicillinase control (indueibility) gene; *p*, penicillinase structural gene; Hg^R, resistance to mercuric chloride; em^R, resistance to erythromycin.

are genotypically $i^{+}p^{+}$. Previous experiments (Richmond, unpublished) have shown that the order of loci on the recombined $\alpha \ldots \gamma$ plasmid is $i.p.Hg.em$ (Fig. 1) and when a cross of the type

$$\left.\begin{matrix}\alpha.i^{+}p^{+}.em^{R} \times \alpha.i^{-}{}_{1}p^{-}{}_{2}.em^{S} \\ \text{donor} \qquad\quad \text{recipient}\end{matrix}\right\} \tag{1}$$

is carried out, the $i^{-}{}_{1}p^{+}.em^{R}$ recombinants occur at about 0·1 % of the total number of erythromycin-resistant transductants. If the location of a micro-mutation lies between $i^{-}{}_{1}$ and $p^{-}{}_{2}$, the number of $i^{-}p^{+}.em^{R}$ recombinants obtained from the cross

$$\alpha.i^{+}.\text{micro}.p^{+}.em^{R} \times \alpha.i^{-}{}_{1}p^{-}{}_{2}.em^{S} \tag{2}$$

should be less than the number obtained from cross (1). This result would also be expected if the micro-loci lay close to *em* but on the far side of that marker with respect to *p*; however, this alternative can be eliminated by a further experiment.

The micro-constitutive strains μ 56.em^{R} and μ 88.em^{R} were therefore used as sources of transducing phage and strain 8325($\alpha.i^{-}{}_{1}p^{-}{}_{2}.em^{S}$) was used as recipient in a transduction experiment. The transductants were selected on erythromycin plates and counted after overnight growth. At the same time the number of magno-constitutive recombinants on the plate was counted. The total number of erythromycin-resistant transductants and magno-constitutive recombinants was also measured in cross (1) as a control. With all the micro-strains tested, the proportion

of the em^R recombinants which were magno-constitutive (i^-p^+) was less than that found in the control cross (1) above (Table 4). This result suggests that the micro-loci of these strains lie between the i^-_1 and p^-_2 loci. The alternative interpretation (that the micro-locus lies close to *em*—see above) can be eliminated by investigation of the products formed by transduction of the micro-mutants to a penicillinase-less strain. When an $\alpha \ldots \gamma$ recombinant plasmid was transduced in this way, the em^R marker remained linked to the penicillinase genes in only about 85% of the recombinants (Novick & Richmond, 1965). When certain of the (micro.em^R) markers

Table 4. *Recombination data for the crosses* $\alpha.i^+p^+.em^R \times i^-_1p^-_2.em^S$, $\alpha.i^+.micro\ 1.p^+.em^R \times \alpha.i^-_1p^-_2.em^S$, *and* $\alpha.i^+.micro\ 56.p^+.em^R \times \alpha.i^-_1p^-_2.em^S$

Cross	Donor	Recipient	Total em^R transductants	$i^-_1p^+.em^R$ recombinants
Control	$\alpha.i^+p^+.em^R$	8325($\alpha.i^-_1p^-_2.em^S$)	27,500	29
1	$\alpha.i^-.\mu1.p^+\ldots\gamma.em^R$	As control	47,000	7
15	$\alpha.i^+.\mu15.p^-\ldots\gamma.em^R$		41,000	8
56	$\alpha.i^+.\mu56.p^-\ldots\gamma.em^R$		52,000	10
89	$\alpha.i^+.\mu89.p^-\ldots\gamma.em^R$		27,000	4

Table 5. *Co-transduction of the* p^+ *and micro loci from the strains* μ *56 and* μ *89 to strain 8325*(N)

Donor	Recipient	Total recipients plated	Total p^+ recipients	Total $p^+.em^R$ trans-ductants	Co-trans-ductants of p^+ and em^R (%)
8325($\alpha.i^+.\mu56.p^+\ldots\gamma.em^R$)	8325(N)	9×10^6	87*	72	86
8325($\alpha.i^+.\mu89.p^+\ldots\gamma.em^R$)	8325(N)	7×10^6	57*	44	77

* Selected with $\frac{1}{10}$th the concentration of penicillin used previously (Richmond, 1965*b*)

were transduced to strain 8325(N), and the transductants selected with penicillin, the micro-properties were co-transduced with the penicillinase locus in all the transductants tested whereas, as expected, there was only about 85% co-transduction of the p^+ and *em* markers (Table 5). The micro-loci in strains μ 88 and μ 56 must, therefore, lie much closer to *p* than to *em* and from the evidence of the previous cross their loci must be between the i^-_1 and p^-_2 loci.

DISCUSSION

The results reported here show that the micro-mutations in these strains do not lie in the penicillinase *i*-gene. If the genetic regions identified in the β-galactosidase system of *Escherichia coli* apply to penicillinase synthesis in *Staphylococcus aureus*, the mutations might be in the structural gene, *p*, or in a region analogous to the 'operator' (Jacob & Monod, 1961, 1965).

As far as the micro-inducible strains are concerned, it is not possible to decide clearly between these alternatives. One possibility is a location in that part of the operator—as originally defined by Jacob & Monod (1961)—which is responsible for controlling the rate of expression of the structural gene. A location in a promoter of the type defined by Jacob & Monod (1965) seems to be ruled out, since the properties

of the micro-constitutive mutants cannot be reconciled with the division of the operator region into two segments (see below and Richmond, 1966). If the micro-inducible mutations lie within the structural gene they must be to the left of the p_2 locus (Fig. 1) and cause a lower rate of enzyme synthesis yet no change in enzymic properties. Theoretically, behaviour of this kind could arise from a structural gene mutation which changed one DNA codon to an alternative codon for the same amino acid or to that of another amino acid whose insertion did not alter enzymic properties. To give the observed phenotypes, however, such a genetic change would have to cause a lower rate of reading of the structural gene whether at the transcription (DNA to RNA) or translation (RNA to protein) level. At the transcription level, the limitation could conceivably arise from a poor supply of the specific transfer RNA required to recognize the 'new' RNA-codon specified by the altered structural gene. An explanation of this kind has already been invoked to explain the properties of the 'polar' mutations in operons concerned with histidine (Ames & Hartman, 1964) and arabinose biosynthesis (Lee & Englesberg, 1963), and with lactose degradation (Signer, Beckwith & Brenner, 1965). Unlike the penicillinase micro-mutations, however, these mutations appear to block completely the synthesis of the protein in whose gene they occur, and only affect the rate of synthesis of subsequent enzymes of the operon (Newton *et. al.* 1965).

As far as the micro-constitutive mutations are concerned, a location in the penicillinase structural gene seems most unlikely since they cause a loss of recognition of the inducer in addition to all the changes found in the micro-inducible strains. Under these circumstances, these mutations probably lie in a region of the operator type and this view is strengthened by their map location between the penicillinase p_2 and i_1 loci. Jacob & Monod's original definition endowed the β-galactosidase operator with two roles—recognition of the *i*-gene product, and initiation (and perhaps rate control) of the expression of the operon (Jacob & Monod, 1961)—and certain mutations in this region might be expected to affect both these properties and thus give rise to a micro-constitutive phenotype. Recent work with the β-galactosidase system, however, has now led Jacob & Monod (1965) to suggest that the two operator functions are separate and to propose a new region— the promoter—which is alone responsible for expression of the operon, while the term 'operator' is retained for the region responsible for recognition of the *i*-gene product. Although the penicillinase micro-inducible mutants could be satisfactorily explained on the basis of a mutation in a promoter, the characteristics of the micro-constitutive mutants are not readily compatible with subdivision of the operator. The divided operator region, which appears to exist in the *lac* segment of *Escherichia coli*, may not therefore occur in the control regions of all inducible enzymes.

I wish to thank my colleagues, Professor M. R. Pollock, F.R.S., and Dr J. F. Collins, for many helpful discussions in connexion with this work. I would also like to thank Mr P. Thompson and Mr K. Frayn for their skilled technical assistance.

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The Effect of Heat on Host-controlled Restriction of Phage λ in *Escherichia coli* K(P1)

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SUMMARY

The growth of phage λ.C (i.e. phage λ grown in *Escherichia coli* C) in *E. coli* K lysogenized by phage P1 is normally restricted so that the efficiency of plating of phage λ.C on K(P1) compared to C is about 10^{-7}. When K(P1) bacteria are heated before infection with phage λ.C this restriction may be decreased as much as a million-fold. The time of exposure to elevated temperature (49° and above) required to achieve this increase in e.o.p. of phage λ.C decreased with increasing temperature up to temperatures which began to inhibit the capacity of bacteria to grow phage. Heated K(P1) bacteria recovered their ability to restrict phage λ.C following the resumption of growth at 37°. Part of this recovery can be inhibited by chloramphenicol. A more dramatic recovery of restriction is observed when heated K(P1) bacteria are resuspended in hypertonic media. Experiments are described which indicate that phage λ.C is restricted at an early step after adsorption and that, if phage escapes this restriction, it can grow in heated bacteria subsequently converted into restricting hosts by resuspension in hypertonic media.

INTRODUCTION

The host range of some bacteriophages is changed after growth in a new bacterial host. This change has been shown to be non-mutational in character and is called 'host-controlled modification' (see Luria, 1953; Arber & Dussoix, 1962). Two distinct host-controlled functions can be recognized: modification and restriction. Modification is a process which acts directly on DNA and probably takes the form of a specific alteration in certain base sequences (Arber, 1965; Klein & Sauerbier, 1965). DNA synthesized in a particular bacterial strain therefore carries a modification pattern specified by the host bacterium. Restriction is a process which can occur when foreign DNA enters a bacterium. When this foreign DNA does not carry a modification pattern which is compatible with the recipient bacterium, it is rapidly degraded to small molecular weight components (Dussoix & Arber, 1962). Mutants which are genetically unable to restrict foreign DNA have been isolated (Glover, Schell, Symonds & Stacey, 1963; Colson, Glover, Symonds & Stacey, 1965; Wood, 1965). However, certain environmental factors are known to decrease the ability

of wild-type bacteria to restrict the growth of unmodified phage particles (Luria, 1953; Lederberg, 1957; Uetake, Toyama & Hagiwara, 1964; Glover & Colson, 1965).

The experiments reported here were designed to elucidate further the mechanism of host-controlled restriction. Host specificity of a phage is indicated by a symbol representing the phage followed by a symbol representing the last host in which the phage was grown (Arber & Dussoix, 1962). The system chosen for study was the restriction of phage λ, grown in *Escherichia coli* C and designated λ.C, by *E. coli* strain K (P1) (i.e. strain K lysogenized by phage P1). The efficiency of plating (e.o.p.) of phage λ.C on *E. coli* K is about 10^{-4} but, when K is made lysogenic for phage P1, the e.o.p. of λ.C on K (P1) decreases to about 10^{-7}. The restricted λ.C DNA is degraded in *E. coli* K and in *E. coli* K (P1) shortly after injection (Arber, Hattman & Dussoix, 1963). It can be asked whether the observed breakdown of the restricted λ.C DNA is the primary cause of the failure of phage λ.C to grow in K (P1) or whether it is a secondary feature consequent upon the failure of phage λ.C to grow in *E. coli* K (P1) for some other reason. To answer this question we have used methods which temporarily decreased restriction in K (P1) and studied the kinetics of the loss of restriction and its subsequent recovery before and after infection with phage λ.C. The results reported here indicate that the initial phase of restriction took place very shortly after adsorption and might be due to a surface-localized nuclease.

METHODS

Bacteria. Escherichia coli K-12 strain C600 (Appleyard, 1954) here designated K, and its P1-lysogenic derivative K (P1); *E. coli* C (Bertani & Weigle, 1953), designated C, were used.

Bacteriophage. Phage λv, a virulent mutant of phage λ (Jacob & Wollman, 1954).

Media. Tryptone broth: 1% (w/v) Difco Bacto tryptone, 0·5% (w/v) NaCl, thiamine 10 μg./ml.; adjusted to pH 7·1. Solid media were formed with 1·5% (w/v) Difco agar.

Media for adsorption of phage λ: 0·01 M-$MgSO_4$ or 0·01 M-$MgSO_4$ in 0·01 M-tris HCl buffer at pH 7·3.

Techniques. The general phage techniques used were as described by Adams (1950). Special techniques relating to phage λ were as described by Arber (1958, 1960). The efficiency of plating (e.o.p.) of infective centres = count on *E. coli* K (P1)/count of phage λ.C stock on *E. coli* C.

RESULTS

The effect of heat on restriction

Uetake *et al.* (1964) showed that exposure of *Salmonella butantan* to temperatures between 44° and 51° for a few minutes before infection with phage ϵ^{15} previously grown on *Salmonella anatum*, increased the e.o.p. of the phage from 2×10^{-2} to 5×10^{-1}. We have studied the effect of heat on the restricting ability of *Escherichia coli* K (P1) by measuring the increase in the e.o.p. of phage λ.C on the heated host bacteria. *E. coli* K (P1) organisms were grown in tryptone broth to a colony count of 10^9 bacteria/ml. The bacteria were harvested by centrifugation and resuspended to a count of 10^{10}/ml. in 0·01 M-$MgSO_4$ and starved for 1 hr at 37°. The bacteria were

heated by diluting the starved bacterial suspension 10-fold into pre-heated 0·01 M-$MgSO_4$ at the chosen temperature. After a given time at the elevated temperature the bacteria were transferred to a tube at 37° containing 1×10^8 phage particles/ml. of the challenge phage λ.C. After 15 min. adsorption at 37°, the infected cultures were diluted and the number of infective centres assayed on *E. coli* K (P1) indicator bacteria. The kinetics of heat-inactivation of restriction by temperatures from 48°

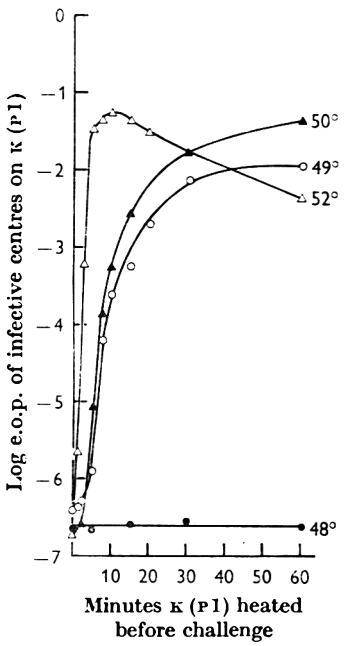

Fig. 1

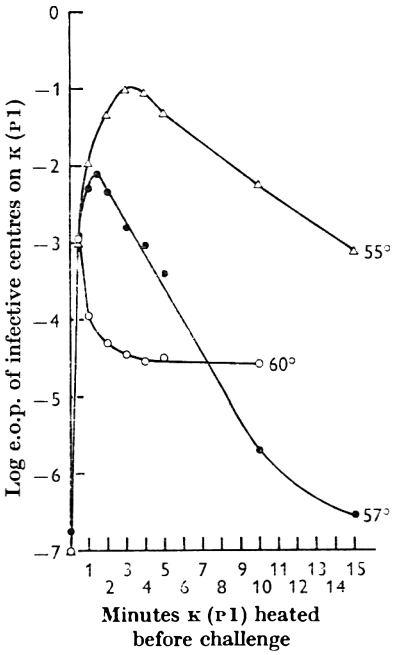

Fig. 2

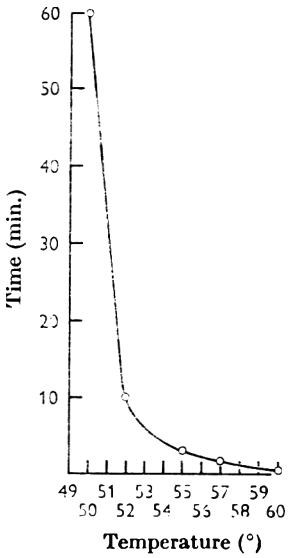

Fig. 3

Fig. 1. Kinetics of the heat inactivation of phage λ.C restriction in *Escherichia coli* K (P1). K (P1) bacteria were grown to colony count 10^9/ml. in tryptone broth, harvested and resuspended to colony count 10^{10}/ml. and starved in 0·01 M-$MgSO_4$. The suspension was heated by making a 1/5 dilution into preheated 0·01 M-$MgSO_4$. After heating the bacteria were returned to 37° and challenged with phage λ.C. The number of infective centres was assayed on *E. coli* K (P1). ●—● 48°; ○—○ 49°; ▲—▲ 50°; △—△ 52°.

Fig. 2. Kinetics of the heat inactivation of phage λ.C restriction in *Escherichia coli* K (P1). Experimental procedure as in Fig. 1. △—△ 55°; ●—● 57°; ○—○ 60°.

Fig. 3. Time of exposure to heat treatment required to obtain the maximum decrease in the restriction of phage λ.C by *Escherichia coli* K (P1).

to 60° are illustrated in Figs. 1 and 2. The e.o.p. of phage λ.C on *E. coli* K (P1) increased by a factor up to 10^6 when the host bacteria were heated before infection. The time of exposure to elevated temperatures required to achieve the maximum effect (the highest e.o.p. of phage λ.C) decreased with increasing temperature (Fig. 3).

The colony count of the cultures did not decrease when they were heated for up to 60 min. at temperatures up to 50°. Above 50° the viability decreased both as a function of the temperature and of the exposure time. Fig. 4 shows that after long exposure times at 57° the decrease in the number of infective centres both for

phage λ.C on *Escherichia coli* C and for unrestricted phage λ.K (P1) on *E. coli* K (P1) followed roughly the same kinetics as the decrease in the number of viable bacteria. The capacity of *E. coli* K (P1) to grow phage λ.C appeared to be rather more heat-sensitive than its capacity to grow phage λ.K (P1). The result could be explained if there were a heat effect on the ability of *E. coli* K (P1) to modify phage λ.C so that a fraction of the infected K (P1) bacteria released bursts of phage λ.C which would not plate on the K (P1) indicator bacteria. However, when infected bacteria taken at this part of the curve were allowed to lyse in liquid media at 37°

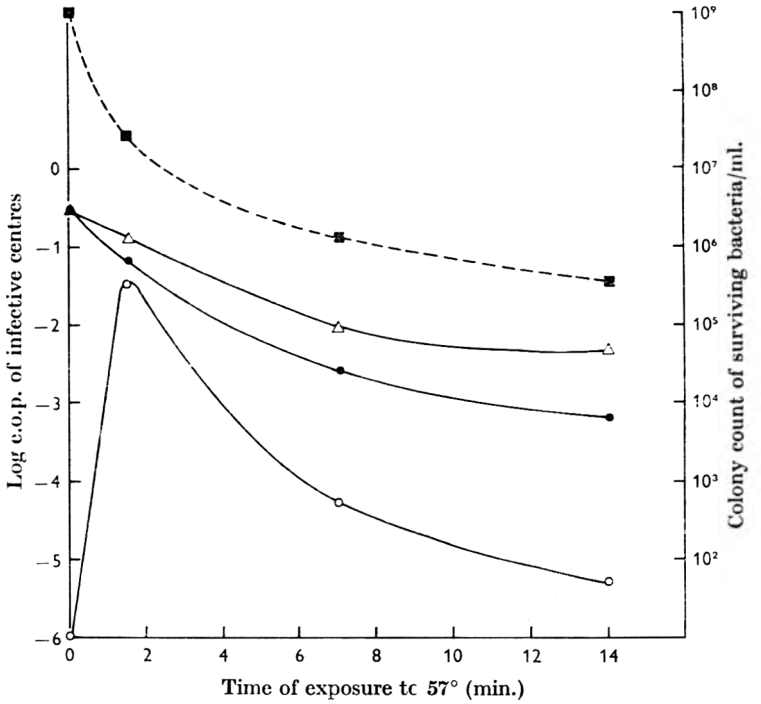

Fig. 4. Effect of heat on the viability and the capacity of *Escherichia coli* K (P1) and *E. coli* C to grow host-modified phage λ. Experimental procedure as in Fig. 1. ■—■ *E. coli* K (P1) colony count; △—△ infection of *E. coli* C with phage λ.C; ●—● infection of *E. coli* K (P1) with phage λ.K (P1); ○—○ infection of *E. coli* K (P1) with phage λ.C.

the bursts consisted entirely of modified λ.K (P1) phage particles. Alterations in the physiological condition of the bacteria before heating, and in the medium in which they were heated, showed that the heat effect was most pronounced with starved stationary-phase bacteria heated in 0·01 M-$MgSO_4$. Bacteria in other phases of growth heated in tryptone broth responded less well.

The recovery of restriction after heat treatment

Figure 5 shows the recovery of restriction after heat treatment under various conditions. In each experiment cultures of *Escherichia coli* K (P1) were grown in tryptone broth, harvested and resuspended in phage λ-adsorption buffer and then heated at 50° for 30 min. Recovery of restriction was measured by returning the

bacteria to 37° and aerating them in the recovery medium for 4 hr; at given intervals 1 ml. samples were withdrawn and challenged with phage λ.C. After 15 min. adsorption, the number of infective centres was assayed on *E. coli* K (P1) indicator bacteria. Curve 1 shows that restriction did not markedly 'recover' when the bacteria were maintained at 37° in phage λ-adsorption buffer after heating. Thus, heat inactivation of the restriction process was not reversed by a simple incubation at

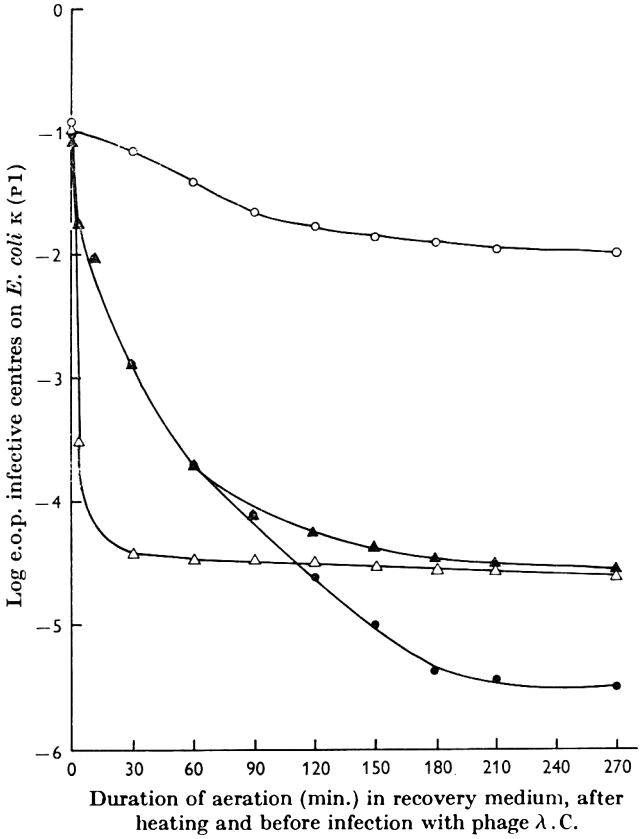

Fig. 5. Kinetics of the recovery of restriction in *Escherichia coli* K (P1) after heat inactivation. *E. coli* K (P1) was grown to colony count 5×10^8/ml. in tryptone broth, harvested and resuspended at colony count 5×10^9/ml. in phage λ-adsorption buffer. The suspension was heated for 30 min. at 50° by making a 1/5 dilution into pre-heated phage λ-adsorption buffer. After heating the bacteria were returned to 37° and again diluted 1/5 into different recovery media to a final colony count of 2×10^8/ml. The suspensions were aerated for 4 hr. and at intervals 1 ml. samples were removed and challenged with 1×10^7 phage λ.C particles. After adsorption for 15 min. the number of infective centres was assayed on *E. coli* on K (P1). Recovery media: ○—○, phage λ-adsorption buffer; ●—●, tryptone broth + 0·01 M-$MgSO_4$ + thiamine 10 μg./ml.; ▲—▲, tryptone broth + 0·01 M-$MgSO_4$ + thiamine 10 μg./ml. + chloramphenicol 50 μg./ml.; △—△, phage λ-adsorption buffer + M-NaCl.

37°. When heated bacteria were allowed to recover in fresh growth medium a marked recovery of restriction occurred (Fig. 5, curve 2). Colony counts made on these bacterial suspensions showed that the organisms began to divide actively only after a lag of 90 to 120 min. The initial and major part of the recovery took place

during this lag and was not dependent upon new protein synthesis, because the addition of chloramphenicol 50 μg./ml. did not influence the recovery process during the first 90 min. (Fig. 5, curve 3). Comparison of the latter parts of curves 2 and 3 shows that, when growth was resumed, renewed synthesis of a factor involved in restriction occurred, because this portion of the recovery was inhibited by chloramphenicol.

Figure 5, curve 4 shows that when heated bacteria were allowed to recover in the presence of M-NaCl there was a dramatic and immediate response. Restriction increased immediately by a factor of 10^4. The main factor in recovery under normal growth conditions (Fig. 5, curve 2) must therefore be the presence of 0·5% (w/v) NaCl in the growth medium.

Table 1. *The effect of NaCl on the recovery of restriction in Escherichia coli* K(P1) *after heat treatment*

E. coli K (P1) was grown to colony count of 5×10^8/ml. in tryptone broth, harvested and starved for 1 hr at 37° in 0·01 M-$MgSO_4$ at colony count 5×10^9/ml. The suspension was heated for 30 min at 50° by making a 1/5 dilution into the above pre-heated media. Restriction was measured, either directly after heat treatment or after a post-heat treatment as indicated, by challenging with $5{\cdot}5 \times 10^7$ phage λ.C particles and assaying the number of infective centres on *E. coli* K (P1) indicator bacteria.

Experiment	Media in which the bacteria were heated	Post-heat treatment before challenge with phage λ.C	Number of infective centres on *E. coli* K (P1)
1	Distilled water	Addition of 0·01 M-$MgSO_4$	$4{\cdot}6 \times 10^6$
2	λ-adsorption buffer	None	$5{\cdot}5 \times 10^6$
3	λ-adsorption buffer + M-NaCl	None	$1{\cdot}5 \times 10^1$
4	λ-adsorption buffer + 2 M-glucose	None	$3{\cdot}3 \times 10^3$
5	Tryptone broth	Addition of 0·01 M-$MgSO_4$	$1{\cdot}3 \times 10^6$
6	Tryptone broth + 1 M-NaCl	Addition of 0·01 M-$MgSO_4$	$1{\cdot}3 \times 10^1$
7	λ-adsorption buffer	M-NaCl	$5{\cdot}5 \times 10^2$
8	No heat treatment	Distilled water for 3 hr	$5{\cdot}5 \times 10^2$
9	No heat treatment	None	$1{\cdot}0 \times 10^1$

The effect of hypertonic medium on the recovery of restriction after heat treatment was further investigated in a series of experiments summarized in Table 1. Comparison of expt. 2 and 3 shows that when bacteria were heated in the presence of M-NaCl there was almost no heat effect on restriction. That this was due to a protection by the hypertonic medium is shown by expt. 7 in which there was an effect of heat on restriction in spite of the fact that M-NaCl was added immediately after heating and before infection. The effect of NaCl was just as apparent when bacteria were heated in tryptone broth (expt. 5 and 6) as when heat treatment was given in distilled water or buffer (expt. 1 and 2). Experiment 4 shows that 2 M-glucose solutions which produced about the same osmotic pressure at 37° as M-NaCl also led to a rapid recovery of part of the restricting ability of heated *Escherichia coli* K (P1). Experiment 8 on the other hand shows that when unheated bacteria were suspended in distilled water for 3 hr their ability to restrict phage λ.C was decreased.

These results suggest that the effect of M-NaCl in bringing about the rapid recovery of restriction may be due to an osmotic phenomenon. This was amply illustrated by the fact that M concentrations of the following salts, Na, K, Li, NH_4, Mg, Mn, Ca as chlorides, sulphates or iodides when added to the adsorption buffer

produced approximately the same increase in restriction, and the addition of 2 M-glycerol to the adsorption buffer gave the same result. Furthermore, the extent to which the restriction of heated bacteria recovered when they were resuspended in hypertonic media was dependent upon the molarity of the added salt. Table 2 illustrates the effect of various concentrations of NaCl on the reappearance of restriction in heated *Escherichia coli* K (P1). It is clear that the extent to which restriction reappeared was proportional to the molarity of the medium in which the heated bacteria were resuspended. This reactivation of restriction in hypertonic media was reversible as might be expected for an osmotic phenomenon. The experiments summarized in Table 3 show that the restricting ability of heated bacteria could be made to fluctuate, depending on the osmotic pressure of the medium in which they were resuspended.

Table 2. *The effect of different concentrations of NaCl on the reappearance of restriction in Escherichia coli K(P1) after heating to 50° for 30 min*

E. coli K (P1) was grown to a colony count of 5×10^8/ml. in tryptone broth, the bacteria harvested and resuspended in phage λ-adsorption buffer at colony count 5×10^9/ml. The suspension was heated for 30 min. at 50° by making a 1/5 dilution into pre-heated phage λ-adsorption buffer. After heating, the bacteria were returned to 37° and the concentration of NaCl adjusted as indicated; $5{\cdot}5 \times 10^7$ phage λ.C particles were adsorbed for 15 min. and the number of infective centres assayed on *E. coli* K (P1) indicator bacteria.

Concentration of NaCl (M)	Number of infective centres	Efficiency of plating corrected for adsorption (e.o.p.)
0	$4{\cdot}6 \times 10^6$	$8{\cdot}3 \times 10^{-2}$
0·05	$1{\cdot}6 \times 10^5$	$6{\cdot}3 \times 10^{-3}$
0·1	$2{\cdot}8 \times 10^4$	$5{\cdot}1 \times 10^{-4}$
0·5	$2{\cdot}0 \times 10^3$	$3{\cdot}7 \times 10^{-5}$
1·0	$4{\cdot}2 \times 10^2$	$8{\cdot}3 \times 10^{-6}$
Control *a**	$1{\cdot}0 \times 10^1$	$1{\cdot}85 \times 10^{-7}$
Control *b*	$1{\cdot}0 \times 10^7$	1·0
Control *c*	$7{\cdot}5 \times 10^6$	1·0
Control *d*	$8{\cdot}1 \times 10^6$	1·0

* *Control a.* Unheated *E. coli* K (P1) were suspended in phage λ-adsorption buffer at colony count 1×10^9/ml. and challenged with $5{\cdot}5 \times 10^7$ phage λ.C particles.

Control b. Heated *E. coli* K (P1) in phage λ-adsorption buffer were challenged with $1{\cdot}0 \times 10^7$ phage λ.K (P1) particles.

Control c. Heated *E. coli* K (P1) in phage λ-adsorption buffer + M-NaCl were challenged with $1{\cdot}0 \times 10^7$ phage λ.K (P1) particles.

Control d. Heated *E. coli* C in phage λ-adsorption buffer + M-NaCl were challenged with $1{\cdot}0 \times 10^7$ phage λ.C particles, and the number of infective centres assayed on *E. coli* C indicator bacteria.

The timing of the restriction process

Escherichia coli K (P1) organisms in which the capacity to restrict the growth of phage λ.C had been impaired by heat treatment instantly recovered most of their restricting ability when they were resuspended after heating in a hypertonic medium. This observation was utilized in an effort to determine as accurately as possible the time after adsorption at which restriction takes place. These experiments are summarized in Table 4. When phage λ.C was adsorbed to heated *E. coli*

Table 3. *Reversibility of the reactivating effect of* M-*NaCl on the restriction of phage* λ.*C by heated Escherichia coli* K(P1)

E. coli K (P1) was grown to colony count 5×10^8/ml. in tryptone broth, the organisms harvested and resuspended to colony count 5×10^9/ml. in phage λ-adsorption buffer. The suspension was heated for 30 min. at 50° by making a 1/5 dilution into pre-heated phage λ-adsorption buffer; after heating the bacteria were returned to 37°.

Experiment 1. Heated bacteria in phage λ-adsorption buffer challenged with $1{\cdot}5 \times 10^7$ phage λ.C particles.

Experiment 2. Heated bacteria in phage λ-adsorption buffer + M-NaCl challenged with $1{\cdot}5 \times 10^7$ phage λ.C particles.

Experiment 3. Heated bacteria in phage λ-adsorption buffer + M-NaCl (from Expt. 2) centrifuged, washed twice in phage λ-adsorption buffer and resuspended in phage λ-adsorption buffer at colony count 1×10^9/ml. and challenged with $1{\cdot}5 \times 10^7$ phage λ.C particles.

Experiment 4. Heated bacteria from Expt. 3 finally resuspended in phage λ-adsorption buffer + M-NaCl and challenged with $1{\cdot}5 \times 10^7$ phage λ.C particles.

Unheated control. Unheated bacteria in phage λ-adsorption buffer challenged with $1{\cdot}5 \times 10^7$ phage λ.C particles.

In all experiments the amount of free phage after adsorption for 15 min. at 37° was measured and the number of infective centres assayed on *E. coli* K (P1) indicator bacteria.

Experiment	Number of infective centres on *E. coli* K (P1)	Free phage	Efficiency of plating corrected for adsorption (e.o.p.)
1	$1{\cdot}18 \times 10^6$	$2{\cdot}0 \times 10^6$	$9{\cdot}1 \times 10^{-2}$
2	$1{\cdot}17 \times 10^2$	$4{\cdot}5 \times 10^6$	$1{\cdot}1 \times 10^{-5}$
3	$4{\cdot}8 \times 10^5$	$2{\cdot}0 \times 10^6$	$4{\cdot}8 \times 10^{-2}$
4	$1{\cdot}0 \times 10^2$	$4{\cdot}9 \times 10^6$	$1{\cdot}0 \times 10^{-5}$
Unheated control	$1{\cdot}1 \times 10^1$	$2{\cdot}01 \times 10^6$	$8{\cdot}3 \times 10^{-7}$

Table 4. *The timing of host-controlled restriction in Escherichia coli* K(P1)

E. coli K (P1) was grown in tryptone broth to colony count 5×10^8/ml., organisms harvested and resuspended in phage λ-adsorption buffer to colony count 5×10^9/ml. The suspension was heated for 30 min. at 50° by making a 1/5 dilution into pre-heated phage λ-adsorption buffer. The bacteria were then returned to 37° and 5 M-NaCl added as indicated to a final concentration of M. The bacteria were then challenged with $1{\cdot}0 \times 10^8$ phage λ.C particles.

In Expts. 1, 2 and 3, 15 min. were allowed for phage adsorption and the number of infective centres was assayed on *E. coli* K (P1) indicator bacteria.

In Expts. 4*a*, 4*b* and 4c, phage λ antiserum was added after the adsorption period and 5 min. later the number of infective centres was assayed on *E. coli* K (P1).

Experiment	Treatment of bacteria before challenge with phage λ.C	Treatment of bacteria after challenge with phage λ.C: NaCl added to M	Efficiency of plating corrected for adsorption (e.o.p.)
1	None	None	$9{\cdot}1 \times 10^{-7}$
2	Heated	None	$8{\cdot}3 \times 10^{-2}$
3	Heated	At time of challenge	$1{\cdot}3 \times 10^{-5}$
4*a*	Heated	2 min. after challenge	$5{\cdot}3 \times 10^{-3}$
4*b*	Heated	5 min. after challenge	$2{\cdot}1 \times 10^{-2}$
4*c*	Heated	15 min. after challenge	$8{\cdot}3 \times 10^{-2}$

K (P1) at the same time as they were suspended in M-NaCl the degree of restriction was high (expt. 3), indicating that NaCl acted very rapidly. When, on the other hand, phage λ.C was allowed a short time to adsorb and to inject into heated bacteria before restriction was restored by the addition of NaCl, a much larger fraction of the infecting phage particles escaped restriction (expt. 4*a*, *b*, *c*). From these results we conclude that an essential step in restriction takes place very soon after adsorption, perhaps when the phage DNA is injected, and that once the phage DNA has been injected into the bacterial cytoplasm it can no longer be restricted.

Table 5. *The restriction of phage λ.C by Escherichia coli* K (P1) *heated after adsorption*

E. coli K (P1) was grown in tryptone broth to colony count of 5×10^8/ml., the organisms harvested and resuspended to colony count 5×10^9/ml. in phage λ-adsorption buffer. The suspension was heated for 1·5 min. at 57° by making a 1/5 dilution into pre-heated phage λ adsorption buffer.

Experiment 1. 5×10^9 bacteria were challenged with $5{\cdot}0 \times 10^8$ phage λ.C particles; after 2 min of adsorption phage λ antiserum was added and the bacteria heated.

Experiment 2. As in Expt. 1 but 15 min. were allowed for adsorption of phage λ. In both experiments the number of infective centres after heating was assayed on *E. coli* K (P1) indicator bacteria. To decrease error due to phage adsorption to the bacteria after heating the suspension was made to M-NaCl by adding NaCl immediately before assaying for number of infective centres.

Unheated control. 5×10^9 bacteria were challenged with $5{\cdot}0 \times 10^8$ phage λ.C particles and after 2 min. adsorption, phage λ antiserum was added and after 5 min. the number of infective centres assayed on *E. coli* K (P1) indicator bacteria.

Heated control. 5×10^9 bacteria were heated, returned to 37° and challenged with $1{\cdot}0 \times 10^8$ phage λ.C particles. After 2 min. adsorption phage λ antiserum was added and after 5 min. the number of infective centres was assayed on *E. coli* K (P1) indicator bacteria.

Expt.	Time of adsorption before heating to 57° (min.)	Number of infective centres on *E. coli* K (P1)	Efficiency of plating corrected for adsorption (e.o.p.)
1	2	$2{\cdot}1 \times 10^3$	$8{\cdot}3 \times 10^{-6}$
2	15	$2{\cdot}6 \times 10^2$	$1{\cdot}0 \times 10^{-6}$
Unheated control	—	$2{\cdot}3 \times 10^2$	$9{\cdot}1 \times 10^{-7}$
Heated control	—	$2{\cdot}5 \times 10^7$	$1{\cdot}0 \times 10^{-1}$

If this conclusion is correct it should be impossible to 'rescue' a restricted phage by inactivating the restriction process in the host bacterium by a brief exposure to high temperature shortly after phage adsorption and injection. The following experiment was made to test this prediction. *Escherichia coli* K (P1) organisms were grown in tryptone broth to a colony count of 5×10^8/ml., and harvested and resuspended to 5×10^9 bacteria/ml. in phage λ-adsorption buffer. The bacteria were then infected at 37° with phage λ.C at a multiplicity of 0·1. After, respectively, 2 and 20 min. adsorption, the amount of free phage was assayed and the infected cultures heated at 57° for 1·5 min. The final number of infective centres produced by the heated bacteria was assayed on *E. coli* K (P1) indicator bacteria. The results of this experiment are summarized in Table 5. Phage λ.C adsorbed to restricting *E. coli* K (P1) organisms for 2 min. before heat treatment yielded 10^4 times fewer infective centres than phage λ.C adsorbed for the same time to heated bacteria. The fact that

they yielded 10 times more infective centres than phage λ.C adsorbed for the same time to unheated bacteria was probably due to phage particles which adsorbed and injected after the heat treatment had begun.

DISCUSSION

The capacity of *Escherichia coli* K (P1) bacteria to restrict phage λ.C is heat-sensitive. The e.o.p. of phage λ.C on *E. coli* K (P1) increased as much as a million-fold when the bacteria were heated before infection. The time of exposure to elevated temperature required to achieve the maximum increase in the e.o.p. of phage λ.C decreased with increasing temperature up to temperatures which inhibited the capacity of the bacteria to grow phage. Uetake *et al.* (1964) concluded that there was a thermolabile factor responsible for the restriction of host-modified ϵ^{15} phage in *Salmonella butantan.* However, it appears that the effect of heat on *E. coli* K (P1) cannot simply be ascribed to the innate thermolability of the host-controlled restriction process, for two reasons. Firstly, when the bacteria are heated in a hypertonic medium, there is very little effect on restriction; secondly, when the heated bacteria are resuspended in a hypertonic medium, there is an immediate restoration of a part of their capacity to restrict. This immediate recovery of restriction is not simply due to a 're-naturation' process at 37°, because the restrictive ability of bacteria kept in phage λ-adsorption buffer after heating did not markedly increase.

However, at least part of the restrictive factor is permanently lost by heated bacteria since they only recovered their full capacity to restrict after growth. This part of the recovery process can be inhibited by chloramphenicol which indicates that protein synthesis is required, presumably the synthesis of an enzyme responsible for restriction. The opposing effects of heat treatment and hypertonic media indicate that the heat effect is in part due to an osmotic phenomenon which results in the partial release of some of the restrictive factor and to the inactivation of the remainder. A hypertonic medium provides protection against both these effects.

That restriction of phage λ.C takes place very quickly after adsorption has been demonstrated by two different kinds of experiment. In the first, phage is adsorbed to bacteria in which restriction has been decreased by heat treatment and then restored by the addition of NaCl very shortly after adsorption. Under these conditions a significant fraction of the phage escaped restriction, indicating that once the phage DNA had been injected into the bacterium it was no longer susceptible to the restriction process. In the second, phage was adsorbed to normal restricting bacteria and then very shortly after adsorption the restriction was decreased by heat treatment. Under these conditions no significant rescue of the phage was observed.

These results can be explained by the following working hypothesis. An essential part of the host-controlled restriction process involves a nuclease located on the surface of the host bacterium, perhaps in the periplasm between cell wall and cytoplasmic membrane. This nuclease can act on DNA which lacks a compatible modification pattern when it passes through the periplasm. Support for this hypothesis has recently been obtained from two sets of experiments. Stationary phase bacteria of *Escherichia coli* strains B, K and C grown to completion in certain media

lose most of their endonuclease I (Shortman & Lehman, 1964). Under the same cultural conditions *E. coli* K (P1) organisms lost a large part of their capacity to restrict phage λ.C (Schell & Glover, 1966*a*). Furthermore, this nuclease has been shown to have a periplasmic location (Hilmoe, quoted by Neu & Heppel, 1964; Cordonnier & Bernardi (1965)). Conditions which result in the release of a number of surface-localized enzymes (Neu & Heppel, 1965) also lead to a decrease in the ability of *E. coli* K (P1) to restrict phage λ.C (Schell & Glover, 1966*b*).

The opposing effects of heat and hypertonic media on the restriction of phage λ.C by *Escherichia coli* K (P1) can be explained in the following way. Heating leads to the partial release of some of the restrictive factor and to the inactivation of the rest. When these heated bacteria are resuspended in hypertonic media plasmolysis occurs and the cytoplasmic membrane separates from the cell wall (see Mitchell, 1961). This separation of cell wall from cytoplasmic membrane increases the volume of the periplasm and could result in an extension of the period during which the phage DNA is accessible to the restricting enzyme and thus leads to a higher degree of restriction. This explanation is supported by the fact that the activation of restriction in hypertonic media is a reversible phenomenon. Phage which infects heated bacteria in a hypertonic medium may be restricted, while phage which infects heated bacteria in normal media is not restricted. The effect is specific for phages normally susceptible to host-controlled restriction and there is no comparable effect of heat or hypertonic media on the ability of phage λ.K (P1) to grow in *E. coli* K (P 1).

The protection obtained in hypertonic media against the effects of heat may also be due to plasmolysis of the cells which prevents the release and inactivation of the restricting enzyme. The host-controlled restriction process in *Escherichia coli* K (P 1) has two components, one under the control of the bacterial genome and one under the control of the prophage P 1. Both these restrictions are sensitive to heat and both can be partially restored in hypertonic media (Schell & Glover, unpublished results). Therefore, these two restriction processes, though different in specificity, behave similarly to heat treatment and may have a common component.

Fukasawa (1964) suggested that a surface localized DNAse is responsible for the restriction of non-glucosylated coliphage T4 DNA by *Escherichia coli* B. This suggestion is supported by the recent results of Molholt & Fraser (1965), who showed that non-glucosylated T-even coliphages were no longer restricted by spheroplasts made from normally restrictive hosts.

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Growth Response of Mycoplasma to Carotenoid Pigments and Carotenoid Intermediates

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SUMMARY

Biosynthetic precursors of carotenoids and hydroxylated carotenoids were substituted for sterol in growth experiments with two representative sterol-requiring mycoplasmas, *Mycoplasma hominis*, strain 07 and avian *Mycoplasma* sp., strain J. Only C_{40} dihydroxyl compounds but not biosynthetic precursors supported growth of *M. hominis*. These results suggested that all enzymes in the biosynthetic pathway to carotenoids were lacking in this organism. A slight response to phytoene and neurosporene indicated that this species may be capable of hydroxylation of C_{40} compounds. Certain precursors of carotenoids and C_{40} dihydroxyl carotenoids supported growth of avian strain J. The phosphorylated mevalonic acids did not support growth of this organism, while isopentenyl pyrophosphate produced a response. These results indicated that an enzymic block occurs between mevalonic acid and isopentenyl pyrophosphate. Phytoene, phytofluene, ζ-carotene, and neurosporene supported growth of this organism indicating that enzymes probably are present for the conversion of certain C_{40} intermediates to dihydroxyl C_{40} compound(s). Lycopene and β-carotene did not support growth probably due to the lack of hydroxylating enzymes for these carotenoids. Monohydroxyl C_{40} carotenoids produced a negligible growth response with both organisms. The growth response of both organisms to sarcinaxanthin and lutein indicated that the presence of two hydroxyl groups in the 3,3′-positions is essential for growth. That the carotenol of *M. laidlawii* strain B supported growth is further proof that a dihydroxyl C_{40} compound is required for growth (the exact locations of the hydroxyl groups are unknown). Since earlier work has suggested analogous functions for sterol and carotenol the sterol requirement of certain mycoplasma species appears to be the result of the inability of these organisms to effect biosynthesis of carotenols.

INTRODUCTION

Growth of sterol-requiring mycoplasmas depends upon the molecular configuration of the available sterol. Smith & Lynn (1958) reported that the sterol must possess an hydroxyl group at C_3 and a hydrocarbon chain at C_{17}. Smith (1964) demonstrated growth responses of sterol-requiring mycoplasmas only to sterols which possess an equatorial 3-hydroxyl group and the A/B rings either in the *trans* configuration or held planar by a Δ^5 double bond. Saprophytic strains of the genus *Mycoplasma* are capable of synthesizing C_{40} carotenoids identified as the hydrocarbon, neurosporene, and carotenol (Smith 1963*a*). Enzymic blocks in the biosynthetic pathway to carotenoids have been demonstrated in the two sterol-

requiring mycoplasma organisms examined (Smith & Henrikson, 1965*a*; Henrikson & Smith, 1965). Sterol and carotenol appear to carry out analogous functions in the genus (Smith, 1963*b*). Hence, a study was made to determine whether biosynthetic intermediates to carotenoids and hydroxylated carotenoids would support growth of sterol-requiring strains.

METHODS

Strains. Mycoplasma sp. strain J, from an avian source, and *M. hominis* strain 07 were used as representative sterol-requiring mycoplasmas.

Assay of growth. The liquid test medium consisted of PPLO broth (Morton, Smith & Leberman, 1951) prepared with ingedients extracted in the dry state with chloroform + methanol (2 + 1, v/v). Desiccated PPLO serum fraction (Difco) was also extracted with chloroform + methanol (2 + 1, v/v), dissolved in distilled water after complete removal of solvent and sterilized by filtration through a Selas 02 filter candle. Growth experiments were made as previously described (Smith & Lynn, 1958) with the following exceptions: inoculum consisted of a 0·5 % (v/v) log-phase culture resulting in an initial concentration of 5×10^6 colony-forming units/ml.; sodium oleate at a final concentration of 1×10^{-6} M was substituted for the phospholipid requirement. The positive controls contained cholesterol; the negative controls were cholesterol free. The concentration of the sterol substitutes ranged from 0·01–0·2 μmole/ml. Cholesterol and sterol substitutes were added as solutions in absolute ethanol prepared immediately before use. Control tubes without sterol contained equivalent amounts of absolute ethanol. Water insoluble compounds were readily dissolved or colloidally dispersed in the culture media due to the presence of oleic acid and protein which aid in solubilization or micelle formation of lipids (Smith & Boughton, 1960). Incubation was carried out in the dark at 37° for 2 days, the time required to give optimal colony counts for the cholesterol-containing control cultures. The colony counts of cholesterol containing tubes were approximately 10^9 colony-forming units/ml. and were arbitrarily set to an arithmetic value of 1·00 (Smith & Lynn, 1958). All other counts were calculated relative to this value. Thus a value of 0·01 represents about 10^7 colony-forming units/ml.; 0·1, 10^8; 1·0, 10^9; 10, 10^{10}, etc. A response giving a count of two logarithms above the control without sterol was considered particularly significant.

Compounds employed as substitutes for sterol. The following compounds were obtained from commercial sources: 5-phosphomevalonic acid, 5-pyrophosphomevalonic acid, isopentenyl pyrophosphate, geraniol, farnesol, nerolidol, phytol, lycopene and β-carotene.

Geranyl pyrophosphate and farnesyl pyrophosphate were synthesized as described by Popjak *et al.* (1962). Geranyl geraniol was a gift from Dr O. Isler (F. Hoffmann-La Roche and Co., Basle, Switzerland).

The remaining compounds were isolated from natural sources. All compounds were used immediately following isolation and characterization. During the few hours required for characterization, the compounds used for incorporation into the culture medium were held in the dark in a nitrogen atmosphere. Phytoene and phytofluene were extracted from fresh tomatoes or commercial tomato paste and purified chromatographically as described by Anderson, Norgard & Porter (1959). The spectral properties of phytoene resembled those described by Goodwin (1952),

and the spectral properties of phytofluene resembled those obtained by Zechmeister & Sandoval (1946).

ζ-Carotene was extracted from tomato paste, saponified, and initially purified by column chromatography as described by Anderson *et al.* (1959). Further purification was accomplished by thin-layer chromatography. Thin-layer chromatographic plates, coated with Silica Gel G, (E. Merck AG, Darmstadt, Germany) 250 mμ thick, were developed in light petroleum (30–60°) containing 5% (v/v) acetone. The pigmented area immediately below the solvent front was removed, eluted with methanol, and dried *in vacuo*. The spectral properties were similar to those described by Nash & Zscheile (1945).

Laidlaw B carotenol and neurosporene were isolated from *Mycoplasma laidlawii* strain B grown in lipid-free tryptose as described by Smith & Henrikson (1965*b*). The organisms were washed and harvested as described by Rothblat & Smith (1961). The wet cell paste was extracted 3 times with 40 volumes of chloroform + methanol (2+1, v/v) and the pooled extracts washed by the method of Folch, Lees & Stanley (1957). The dried lipid extract was redissolved in 10 ml. *n*-hexane and applied to a column of activated silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.). The pigmented compounds were separated using as eluents: benzene + hexane (6+94 by vol.) 200 ml.; benzene + hexane (1+4 by vol.) 250 ml.; benzene + hexane (6+4 by vol.) 250 ml.; benzene, 200 ml.; chloroform + benzene (3+1 by vol.) 250 ml.; chloroform, 200 ml.; chloroform + methanol (20+1 by vol.) 200 ml. Approximately 10 ml. fractions were collected. Neurosporene was eluted with benzene + hexane (6+94 by vol.) whereas the carotenol was eluted with chloroform + benzene (3+1 by vol.).

Rubixanthin was obtained from dried rose hips (*Rosa rubiginosa*) which were extracted successively with methanol, acetone and light petroleum (30°–60°). All extracts were pooled and concentrated to 50 ml. *in vacuo* and saponified for 30 min. with an equal volume of N-potassium hydroxide. The pigments were extracted from the saponified mixture with light petroleum (30°–60°) after the addition of about 100 ml. alkaline 10% (w/v) sodium chloride. The light petroleum extract was concentrated *in vacuo* and chromatographed on MgO (Sea-Sorb, Fisher Scientific Co., Fairlawn, N.J.) + Hyflo SuperCel (1+2, w/w). The column was developed with increasing amounts of acetone in light petroleum. Rubixanthin was eluted with light petroleum containing 20–30% (v/v) acetone. The spectral properties coincided with those reported by Karrer & Jucker (1950).

Lutein was extracted from yellow corn meal (1 lb.) with 3 l. methanol. The methanol extract was diluted with aqueous sodium chloride and extracted three times with 1 l. volumes of light petroleum. Light petroleum extracts were pooled, dried *in vacuo*, and saponified by refluxing in 75 ml. ethanol + 30 ml. of 10% ethanolic KOH for 30 min. The saponified mixture was extracted several times with light petroleum. The extracts were concentrated *in vacuo* and chromatographed on a column of 10 g. MgO + Hyflo SuperCel (1+1, w/w). The column was developed with increasing amounts of acetone in light petroleum. Lutein was eluted with light petroleum containing 85% (v/v) acetone. The spectral properties were similar to those described by Deuel (1951). Infra-red spectra demonstrated a strong hydroxyl absorption.

Solanesol was extracted from ripened Burleigh tobacco and initially isolated on a

Unisil column and rechromatographed on an alumina column as described by Rowland, Latimer & Giles (1956). The quantities of solvents used for development of the alumina column were as follows:

fraction 1, 500 ml. benzene + chloroform (2 + 1 by vol.);
fraction 2, 300 ml. benzene + chloroform (1 + 2 by vol.);
fraction 3, 300 ml. benzene + chloroform (1 + 2 by vol.);
fraction 4, 300 ml. benzene + chloroform (1 + 2 by vol.);

fractions 3 and 4 were rechromatographed individually. The melting point of the isolated compound was 43° compared to 41·5°–42·5° as reported by Rowland *et al.* (1956).

Chloroxanthin was obtained from the green mutant of *Rhodopseudomonas spheroides* (Griffiths & Stanier, 1956) kindly supplied by Dr R. Y. Stanier (University of California, Berkeley). The organisms were grown and extracted and the chloroxanthin purified as described by Nakayama (1958) with the exception that elution was accomplished with light petroleum (30°–60°) containing 15% (v/v) acetone. The spectral properties were similar to those obtained by Nakayama (1958).

Sarcina lutea, the source of sarcinaxanthin, (Nakayama, 1958) was grown on nutrient agar in Roux flasks at 37° for 48 hr. The washed wet cell paste was treated with 5% (w/v) trichloroacetic acid and extracted with acetone under reflux. The acetone extract was dried *in vacuo*, dissolved in a minimal volume of light petroleum and chromatographed on a column of MgO + Hyflo SuperCel (1 + 1, w/v) with increasing amounts of acetone in light petroleum. Sarcinaxanthin was eluted with light petroleum containing 70% (v/v) acetone. The pigment appeared hypophasic in methanol + light petroleum as described by Nakayama (1958) and the spectral properties also coincided.

Dolichol was isolated from 7 lb. pig liver and purified chromatographically as described by Burgos, Heming, Pennock & Morton (1963).

All isolated compounds each produced only one spot on thin layer chromatographs after final purification. Hence, they can be considered chromatographically pure. The absence of 3-β-hydroxy sterols, the only steroids which support growth of the mycoplasma species tested, was verified by the absence of any digitonin-precipitable or Lieberman–Burchard positive substance in any of the compounds used. Although carotenoids are unstable, efforts to prevent isomerization and/or oxidation during incubation in the culture medium included the use of deep stationary tube cultures and the exclusion of light.

RESULTS

Tables 1 and 2 present the results of the growth experiments. *Mycoplasma hominis* strain 07 exhibited a negligible growth response to all phosphorylated intermediates in the pathway of carotenoid synthesis. Among the C_{40} hydrocarbons used, a slight growth response was exhibited to phytoene and neurosporene and a negligible response to phytofluene, ζ-carotene, lycopene and β-carotene. The results indicate that *M. hominis* probably contains none of the enzymes in biosynthetic pathway to phytoene but may be capable of hydroxylation of C_{40} compounds.

The sterol requirement for avian strain J was not met with the phosphorylated

mevalonic acids; however, with isopentenyl pyrophosphate a slight growth response occurred at 0·1 μmole/ml. Geranyl and farnesyl pyrophosphate produced a negligible growth response. Phytofluene produced its greatest growth response at 0·05 μmole/ml. Lycopene and β-carotene did not affect growth. The results indicate that an enzymic block occurred between mevalonic acid and isopentenyl pyrophosphate,

Table 1. *Growth response of Mycoplasma hominis strain 07 and Mycoplasma sp., avian strain J, to biosynthetic intermediates of carotenoids*

Compound	Structure A*	B	C	D	Concn. (μmole/ml.)	Response† Strain 07	Strain J
Cholesterol (control)	—	—	—	—	0·03	1·00	1·00
No sterol (control)	—	—	—	—	—	0·01–0·05	0·01–0·05
5-Phosphomevalonic acid	6	0	0	0	0·01–0·20	0·03	0·08–0·12
5-Pyrophosphomevalonic acid	6	0	0	0	0·01–0·20	0·02–0·03	0·08–0·09
Isopentenyl pyrophosphate	5	1	0	0	0·01	0·05	0·06
					0·05	0·04	0·06
					0·10	0·04	0·69
Geranyl pyrophosphate	10	2	0	0	0·01–0·20	0·009–0·02	0·01–0·03
Farnesyl pyrophosphate	15	3	0	0	0·01–0·20	0·003–0·01	0·01–0·02
Phytoene	40	9	3	0	0·01	0·30	0·71
Phytofluene	40	10	5	0	0·01	0·04	1·37
					0·05	0·001	21·2
ζ-Carotene	40	11	7	0	0·01	0·01	0·68
Neurosporene	40	12	9	0	0·01	0·24	0·79
Lycopene	40	13	11	0	0·01–0·20	0·003–0·10	0–0·02
β-Carotene	40	11	11	2	0·01–0·20	0–0·05	0–0·006

*A = Carbon chain length; B = no. double bonds; C = no. conjugated double bonds; D = no. aliphatic rings.

† = Colony-forming units/ml., with test substance:colony-forming units/ml. with cholesterol control.

as recently demonstrated with labelled intermediates in our laboratory (Henrikson & Smith, 1965). The growth response to phytoene, phytofluene, ζ-carotene and neurosporene indicate that these compounds may be precursors of carotenol.

Among the hydroxylated compounds employed as the sterol substitutes, little or no response was obtained with geraniol, farnesol, nerolidol, phytol geranyl geraniol, rubixanthin, chloroxanthin, solanesol or dolichol (Table 2). Both strains responded to lutein and sarcinaxanthin. Increasing the concentration of lutein above 0·05 μmole/ml. inhibited growth of *Mycoplasma hominis* strain 07. Both strain 07 and avian strain J showed an increasing growth response to Laidlaw B carotenol up to 0·05 μmole/ml. beyond which concentration growth inhibition occurred. This inhibition was analogous to the inhibitory effects of cholesterol at concentrations exceeding the optimum (Edward & Fitzgerald, 1951). Thus, it can be generalized that both organisms require a C_{40} compound of *trans* configuration with two hydroxyl groups. Cyclization and number and conjugation of double bonds are not important.

Confirmation of the results of growth experiments was attempted by an analysis

of the unsaponifiable lipid of avian strain J grown in the presence of the unsaponifiable lipid of *Mycoplasma laidlawii* strain B. Avian strain J was passed through about seven transfers in lipid-free culture medium supplemented with the 'Laidlaw B' lipid. Thallium acetate (1/4000, w/v) inhibited growth under these conditions in contrast to no inhibition in cholesterol-supplemented medium. Five l. of a culture

Table 2. *Growth response of Mycoplasma hominis strain 07 and Mycoplasma sp. avian strain J to hydroxyl compounds as substitutes for sterol*

Compound	Structure *A	B	C	D	E	F	Concn. (μmole/ml.)	Response† Strain 07	Strain J
Cholesterol (control)	—	—	—	—	—	—	0·03	1·00	1·00
No sterol (control)	—	—	—	—	—	—	—	0·01–0·05	0·01–0·05
Geraniol	10	2	0	0	1	1	0·01–0·20	0–0·09	0–0·006
Farnesol	15	3	0	0	1	1	0·01–0·20	0	0–0·23
Nerolidol	15	3	0	0	1	3	0·01–0·05	0	0·002–0·007
Phytol	20	1	0	0	1	1	0·01–0·05	0	0
Geranyl geraniol	20	4	0	0	1	1	0·01–0·05	0–0·01	0–0·07
Laidlaw B carotenol	40	(11)	(8)	0	2	?	0·01	0·66	0·96
							0·02	1·00	0·98
							0·03	0·71	4·56
							0·04	1·05	10·30
							0·05	2·55	4·90
							0·10	1·84	0·21
							0·20	0	0
Lutein	40	11	10	2	2	3,3′	0·01	0·16	0·10
							0·05	2·82	0·08
							0·10	0·44	20·7
							0·20	0·05	30·7
Chloroxanthin	40	12	9	0	1	2	0·01–0·20	0·002–0·02	0·01–0·04
Sarcinaxanthin	40	12	9	0	2	3,3′	0·01	0·10	0·02
							0·05	0·55	0·22
							0·10	0·55	0·88
							0·20	0·20	1·10
Rubixanthin	40	12	11	1	1	3	0·01–0·20	0·001–0·03	0–0·24
Solanesol	45	9	0	0	1	1	0·001–0·02	0–0·10	0–0·54
Dolichol	100	19	0	0	1	1	0·01–0·05	0·02	0·01

*A = carbon chain length; B = no. double bonds; C = no. conjugated double bonds; D = no. aliphatic rings; E = no. hydroxyl groups; F = position of hydroxyl group(s).

† = Colony-forming units/ml., with test substance:colony-forming units/ml., with cholesterol control.

of avian strain J grown in the presence of the 'Laidlaw B' lipid (from Laidlaw B organisms harvested from 30 l. of culture) were harvested, extracted and saponified by the procedures of Rothblat & Smith (1961). Spectrophotometric examination of the unresolved lipid showed absorption maxima at 440, 415, 395 mμ similar to those (446, 422, 402) of the carotenol of *M. laidlawii* (Smith, 1963*a*). The shift to lower wavelengths probably indicates some degree of trans → cis isomerization. Thin layer chromatography on Silica Gel G with 5% (v/v) methanol in light petroleum as developing solvent yielded spots compatible with those found in the 'Laidlaw B'

lipid, i.e. for distance of flow of 13 cm. colourless spots at R_F values 0·5 to 0. Analyses for cholesterol indicated a concentration of less than 0·2% dry wt. at compared with 8% in cholesterol-grown organisms.

DISCUSSION

Two objections to these results can be raised, namely, that the carotenoids used were contaminated with other lipids which satisfied the sterol requirement for the mycoplasmas, and that the carotenoids underwent chemical change during the incubation period. With regard to the first point, the carotenoids used were isolated by known procedures for the specific compounds in question, produced only one spot on thin layer chromatograms, and exhibited absorption spectra typical of the pure compounds. Furthermore, no 3-β-hydroxy sterol was detected in the preparations. It is highly probable that carotenoids are the active factors since: (1) only 3-β-hydroxy planar sterols satisfied the requirement for unsaponifiable lipid; (2) the major if not the only compound added in the carotenoid preparations was carotenoid; (3) the concentrations which gave optimal growth responses were similar to the optimal concentration of sterol. Caution was exercised to reduce chemical changes of the carotenoids during isolation and use by maintenance of a nitrogen atmosphere where feasible, elimination of light and use of deep static cultures deficient in oxygen. In spite of these precautions, changes did occur as evidenced by loss of colour of the Laidlaw B carotenoids during growth of avian strain J, and a spectral shift indicative of a *trans* → *cis* isomerization. Although some cholesterol was detected in the organisms after several transfers in a cholesterol-free culture medium, no growth occurred when neither carotenols nor sterols were added to the medium. This cholesterol undoubtedly represented carry-over from the large inoculum (10%, v/v) used in growing the larger batch. It is probable that the carry-over of cholesterol was considerably less in the growth experiments in which a much smaller inoculum (0·5%, v/v) was used.

The present work has shown that certain precursors of carotenoids and C_{40} dihydroxyl compounds supported growth of *Mycoplasma* sp. avian strain J. Only C_{40} dihydroxyl compounds supported growth of *M. hominis* strain 07. The data indicate that *M. hominis* lacks all enzymes in the biosynthetic pathway of carotenoids beginning with mevalonic acid kinase. This organism also lacks β-hydroxy-β-methyl-glutaryl coenzyme A condensing enzyme and β-hydroxy-β-methyl-glutaryl coenzyme A reductase, while avian strain J possesses all enzymes necessary for the synthesis of mevalonic acid (Smith & Henrikson, 1965*a*). Radioisotopic data demonstrated an enzymic block between mevalonic acid and isopentenyl pyrophosphate in avian strain J (Henrikson & Smith, 1965). The present data confirm such an enzymic block since the phosphorylated mevalonic acids failed to substitute as the sterol source whereas isopentenyl pyrophosphate produced a significant growth response. The negligible response to geranyl pyrophosphate and farnesyl pyrophosphate could be attributed to a lack of permeability due not only to their phosphorylated nature but also to increasing carbon chain length. All intermediates in the form of free alcohols also produced negligible responses. These findings would be expected, for phosphorylation occurs only at the level of mevalonic acid.

The growth response of *Mycoplasma* sp. avian strain J to phytoene, phytofluene,

ζ-carotene and neurosporene indicated that enzymes probably were present for the synthesis of dihydroxyl C_{40} compound(s). The present work does not show whether another carotenol is synthesized from these hydrocarbons, or whether each of the C_{40} hydrocarbons merely is hydroxylated. That lycopene and β-carotene did not support growth might be attributed to the lack of hydroxylating enzymes for these carotenoids which occur later in the biosynthetic pathway in some organisms.

The negligible growth response of both Mycoplasma strains with rubixanthin and chloroxanthin as compared with the enhanced growth of both organisms with lutein and sarcinaxanthin indicated that the presence of two hydroxyl groups in the 3,3′-position (Karrer & Jucker, 1950; Nakayama, 1958) is essential for growth. That 'Laidlaw B' carotenol supported growth is further proof that a dihydroxyl C_{40} compound is required, although the exact locations of the hydroxyl groups are not known. Cyclization and the number of conjugated double bonds are not specific requirements.

Smith (1963*b*) proposed that the functional role of sterols could be satisfied by dihydroxyl C_{40} compounds. Both sterol and carotenol are found almost exclusively in the mycoplasma cell membrane (Smith & Rothblat, 1962; Pollack, Razin, Pollack & Cleverdon, 1965). Only planar 3-hydroxy sterols containing an 8 or 9 carbon side chain or planar 3,3′-dihydroxy C_{40} polyterpenes support growth. Two differences in molecular configuration are the shorter overall length and the presence of a polar group only at one end of the sterol. However, if two cholestanol molecules were oriented so that the 3-hydroxyl groups were terminal and the hydrocarbon side chains overlapped, a 'dimer' of the molecular configuration and dimensions of a 3,3′-dihydroxy C_{40} polyterpene would be produced. By use of Drieding molecular models, the lengthwise dimensions of the overlapping cholestanol molecules is 29·6 Å and of an all *trans* dihydroxyl C_{40} polyterpene, 31·2 Å, exclusive of the hydroxyl groups. These dimensions are compatible with the width (35 Å) of the electron transparent layer of the unit membrane of mycoplasmas as determined in ultrathin sections (van Iterson & Ruys, 1960), thereby presenting the proper space for the lengthwise orientation of the molecules across the unit membrane in the model proposed by Davson & Danielli (1952). Although the molecular dimensions of particles comprising the cell membrane as envisioned in the corpuscular theory (Benson, 1964) are not known, the lipid structure conceivably could lie across a hydrophobic area of the protein (Green & Fleischer, 1963) with the hydroxyl groups oriented at the lateral surfaces. In either orientation, the hydroxyl groups would be available for enzymic reactions.

It has been reported that sterol-requiring mycoplasmas are able to grow underneath colonies of *Staphylococcus albus* and *Proteus vulgaris* in serum-free medium (Morton, Smith & Leberman, 1949). It is possible that growth occurs under these conditions because mycoplasmas are able to utilize carotenols produced by the bacteria in question. Carotenoids have been isolated from strains of *Staphylococcus aureus* by Sobin & Stahly (1943) (rubixanthin and Δ-carotene), by Chargaff (1933) (zeaxanthin) and by Suzue (1959). Colourless hydroxylated C_{40} polyterpenes probably also occur (Jensen, Cohen-Bazire, Nakayama & Stanier, 1958). Possibly carotenoids or analogous compounds capable of substituting for sterols also are present in *P. vulgaris*.

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The Structure and Infective Process of a *Pseudomonas aeruginosa* Bacteriophage Containing Ribonucleic Acid

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SUMMARY

The electron microscope shows that there are a number of different morphological types of bacteriophages which grow on *Pseudomonas aeruginosa*. Some are conventional ones with contractile or non-contractile tails, but the most interesting is a tail-less phage containing RNA. The structure of both conventional and RNA phages is described. It is shown that the RNA phage probably infects the cell via polar pili. Intracellular multiplication and lysis by the RNA phage is followed in thin sections of infected cells. In the early stages, the nuclear region is much reduced and dense granular areas appear. These subsequently differentiate into crystalline aggregates of virus particles; at the same time a large bulge, identical to that found associated with spheroplast formation, appears. The crystals continue to increase in size until the spheroplast ruptures and lysis occurs.

INTRODUCTION

The specific bacteriolytic agents of the genus *Pseudomonas* consist of several morphological types of bacteriophages and a number of bacteriocins. The natural habitats of *Pseudomonas aeruginosa* include the intestinal canal, water, sewage, pus and the human skin. The habitat of its bacteriophages will of course be the same, and it has been found that large numbers can be obtained from sewage. In the present paper, isolates from this source are illustrated and compared with phages of other species in the genus. *P. aeruginosa* phages are represented by four basic morphological types (Bradley, 1965*a*); those with contractile tails, those with long non-contractile tails, tail-less phages with small capsomeres and containing ribonucleic acid (RNA), and a rod-shaped or filamentous type (Takeya & Amako, 1966).

Pseudomonas aeruginosa, unlike other members of the genus, is mildly pathogenic to man and animals and has been well studied; its bacteriophages are therefore of special interest. In addition, a fertility factor, known as FP, has been demonstrated for it (Holloway & Jennings, 1958; Holloway & Fargie, 1960); it is thus of some importance to determine whether or not its RNA phages infect via male-specific pili as appears to be the case with those of *Escherichia coli* (Brinton, 1965; Edgell & Ginoza, 1965). With *E. coli* the pili are coded for by the F factor (Brinton, Gemski & Carnahan, 1964) and the RNA phages adsorb to them (Crawford & Gesteland, 1964; Bradley, 1964).

Since RNA phages have only been found for three genera of bacteria, *Escherichia*, *Pseudomonas* and *Caulobacter* (Schmidt & Stanier, 1965), and intracellular

multiplication has only been studied for the first of these, it is important to follow the infective process in the other two. It is by no means certain that the same cytological changes in infected cells will be observed in all three cases. For this reason a detailed electron-microscopic study has been made of the whole infective process of a Pseudomonas RNA phage from adsorption to lysis.

METHODS

Culturing media and methods. Oxoid nutrient broth was used for both plate and broth cultures, the former containing 2% (w/v) agar. Phages were grown by confluently lysing bacteria in double agar layer plates (Adams, 1959), the soft agar layer containing 1% (w/v) agar + 1% (w/v) peptone.

Isolation and purification of bacteriophages. Untreated sewage influent was plated by the double agar layer method with *Pseudomonas aeruginosa* strain 1. Each ml. of sewage contained between 100 and several thousands of phage particles so that discrete plaques were produced with 0·1 ml. to 0·01 ml. of sewage per plate. After incubation at 37° overnight, plaques of differing morphology were picked and suspended in 1·0 ml. broth. Subsequent purification was achieved by several cycles of plaque-picking. High-titre phage preparations were obtained from successive single plaque isolations by the confluent lysis of bacteria growing on double agar layer plates using 0·25 ml. plaque eluate per plate. The plates were extracted with 0·1 M-ammonium acetate solution (neutral) for electron microscopy and purified by alternate high- and low-speed centrifugation.

Sources of host bacteria and phages. Pseudomonas aeruginosa strain 1 and the RNA phage 7S were kindly supplied by Dr T. Feary. Strain c 10 was obtained from Dr L. Dickinson together with phage Pc. Other pseudomonas species were provided by Dr E. Billing. The pyocin indicator, *P. aeruginosa* strain 1050 was provided by Dr Y. Hamon (for addresses see Acknowledgements). Other phages were obtained from various sewage samples and labelled as follows. The prefixes PP, PB and PL denote the source locations Pangbourne (Berks), Basingstoke (Hants) and Linlithgow (West Lothian) respectively. The isolate PP 7, an RNA phage from Pangbourne, is the most important described here.

The isolation of phage-resistant bacteria. It was found that the RNA phages 7S and PP 7 easily contaminated the laboratory in a short time. While care was taken to only work with one at a time, the growth of other phages on the host *Pseudomonas aeruginosa* strain 1 was facilitated by the isolation of a mutant resistant to the RNA phages. The spot-test method was used: a loopful of RNA phage suspension was placed on the surface of a double agar layer plate of host bacterium, and resistant clones obtained from the resulting plaque. One of these was designated *P. aeruginosa* strain 1/7; though it was obtained with phage PP 7 it was also resistant to phage 7S (see Table 1).

Lytic activity tests. The various phages isolated on *Pseudomonas aeruginosa* strain 1 were tested against three pseudomonas species and other strains of *P. aeruginosa* for lytic activity using the spot-test as described above. In addition, the activity of phages on double agar layer plates of host bacterium (*P. aeruginosa* strain 1) containing 1 mg./ml. of ribonuclease (RNAse) in the top layer of agar was tested.

Acridine-orange staining. The type of nucleic acid in phage PP 7 was identified as single-stranded RNA (1-RNA) using a modified fluorescent staining procedure (Bradley, 1965*b*, 1966) based on that of Mayor & Hill (1961). A suspension of the phage was obtained by extracting confluently lysed soft agar plates with phosphate buffered saline (g./l.: Na_2HPO_4, 1·27; KH_2PO_4, 0·41; NaCl, 7·36; pH 7·2). This was partly purified by cycles of alternate high- and low-speed centrifugation and also treated with deoxyribonuclease (DNAse) and RNAse at concentrations of about 10 γ/ml. and 100 γ/ml. respectively for 1 hr at 37°. Droplets of phage suspension were dried on microscope slides, fixed in Carnoy's fluid for 5 min. and rinsed in absolute alcohol. After drying in a stream of warm air they were stained in 0·01 % acridine orange in modified McIlvaine's buffer (0·1 M-citric acid, 6·0 ml.; 0·15 M-Na_2HPO_4, 4·0 ml.; pH 3·8) for 5 min. They were treated with 0·15 M-Na_2HPO_4 (for 15 min. and finally viewed under 2570 Å ultraviolet light. A post-stain treatment was carried out by immersing the slides in solutions of molybdic or tartaric acids as described elsewhere (Bradley, 1966), the colours under u.v. also being noted.

Lysis of broth culture by phage PP 7. Thin sections of RNA phage-infected cells were obtained from a culture of *Pseudomonas aeruginosa* strain 1 as follows. A log-phase broth culture was diluted so that 250 ml. contained about $1{\cdot}5 \times 10^8$ bacteria/ml., 50 ml. of this was removed to serve as a control culture and the remaining 200 ml. were infected with 15 ml. of a phage suspension containing 1×10^{11} plaque-forming units (p.f.u.) per ml. This gave a multiplicity of infection of about 50:1, a sufficiently high value to ensure the nearly simultaneous infection of all the host cells. Approximately 15 ml. samples were removed for fixation, embedding and sectioning at 15, 35, 50 and 75 min. after infection. In addition, bacterial concentration was estimated by optical absorption at intervals for both cultures so that growth curves could be obtained.

Fixation, embedding and sectioning of bacteria. About 15 ml. of a culture of bacteria previously infected with phage PP 7 as above was centrifuged at 7000 *g* for 3 min. and the pellet resuspended in 5 ml. of 6·25 % (w/v) glutaraldehyde in Sørensen's buffer at pH 7·2 (Sabatini, Bensch & Barrnett, 1963). After 1¼ hr at room temperature the bacteria were washed twice by centrifugation in buffer. The pellet was resuspended in 1 % (w/v) osmic acid in buffer (Sørensen's, pH 7·2) and post fixed at room temperature for 1½ hr. It was found that, if required, the bacteria could be stored overnight at this stage at 2° in buffer; no ill effects were observed in the final sections. The bacteria were again centrifuged into pellets at 7000 *g*; these were sufficiently hard to be passed through dehydration, etc., using a wire loop without breaking.

The pellets were then dehydrated for 20 min. in each concentration of 30 %, 50 %, 75 % and 100 % acetone; the first three solutions contained 1 % (w/v) uranyl acetate for staining. Two further treatments for ½ hr and 1 hr in 100 % acetone were then carried out, the acetone having been dehydrated overnight with anhydrous calcium chloride. The pellets were next transferred to a 50 + 50 (v/v) mixture of acetone + Vestopal in a shallow dish. After about 2 hr at room temperature, a stream of warm air was passed over the dish from a hair drier for another 2 hr to ensure that all the acetone had evaporated. The pellets were next transferred to 100 % Vestopal containing 1 % (v/v) each of initiator and accelerator, where they remained overnight at room temperature. The following morning they were transferred to fresh

Vestopal mixture for 7 hr. The pellets were finally broken into two or three pieces and transferred to gelatine capsules containing Vestopal mixture; these were baked with the lids off at 60° until hard (18–36 hr). Sections were cut on a Huxley ultramicrotome with a diamond knife; they were mounted on grids unsupported, and then stained for 2–5 min. in lead citrate (Reynolds, 1963) before examination in the electron microscope.

Negative staining for electron microscopy. Phage suspensions prepared by extracting confluently lysed plates with neutral 0·1 M-ammonium acetate were mixed with an equal volume of neutral potassium phosphotungstate (2%, w/v) solution. A carbon-coated grid was touched on to the surface of the mixture and dried under a lamp after removing excess liquid by touching the edge with a filter paper.

Preparation and use of anti-7s serum. Anti-serum for phage 7s was prepared in order to determine the serological relationship between phages 7s and PP 7. A rabbit was inoculated intravenously with about 5×10^{11} p.f.u. phage 7s in 0·5 ml. phosphate buffered saline three times over a period of 1 month. About 1 week after the last inoculation 10 ml. of blood was removed by superficial venesection of the ear. The serum was prepared in the normal way and complement removed by heating at 56° for ½ hr. About 5×10^5 p.f.u. of phage were then added to 10 ml. broth containing 0·1% (v/v) anti-serum. The mixture was incubated with shaking at 37° and the phage particles counted at intervals over 1 hr. A graph was then plotted of percentage survivors (on a log scale) against time, for the two phages in question.

RESULTS

Phages with contractile tails

Plate 1, figs. 1, 4 illustrate the octahedral shape of the head of phage BP 1, which is 750 Å in size. The tail, which is 1500 Å long, has a fine subunit structure, the organization of which is not clear. The lower portions of the tails of phage PP 1 in Pl. 1, figs. 2, 3 appear to be thickened, and in Pl. 1, fig. 4 this can be seen to be due to fibres folded up against the sheath for the last 600 Å of its length. Plate 1, fig. 5 shows a PP 1 virion without a head but with a contracted tail sheath. The top of the tail has a double disc arrangement similar to that found in coliphage ZG3A (Bradley, 1963), a *Pseudomonas syringae* phage (Matthews & Bradley, 1964), and a bacillus killer particle (Bradley, 1965*c*). In Pl. 1, fig. 6, two PB 1 virions can be seen adsorbed to a piece of debris. Their sheaths are contracted and the tail fibres are just visible, no longer folded back. A change in subunit packing has taken place in the sheath on contraction since the fine structure of Pl. 1, fig. 4 has been replaced by parallel longitudinal striations. This sort of change has been observed in other phages of similar morphology (Bradley, 1963).

Phages with non-contractile tails

This morphological group is divided into two: phages with regular heads and phages with elongated heads. Two of the former type are shown here, differing only in the structure of the tail tip. Phage Pc (Pl. 1, figs. 9, 10) has a distinct knob on the end of the tail. This can be seen to have a hole in it in the case of an empty virion (Pl. 1, fig. 9), presumably where the tail core passes through. With a full virion, the knob, as well as the tail, is solid, doubtless filled with a strand of nucleic acid.

This phage has been described in detail elsewhere (Bradley & Kay, 1960) but is included here for comparison with the rather similar phage PP 4. Again the head has a regular hexagonal outline and is probably octahedral (Pl. 1, fig. 7). The tail, however, is quite different, the tip resembling a cross (Pl. 1, fig. 8). It appears to be identical to the *Pseudomonas syringae* phage PS 4 (Matthews & Bradley, 1964). A complete virion is shown in Pl. 2, fig. 11. The diameter of the head is 600 Å and the tail is 1950 Å long. Phage Pc has a head of similar dimensions but the tail is rather shorter (1650 Å).

The phage with a non-contractile tail and an elongated head (isolate PB 2) is shown in Pl. 2, fig. 12. The head is 1000 Å long and 700 Å wide with an appearance very similar to that of the T-even coliphages. The tail is similar to that of phage PP 4 though shorter (1750 Å); it exhibits a helical structure (Pl. 2, fig. 12, top left particle and inset) and like PP 4 has a crossed tip. In many respects it is similar to the coliphage ZG3A (Bradley, 1963).

The RNA phages

Because of the importance of the RNA *Pseudomonas aeruginosa* phages both the structure and the infective process of one of these (PP 7) have been studied in detail.

Structure. In Pl. 2, fig. 13, virions of the isolate PP 7 are illustrated in a micrograph which is close to focus and so has rather low contrast. They have the characteristic transparent appearance of other RNA phages specific to *Escherichia coli* and caulobacter species with a regular hexagonal outline. Apart from this, no other details can be seen. In Pl. 3, fig. 16, 17 the electron microscope was slightly underfocused to increase contrast, and some fine detail can be discerned. In Pl. 3, fig. 16, a number of particles show a dark spot on their surface suggesting a pore (two examples are arrowed *P*). One virion also has a number of distinct white spots on it (arrowed *C*), probably representing capsomeres. These are clearer along an edge of a virion in Pl. 3, fig. 17 (arrowed *C*); here they have a centre-to-centre spacing of 30 Å. An empty virion can be seen on the right in this figure. The size of phage PP 7 is about 250 Å.

Adsorption. Plate 3, fig. 14 shows a cell of *Pseudomonas aeruginosa* heavily infected with phage PP 7. It can be seen that the virions are lying in dense aggregates extending from one pole of the bacterium, and also that one or two of them have adhered to the cell surface. A close examination at higher magnification (Pl. 3, fig. 15) reveals that the aggregates are interlaced with very fine strands, which in fact arise from the bacterium. Plate 2, fig. 13 and Pl. 3, fig. 17 show that there is undoubted adsorption to the surface of these filaments, and that they cause the aggregates. Little structure can be seen in the filaments themselves; a portion to the right of the arrow in Pl. 3, fig. 17 appears to be hollow but there is no helical structure visible. The diameter of the strands is about 45 Å. The micrographs thus show two kinds of adsorption: the majority of phages adhere to the filaments and a very small minority to the cell surface. The cell illustrated looks rather transparent for a healthy bacterium and has probably undergone cytological changes associated with phage infection.

Intracellular multiplication. A broth culture infected at a multiplicity of infection of about 50:1 was used to prepare the sections illustrated. As stated in Methods above, cell concentration estimates were taken of this culture so that suitable

sampling times could be determined. These estimates, together with those of the control culture, were plotted against time and the curves obtained are shown in Fig. 1. The sampling positions on the curve are marked C, D, E, F (A and B being control samples of an uninfected culture); these letters correspond to those on the blocks from which the sections were cut (see explanation of plates).

The electron micrographs of sections taken from the blocks designated C, D, E and F on the curve in Fig. 1 follow the morphological changes during the intracellular multiplication of the RNA phage. In Pl. 4, fig. 18 an uninfected cell of *Pseudomonas aeruginosa* strain 1 is seen to have well dispersed nuclear material (nucleoplasm), the DNA fibres being barely visible in this micrograph. There are also numerous dark particles which may be ribosomes. In sample C, only 15 min. after infection, definite changes can be seen (Pl. 4, figs. 19, 20). Dense areas (marked *P*) appear at the poles of the cell, and also at the centre. This has the effect of reducing the area occupied by the nucleoplasm most markedly (see Pl. 4, fig. 20). The latter micrograph also shows a mesosome (arrowed *M*; Fitz-James, 1960; Salton & Chapman, 1962; Vanderwinkel & Murray, 1962). The appearance of sections of cells from block C was noticeably uniform but cells from the remaining blocks showed differences between individual bacteria as would be expected if each had reached a different stage in infection.

Plate 5, fig. 21, which was obtained from block F, shows the appearance of an infected cell at a later stage in the infective cycle than that in block C (see Fig. 1). The shape of the cell, which is typical of a spheroplast, will be discussed below; it is pointed out, however, that it appeared (from light-microscope observations) about $\frac{1}{2}$ hr after infection and increased in frequency thereafter up to lysis. In Pl. 5, fig. 21 the dense patches of undifferentiated viral RNA and protein can be seen at the periphery of the bulge, and in the ends of the two undistorted lengths of cell, often called 'rabbit's ears', and also where these protrusions join. A patch of material can be seen to possess organization in Pl. 5, fig. 22. The plane of this section lies lengthwise through one of the 'ears' and the bulge. The periodicity visible in the phage material is small (about 80 Å) compared with the size of the intact virion. The cell wall shows indications of a break at the top of the bulge.

Many cells in block D had crystalline inclusions of virus similar to those found in *Escherichia coli* by Schwartz & Zinder (1963) though generally larger. In Pl. 5, fig. 23 the cell is beginning to lyse and the phage particles are just breaking away from the right of the crystal. In Pl. 5, fig. 24 a higher magnification shows two different orientations in a crystal, perhaps analogous to twins in metals. In Pl. 6, fig. 25 the cell has not lysed so that the bulge is intact. Two 'ears' can be seen, one tapering away from the plane of the section and one completely filled with a crystal of virions. There are some 30 rows of them, each containing about 40 phage particles making a total of 1200 in this section. In Pl. 7, fig. 26 the earliest stage of crystal formation can be seen; a few spheres are beginning to line up against the cell wall.

Lysis. The moment of lysis is shown in Pl. 7, figs. 27, 28. It is emphasized that the plane of the section is passing through only one 'ear' in Pl. 7, fig. 27; a section through the other 'ear' would probably look similar. A small crystal is present in the 'ear', and a number of virions have passed from a second, now disorganized crystal into the broken bulge. Together with cytoplasmic debris and nucleic acid they are passing through the massive rupture in the cell wall. Many sections have

shown that this is typical and that lysis is achieved by the breakdown of the wall of the spheroplast. In Pl. 7, fig. 28 the same stage is shown in transverse section through the bulge. There are two disorganized crystals, presumably one from each 'ear'. It can be seen here and in other sections, that the spheroplast wall is apparently complete with its various layers and that lysis is caused by fracture rather than general disintegration.

Lytic activity of Pseudomonas aeruginosa phages

Table 1 shows the results of lytic activity tests described above. It can be seen that the RNA phages lyse only *Pseudomonas aeruginosa* strain 1, but other phages have a wider range of activity. In addition lytic activity by the RNA phages on their host is inhibited by the presence of RNAse in the top layer of agar in the double layer plates used for the test. This suggests that both phages contain 1-RNA.

Table 1. *Lytic activity of Pseudomonas aeruginosa phages*

	Bacteriophages							
	Contractile		Non-contractile				RNA	
Host	PP 1	PB 1	PP 4	Pc	PB 2	PL 2	7S	PP 7
P. aeruginosa, 1	+	+	+	+	+	+	+	+
P. aeruginosa, 1 with RNAse	+	+	+	+	+	+	–	–
P. aeruginosa, 1/7	+	+	+	–	+	–	–	–
P. aeruginosa, c10	+	+	–	+	+	+	–	–
P. aeruginosa, 1050	–	–	–	–	–	–	–	–
P. syringae, (NCPPB 1072)	–	+	–	–	(+)	+	–	–
P. fluorescens, (NCTC 10038)	–	–	–	–	–	–	–	–
P. phaseolicola, (NCPPB 52)	–	+	(+)	–	+	+	–	–

NOTE. A + signifies strong phage activity (a clear area), (+) weak activity (a veiled area), and – no activity.

Table 2. *Acridine-orange staining of phages of 7S and PP7*

		Colours		
Phage	Nucleic acid	After Na_2HPO_4	After molybdic acid	After tartaric acid
T 4	2-DNA	Bright green	Bright green	Bright red
WTV RNA	2-RNA	Bright green	Stayed green but faded out	Pale red
ZJ/2	1-DNA	Bright red	Pale green	Pale green
ZIK/1	1-RNA	Bright red	Pale red	Pale red
7 S	1-RNA	Bright red	Pale red	Bright red
PP 7	?	Bright red	Pale red	Bright red

NOTE. WTV stands for wound tumour virus. Coliphages ZJ/2 and ZIK/1 have been described by Bradley (1964).

Acridine-orange staining

The object of this test was to confirm the presence of 1-RNA in phage PP 7. The results are given in Table 2 with other representative types of nucleic acid for comparison. It can be seen that the colours agree with other phages known to contain 1-RNA.

Inactivation of phage PP 7 by anti-7S serum

The graph in Fig. 2 shows that there is a definite reaction between anti-7S serum and phage PP 7. The rate of inactivation is not, however, as great as with the homologous phage. There is therefore a definite serological relationship between phage 7S and phage PP 7, but they are not identical.

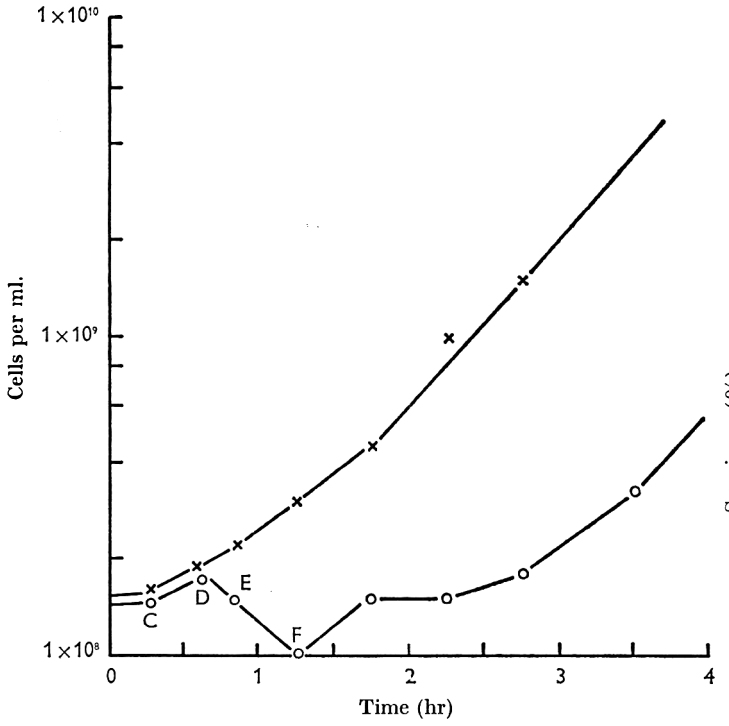

Fig. 1

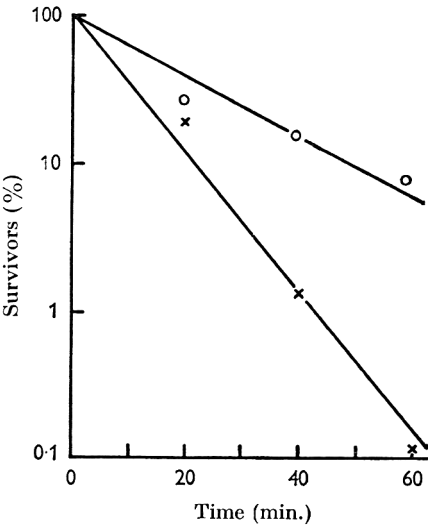

Fig. 2

Fig. 1. Rate of bacterial growth in non-infected (×—×) and PP 7-infected (O—O) broth cultures of *Pseudomonas aeruginosa* strain 1.

Fig. 2. Rate of inactivation by anti-7S serum (dilution 1000:1) of phages 7S (×—×) and PP 7 (O—O).

DISCUSSION

Tailed phages

The tailed phages of *Pseudomonas aeruginosa* are similar in appearance to those associated with other genera of bacteria, the form with contractile tail and octahedral head being particularly common especially amongst coliphages. The electron

micrographs shown here have, however, provided a useful observation. The coliphage E1 (one of the common forms mentioned), which is almost identical with phages PP 1 and PB 1, has four tail fibres (Bradley, 1963). These can usually be seen splayed out at the tip of the sheath. Other contractile types seemed to lack these fibres, but it is shown here that they are in fact folded up against the sheath. On adsorption they appear to splay out (Pl. 1, fig. 6). It would seem that they play a role in adsorption as is the case with the T-even coliphage tail fibres (Kellenberger *et al.* 1965); with T4 they form a network round the sheath and are attached by a reversible bond to the collar under conditions when adsorption is not possible; the bond breaks under favourable circumstances and the fibres are released and assist attachment in some way (see also Bradley, 1963). It seems highly probable from observations shown here that the tail fibres of other contractile phages have a similar function. The non-contractile types have only one unique feature: their tail tip. This cross-like form is common amongst phages specific to other pseudomonas species. The non-contractile phages with regular hexagonal heads seem to be octahedral.

Comparatively few *Pseudomonas aeruginosa* phages have been described in the literature but three examples are given by Slayter, Holloway & Hall (1964). Phage B3 is similar to phage PP 4 and phage E79 to PP 1 and PB 1 save for slight dimensional differences. The third phage they described (F 116) had an octahedral head with an unstable non-contractile tail; this form has not been found in any sewage samples to far. In addition to these isolates, Takeya & Amako (1966) have described a filamentous type similar in many respects to those specific for *Escherichia coli*. These authors give no indication as to how or where this phage was isolated.

RNA phages

Nucleic acid. The size (about 250 Å) and appearance of all 1-RNA phages so far isolated has been generally similar and they are readily recognizable in the electron microscope as small semi-transparent spheres. Nevertheless, once such a phage has been isolated, it is important to show that it does, in fact, contain 1-RNA. Apart from the morphology of the virion, the ability of RNAse to inhibit plaque formation (see Table 1) is valuable evidence in favour of the nucleic acid being 1-RNA. In spite of this apparent certainty, it is obviously desirable to have a further confirmatory test, and acridine-orange staining (Mayor & Hill, 1961; Bradley, 1965*b*; Bradley, 1966) provides this. The results given here: morphology, the effect of RNAse, and acridine-orange staining, confirm without doubt that PP 7 contains 1-RNA.

Structure. As has been stated, micrographs of the RNA phages all look similar regardless of host genus. There is, however, one exception, namely 7 S. Feary, Fisher & Fisher (1964) show a micrograph illustrating a high degree of organization within the capsid. The present author has not found such organization in 7 S, PP 7 or indeed any other RNA phage. If the micrographs of Feary *et al.* (1964) are compared to those shown here, it becomes immediately obvious that the virions in the former are better preserved. This would mean that capsomere organization is not disturbed. Lack of preservation will probably be a function of the negative staining material, phosphotungstic acid being known to vary in efficiency according to source. Thus it may be worth while fixing RNA phages for negative staining, a practice not normally

used. In the present case, simple formaldehyde fixation was attempted but with no effect.

The micrographs of Feary *et al.* (1964) appear to show the structure produced by the faces of the capsid rather than individual capsomeres. This appearance is in agreement with an icosahedral form, which has been observed for the RNA coliphage ZIK/1 (Bradley, 1964). The latter had 92 capsomeres with a centre-to-centre spacing of about 45 Å; PP 7 has smaller ones spaced at 30 Å. They are more difficult to see, but being smaller will provide a sharper outline to the faces of the capsid, which perhaps accounts for the appearance of the pictures of Feary *et al.* (1964). A 30 Å spacing in an edge length of 180 Å on an icosahedron would make the total number of capsomeres 252 (6 per side). Since it is difficult to be accurate about the edge length, 5 capsomeres per side, making a total of 162, is another possibility. It is certainly evident from these observations that the RNA coliphages are not structurally identical with the pseudomonas ones. One feature which does seem to be shared is the pore. This is a point of asymmetry in the capsid, formed perhaps by a missing capsomere. In Pl. 3, fig. 17 the pore does not seem to be associated with adsorption to pili since some can be seen on virions attached to the filament.

Adsorption. There is little doubt that adsorption takes place to fine polar pili. Whether or not this is associated with infection is another question. In the case of coliphages all the evidence is in favour of this (Brinton, 1965; Edgell & Ginosa, 1965) and by analogy one can justifiably assume that the same is likely to be the case with pseudomonas phages. The appearance of the electron micrographs of Crawford & Gesteland (1964), Bradley (1964) and others is remarkably similar to those shown here. In a previous observation (Bradley, 1965*a*) no virions could be seen attached to the *Pseudomonas aeruginosa* cell wall. In that case the bacterium was not yet morphologically changed by the phage infective process and appeared typically dense in the electron micrograph. Here (Pl. 3, fig. 14), the bacterium is apparently damaged, a factor which might produce non-infective adsorption to the cell wall. Another explanation is that some pili, with virions attached, have folded back across the bacterium.

The pili themselves are of special interest. Similar ones have been demonstrated in *Escherichia coli* to be unique and to be associated with and coded for by the F (fertility) factor (Brinton *et al.* 1964; Brinton, 1965). They are thus called F-pili (Brinton, 1965; Valentine & Strand, 1965). Now an infectious fertility factor (FP) has been demonstrated for *Pseudomonas aeruginosa* (Holloway & Jennings, 1958; Holloway & Fargie, 1960): the pili observed here (Pl. 3, figs. 15–18) are probably the same, being adsorbed to by RNA phages. In fact it is the phage particles which have revealed their presence. Negative staining of uninfected cells does not reveal the pili; perhaps they stick to the cell surface or wind round the polar flagellum. The RNA phages thus have the effect of delineating them as is the case with *E. coli* F-pili (Brinton, 1965). The *P. aeruginosa* pili are rather thinner than those of *E. coli*: about 45 Å as opposed to 70 Å. They appear to be hollow in places and so could act as a tube for the transfer of infective RNA from the phages, in which case they should be called FP-pili. They also resemble the filamentous bacteriophages of *E. coli*. These viruses are so similar in appearance to *E. coli* F-pili that one cannot but wonder whether the latter evolved in some way into an infective viral form. The

recently discovered filamentous *P. aeruginosa* phage (Takeya & Amako, 1966) is thicker than the pili illustrated here.

Intracellular multiplication. While the fundamentals of intracellular multiplication appear to be similar to those encountered in *Escherichia coli* (Schwartz & Zinder, 1963), the formation of a spheroplast is quite unique.

The early stages of the infective cycle are marked by the appearance in characteristic positions in the cell of dark masses of material which subsequently crystallize into virions. These crystals seem rather bigger than those illustrated for *Escherichia coli*, particularly in Pl. 6, fig. 25. The number of particles present in this single slice (1200) reflects the huge burst size of the phage. If one assumes that the thickness of the section includes one layer of phages in a perpendicular plane (this would be about 300 Å) then the total number in the crystal would be about 30×1200, assuming a circular cross-section. A burst size of 36,000 is, if anything, rather bigger than that usually quoted for RNA coliphages.

Lysis. The apparent maintenance of the integrity of the cell wall until lysis strongly suggests that the rupture of the spheroplast is brought about by osmosis, the way in which normal spheroplasts lyse. This process is quite different from that associated with *Escherichia coli* with any phage: here the cessation of cell-wall synthesis together with the release of lysozyme causes lysis (Cota-Robles, 1964).

The rapid growth of resistant bacteria after the lysis of a broth culture (Fig. 1) shows that a fairly large proportion of the original bacterial population is resistant to infection; indeed, it is extremely easy to obtain resistant clones. The important question is whether or not resistance is due to the absence of pili. This can only be found out when a method is devised for detecting pili on uninfected cells.

The taxonomy of Pseudomonas aeruginosa phages

The morphological groups fit in with the scheme of classification proposed by Bradley (1965*a*), there being examples of contractile phages, phages with long non-contractile tails, tail-less phages with small capsomeres containing RNA, and a filamentous form containing 1-DNA (Takeya & Amako, 1966). It is interesting to note from Table 1 that the lytic activity of phages isolated on *Pseudomonas aeruginosa* extends to other species of the genus *Pseudomonas* save in the case of the RNA types.

Serological characteristics give a very fine distinction between phages. Thus the fact that there is a definite serological relationship between the two RNA phages 7 s and PP 7 indicates that they have only small chemical differences in the capsid. Similar relationships have been found for RNA coliphages (Bishop & Bradley, 1965; Scott, 1965).

CONCLUSION

The observations described here have revealed a number of fundamental features about *Pseudomonas aeruginosa* and its phages. Perhaps the most important is the presence of what are probably FP-pili. It is clear that much more should be learnt about these appendages to see if they perform the same functions as those of *Escherichia coli*, namely to act as a tube for the passage of DNA and the fertility factor in the conjugation process (Hayes, personal communication).

The second major feature is the association of spheroplast formation with lysis.

A study of lysis by other *Pseudomonas aeruginosa* phages, to see if the same process takes place, would be of particular interest. *Pseudomonas aeruginosa* is a sufficiently common and important organism to warrant more detailed study, and it is hoped that the present work will stimulate this.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Phage PB 1, × 165,000.

Figs. 2, 3. Phage PP 1, × 165,000.

Fig. 4. Phage PB 1, × 275,000.

Fig. 5. Headless phage PP 1, × 275,000.

Fig. 6. Phage PB 1 adsorbed to debris, × 275,000.

Fig. 7. Head of phage PP 4, × 275,000.

Fig. 8. Tail of phage PP 4, × 275,000.

Fig. 9. Empty virion of phage Pc, × 165,000.

Fig. 10. Intact virion of phage Pc, × 275,000.

PLATE 2

Fig. 11. Phage PP 4 intact, × 275,000.

Fig. 12. Phage PB 2 (inset tail of phage PL 2), × 165,000.

Fig. 13. Phage PP 7 particles adsorbed to pilus; near focus electron micrograph, × 275,000.

PLATE 3

Fig. 14. Phage PP 7 adsorbed to host cell, × 37,500.

Fig. 15. Detail of the same, × 75,000.

Figs. 16, 17. Phage PP 7 underfocused; *P* are pores and *C* capsomeres, × 275,000.

PLATE 4

Fig. 18. Section of an uninfected cell of *Pseudomonas aeruginosa*, strain 1, × 37,500.

Figs. 19, 20. Sections of PP 7-infected cells from sample C (Fig. 1), × 37,500.

PLATE 5

Fig. 21. Section of PP 7-infected cell of *P. aeruginosa*, strain 1 from sample F (Fig. 1), × 12,500

Figs. 22, 23. Sections of infected cells from sample D (Fig. 1), × 50,000.

Fig. 24. Crystal from sample D, × 83,000.

PLATE 6

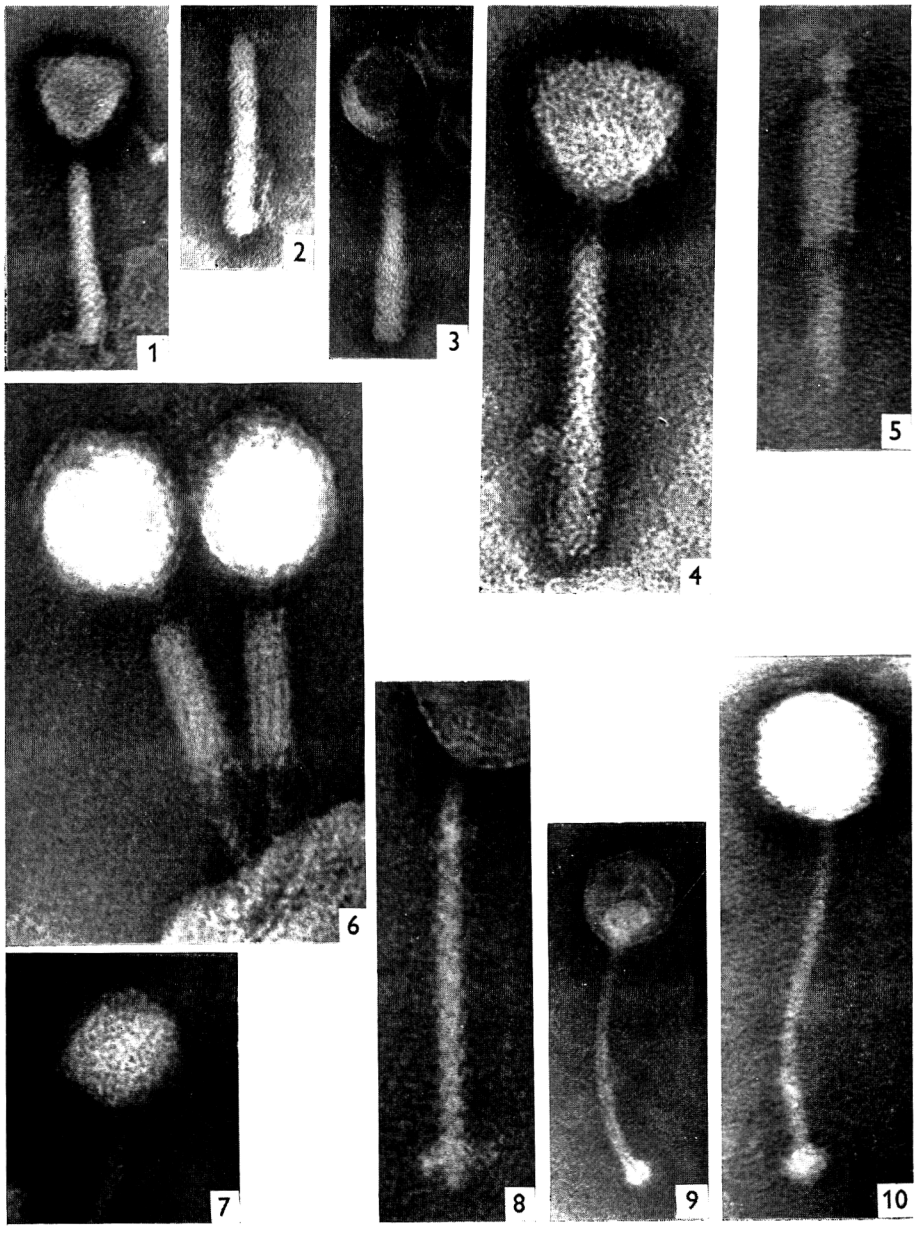
Fig. 25. Sectioned PP 7-infected cell of *P. aeruginosa* strain 1 from sample F, × 37,500.

PLATE 7

Fig. 26. Section of part of PP 7-infected cell from sample C (Fig. 1). × 83,000.

Fig. 27. Section of lysing cell from sample E, × 50,000.

Fig. 28. Section of lysing cell from sample D, × 37,500



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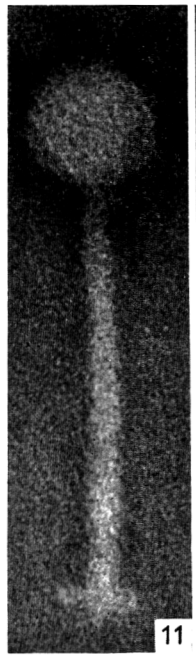
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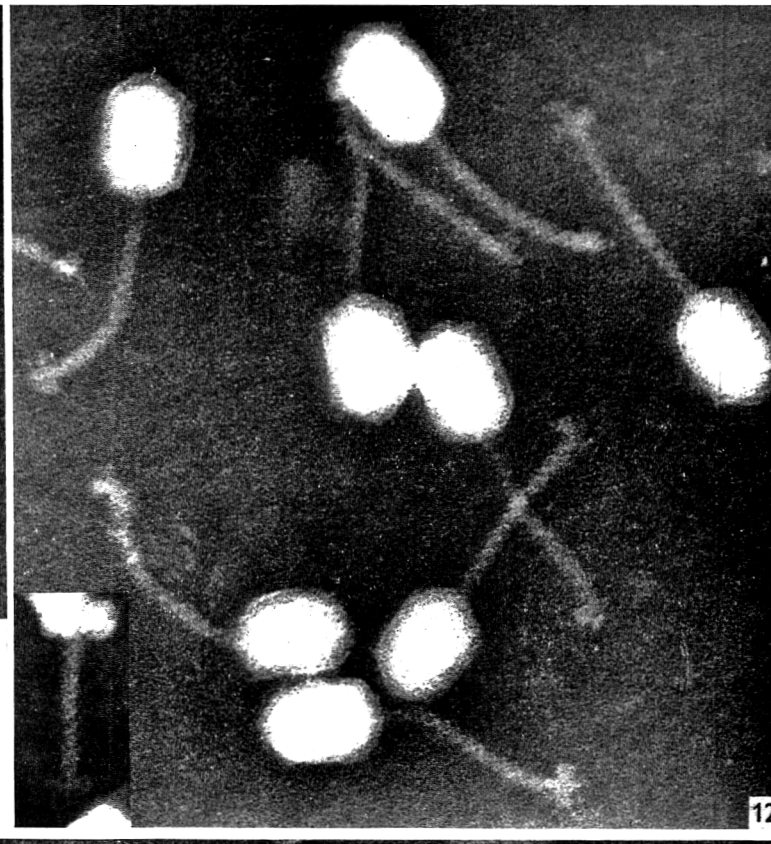
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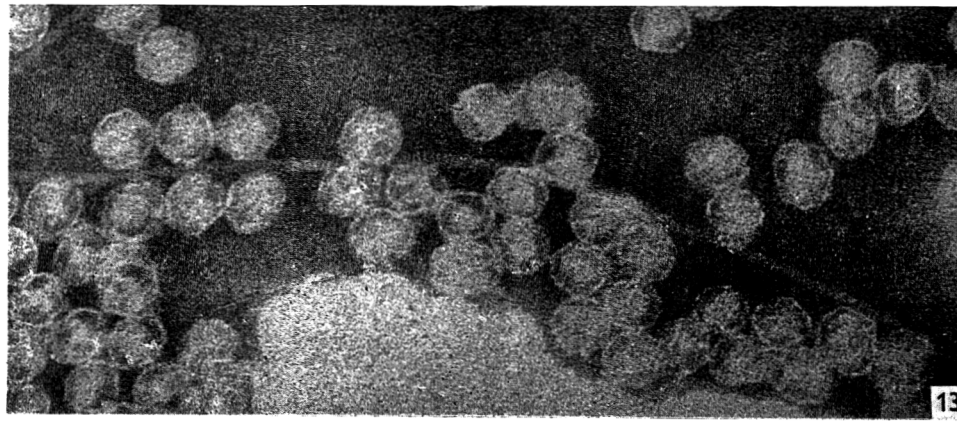
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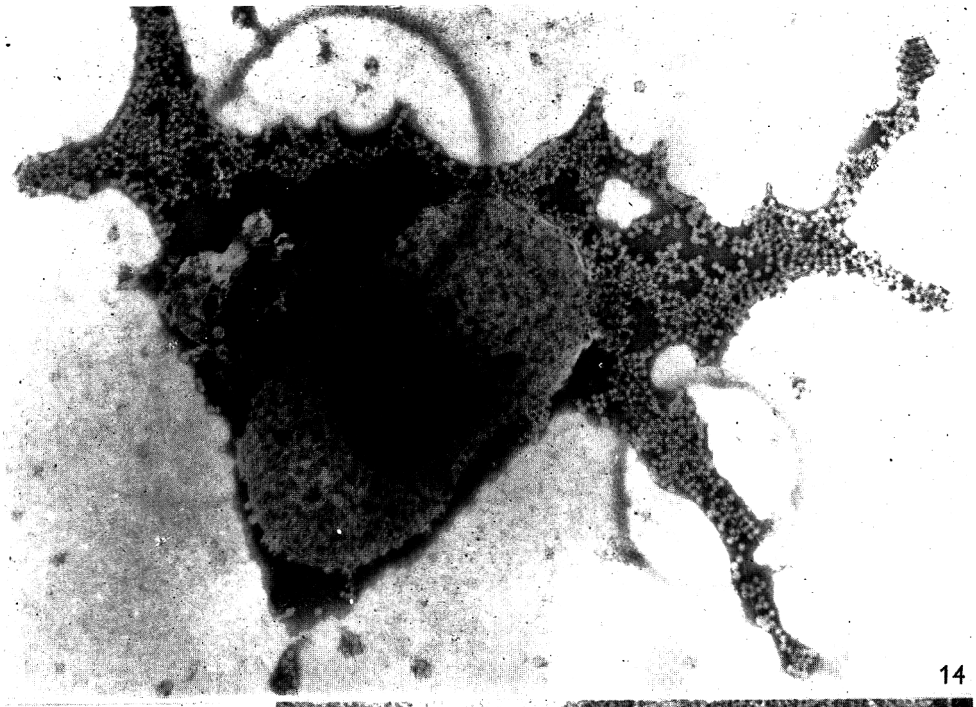
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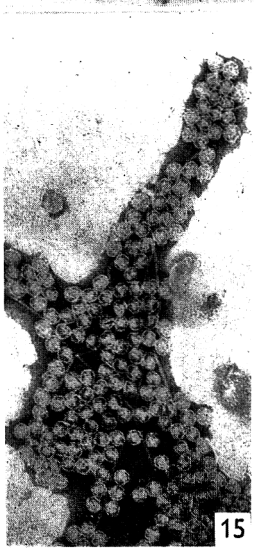
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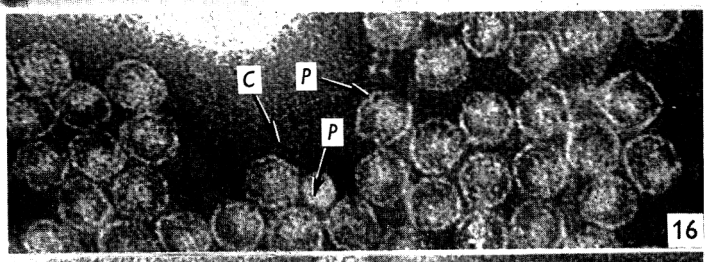
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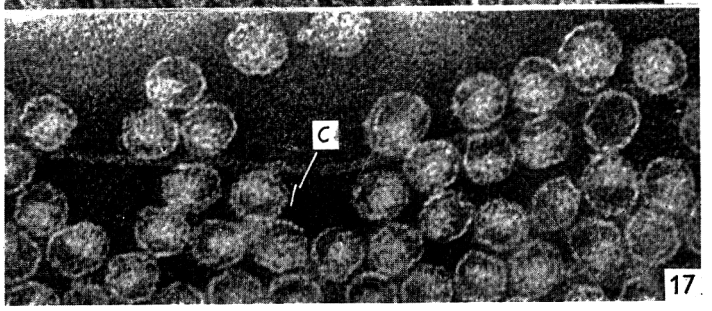
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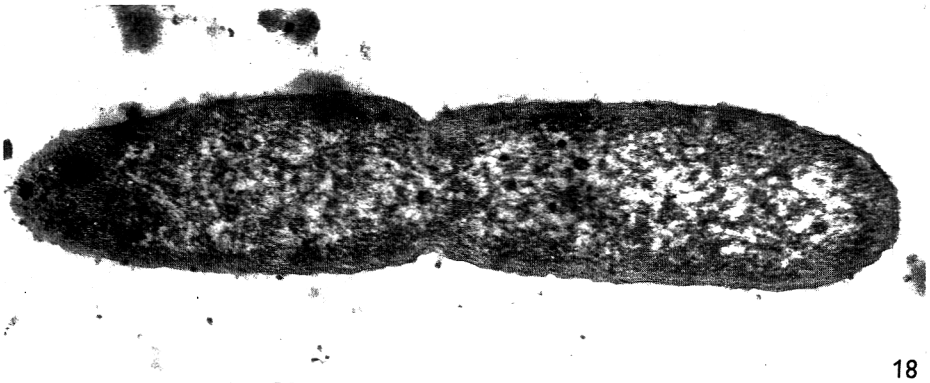

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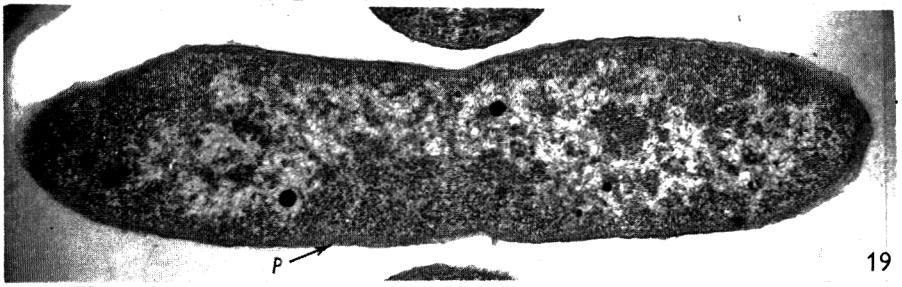

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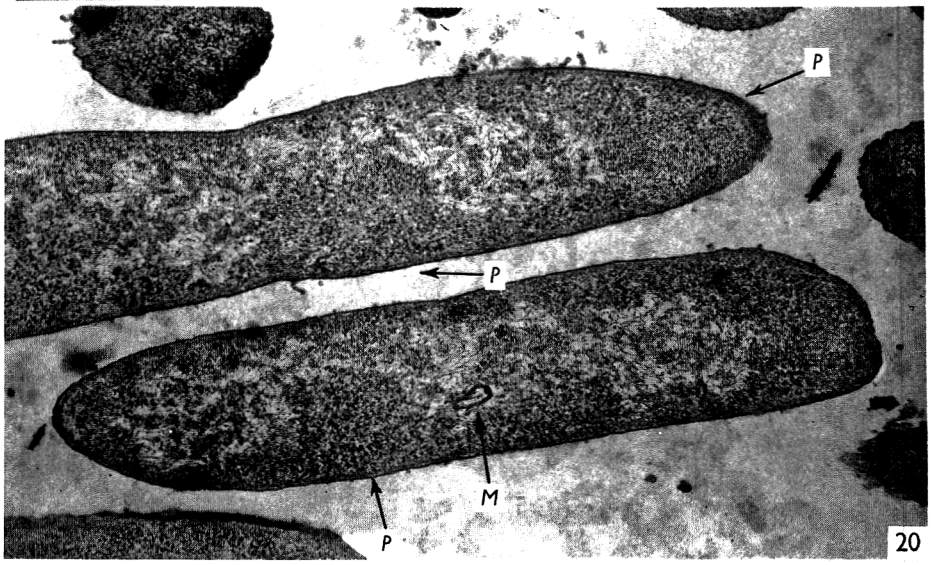

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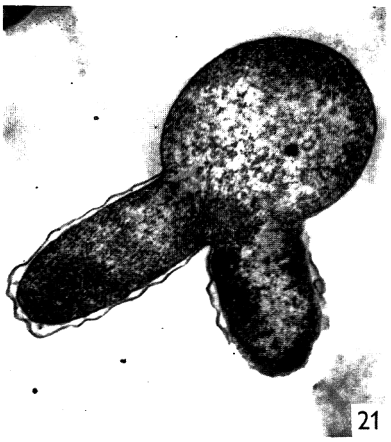
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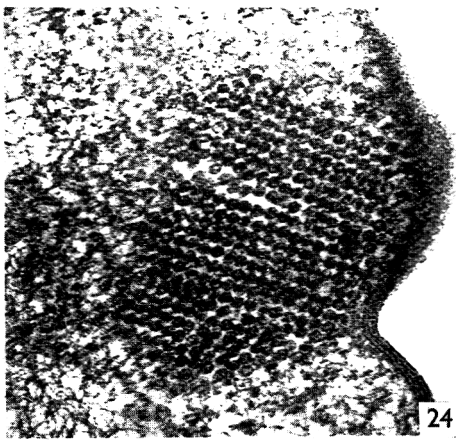
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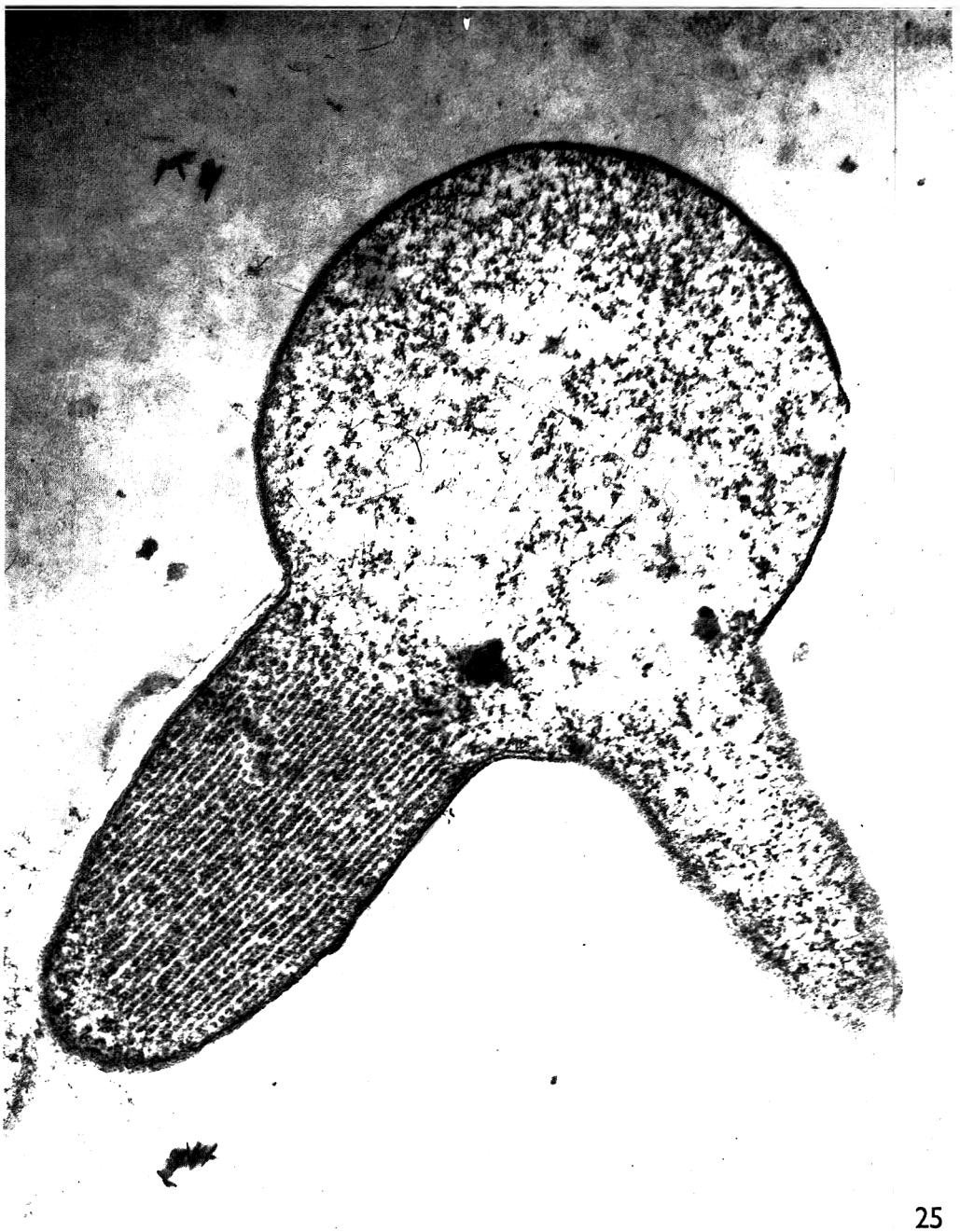

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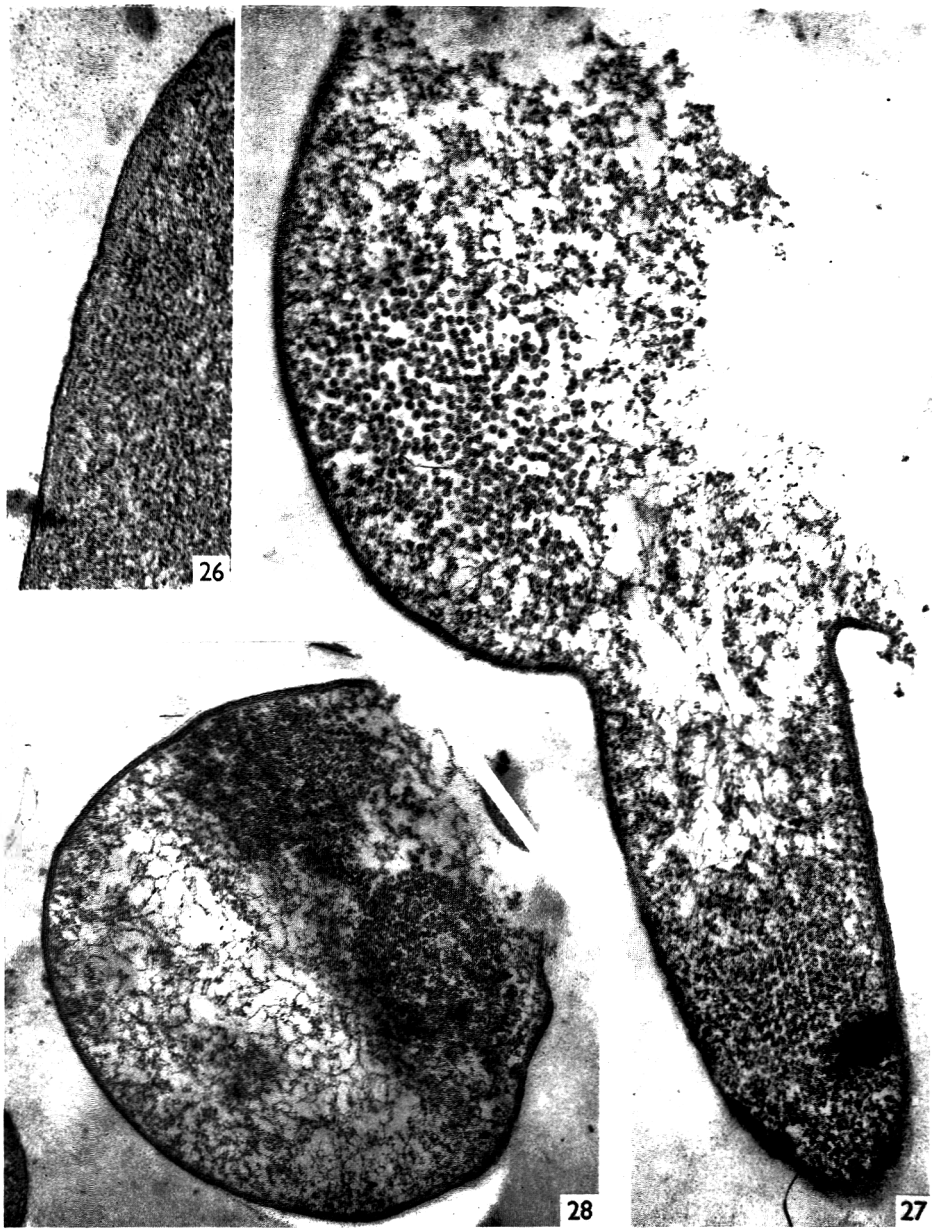
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Restoration of 5-Bromouracil-sensitized *Escherichia coli* Strain B after Exposure to Ionizing or Ultraviolet Radiation

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SUMMARY

Escherichia coli strain B was sensitized to the action of γ-radiation or ultraviolet (u.v.) radiation by incorporating 5-bromouracil into the DNA of the bacteria. Most sensitization was observed after u.v. irradiation, less after anoxic γ-irradiation and least after aerobic γ-irradiation. Incubation of the bacteria for a few hours after irradiation on a nutrient medium which included chloramphenicol generally resulted in extensive restoration of colony-forming ability whether or not the bacteria contained 5-bromouracil. Only after the aerobic γ-irradiation of bacteria containing no bromoruacil was little restoration obtained, After aerobic or anaerobic γ-irradiation the 'rescue' of bacteria containing 5-bromouracil was relatively larger than that observed for bacteria containing no bromouracil. Maximum restoration was obtained after u.v. irradiation and this occurred to about the same extent, whether or not bromouracil had been incorporated into the bacteria. The results suggest that treatment with chloramphenicol decreases the expression of radiation-induced lesions which occur in the bacterial DNA; this may account for the mechanism of action of the inhibitor.

INTRODUCTION

It is well known that the amount of lethal radiation damage which is expressed in bacteria is partly dependent on the manner in which the organisms are treated after irradiation. Treatments which modify radiation response do so, in many instances, by affecting the rate of metabolic processes in the irradiated organisms. With *Escherichia coli* strain B and some of the strains derived from it, the rate of protein synthesis is critical in determining whether or not a considerable proportion of the irradiated bacteria survive as colony formers. Thus, marked restoration of the colony-forming ability of *E. coli* B after exposure to ultraviolet (u.v.) or ionizing radiation can be effected by incubating the bacteria for the initial period after irradiation with chloramphenicol (Gillies & Alper, 1959), or by temporarily depriving irradiated auxotrophic strains of *E. coli* B of an essential amino acid (Gillies, 1961; Forage & Gillies, 1964). It is believed that this effect is due to the specific inhibition of protein synthesis. On the other hand, Alper (1963) showed that the survival of the radiation-resistant strain *E. coli* B/r is decreased when it is treated with chloramphenicol after irradiation. The expression of some types of mutational change in bacteria is also dependent on synthesis of protein after irradiation (Witkin, 1956; Doudney & Haas, 1958; Witkin & Theil, 1960) and in general, conditions

which slow or completely prevent protein synthesis lead to a decrease in the yield of mutants.

In general, conditions which prevent growth or decrease the rate of growth of *Escherichia coli* B cause restoration of colony-forming ability of a fraction of the irradiated population (Alper & Gillies, 1958, 1060) and in broad terms an explanation of the action of chloramphenicol in promoting this restoration can be offered. However, nothing is known about the mechanism by which damage, which would otherwise have developed irreversibly, is removed during a period of arrest of protein synthesis immediately after irradiation. The solution of this problem is hampered by lack of knowledge of the nature of the critical lesions caused by radiations in cells, but there is at least one type of damage induced by u.v. and γ-radiation which appears to be identifiable with a site or sites in the DNA of cells. This is the additional lesion, first detected by Greer (1960) in u.v. irradiated *E. coli* 15T, which shows up when a fraction of the thymine of the cellular DNA is replaced by the halogenated derivative of thymine, 5-bromouracil (5-BU). It therefore appeared worth while to determine whether or not bacteria which had been sensitized to either u.v. or ionizing radiation by the presence of 5-BU in their DNA could be restored by incubation with chloramphenicol after irradiation. By this approach information seemed likely to be gained about the mode of action of chloramphenicol in restoring the colony-forming ability of irradiated bacteria, and possibly also on the mechanism of the sensitization brought about by 5-BU.

METHODS

Organism. The strain B of *Escherichia coli* was the same as that used in previous work (Alper & Gillies, 1958, 1960; Gillies & Alper, 1959). It was maintained on slopes of Oxoid nutrient agar stored at 4°.

Growth and preparation of bacteria for irradiation. Bacteria required for experiment were first grown in a minimal medium by subculturing into an inorganic salts glucose medium (Lederberg, 1950) followed by overnight incubation at 37° for 15 hr. Incorporation of 5-bromouracil (5-BU) into the bacteria was effected by inoculating about 4×10^7 organisms from the overnight culture into 20 ml. of a defined medium containing 5-BU in a 100 ml. bottle which was rotated on an angled turntable at 37° for 6 hr. In this way adequate aeration of the culture was obtained; this was essential for rapid growth and for measurable radiation-sensitization of the bacteria to occur. The concentration of the suspension at the end of the incubation period was about 5×10^7 bacteria/ml. The composition of the defined growth medium is shown in Table 1; it is based upon the medium used by Kaplan, Smith & Tomlin (1962). It contained 5-BU (75 μg./ml.) and also sulphanilamide (0·2%) to inhibit the action of folic acid which is required for *de novo* synthesis of thymidylic acid. The control population of bacteria which did not contain 5-BU was grown at the same time in the same medium except that thymine (75 μg./ml.) replaced the 5-BU. The bacteria were harvested by centrifugation, washed twice in M/15-phosphate buffer (pH 7) and finally resuspended for irradiation in buffer at a concentration of about 6×10^6 bacteria/ml.

Irradiation of bacteria

With γ-rays. Bacteria were exposed to radiation from a ^{60}Co source (Vickers-Armstrong, Mark IV Hotspot Irradiation Unit) in the vessel described previously (Forage & Gillies, 1964). Either oxygen or oxygen-free nitrogen ('white spot') was bubbled through the suspension as required. The dose rate delivered in the vessel, as measured by ferrous sulphate dosimetry (Miller & Wilkinson, 1952), was 2·2 krad./min. Irradiations were performed at room temperature.

Table 1. *Composition of the defined sulphanilamide medium in which Escherichia coli* B *was grown before irradiation*

Sulphanilamide was used to inhibit the endogenous synthesis of thymidylic acid. 5-bromouracil (5-BU) or thymine was added as required. Adjusted to pH 7·3.

Constituent	% (w/v)	Constituent	% (w/v)
K_2HPO_4	0·7	Xanthine	0·003
KH_2PO_4	0·2	Pyridoxine	0·0001
Na citrate.$2H_2O$	0·004	Thiamine	0·0001
$MgSO_4.7H_2O$	0·001	Glucose	0·3
$(NH_4)_2SO_4$	0·01	Sulphanilamide	0·2
DL-methionine	0·003	Thymine or 5-bromouracil	0·0075
DL-serine	0·003		
DL-valine	0·003		
L-histidine	0·003		

With X-rays. In a few experiments X-rays (190 kV., 3 mA.) from a General Electric X-ray Corporation KX-10 Unit were used. The bacteria were irradiated under controlled gas conditions in the type of vessel described by Alper (1955). The dose rate of unfiltered radiation delivered to the suspension in the vessel was 0·4 krad./min.

With ultraviolet radiation. Ten ml. of the bacterial suspension was placed in an open glass dish (diam. 8·5 cm.) and exposed, at room temperature, to u.v. radiation from a 15 W. Hanovia water-jacketed bactericidal tube which delivered more than 90% of its radiation at 2537 Å. The dish was gently shaken during exposure. The dose rate was 155 ergs/mm.^{2}/min., as measured with a u.v. sensitive photometer originally calibrated by Dr R. Latarjet.

Treatment of bacteria after irradiation

To determine the effect of temporarily treating bacteria containing thymine (thymine bacteria) and bacteria containing 5-BU (5-BU bacteria) with chloramphenicol, the technique of growing irradiated bacteria on cellophan carriers lying on the incubation medium was used (Alper & Gillies, 1958). This allowed bacteria to be transferred quickly from one medium to another. Suitable dilutions of the irradiated bacteria were dispensed on cellophan carriers lying on the surface of a series of plates of Oxoid nutrient agar containing chloramphenicol (5 μg./ml.), pre-warmed to 37°. The inoculated plates were returned to the incubator, and at intervals a plate was removed and the bacteria transferred on their carriers to fresh pre-warmed plates of Oxoid nutrient agar containing no inhibitor. The macrocolonies which subsequently developed after overnight incubation at 37° were

counted. Samples of un-irradiated bacteria were treated in the same fashion. The number of macrocolonies which grew after a given treatment was determined as the average count obtained from bacteria seeded on six replicate pieces of cellophan. Where errors are indicated these represent the 95% confidence limits of the colony count.

RESULTS

Under the conditions used the rate of growth of *Escherichia coli* B, as determined by colony count, was the same for the first 6 hr of incubation whether thymine or 5-BU was included in the sulphanilamide medium (Fig. 1). However, after that

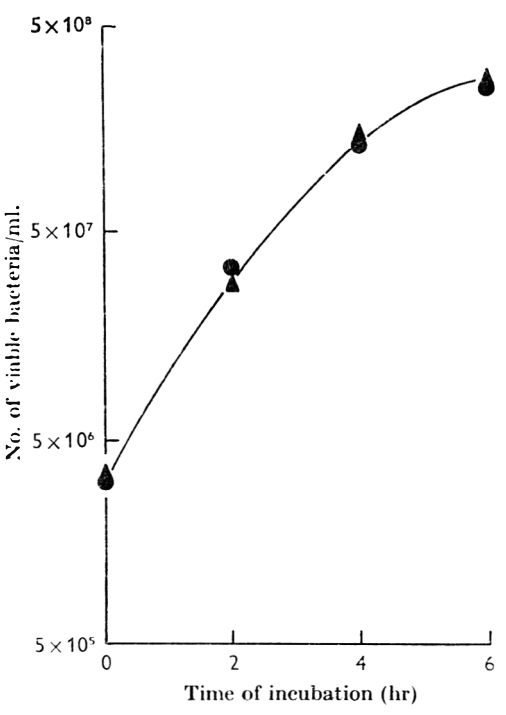

Fig. 1

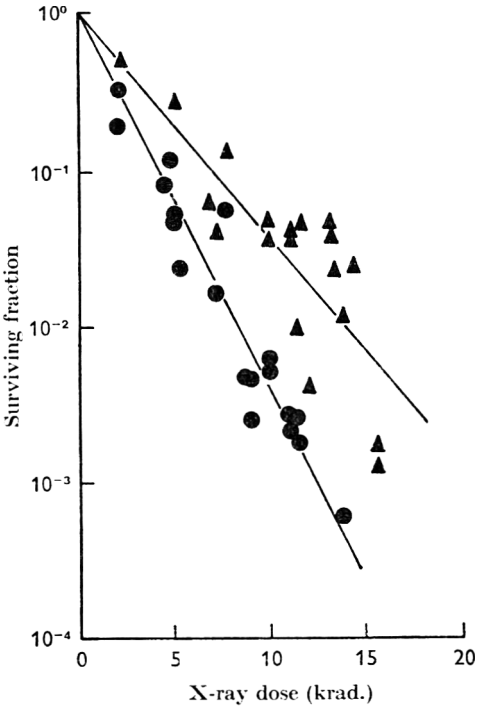

Fig. 2

Fig. 1. Growth of *Escherichia coli* strain B in sulphanilamide medium containing: ●, 5-bromouracil (5-BU; 75 μg./ml.); ▲, thymine (75 μg./ml.)

Fig. 2. Survival of *Escherichia coli* strain B after exposure to γ-radiation under aerobic conditions. Bacteria were grown before irradiation in: ●, sulphanilamide medium + 5-BU; ▲, sulphanilamide medium + thymine.

time the rate of growth of 5-BU bacteria decreased below that of thymine bacteria. This was probably due to the lethal action of high concentrations of 5-BU and possibly also to the inhibition of division as the bacteria accumulated more 5-BU. Since significant radiation-sensitization of the 5-BU bacteria occurred by 6 hr, the bacteria were harvested at that time, and irradiated as a suspension in buffer (pH 7·0).

In Figs. 2 and 3 data are presented on the γ-radiation sensitivities of 5-BU and of thymine bacteria irradiated in the presence and absence of oxygen, respectively.

Each point, derived from a separate experiment, represents the surviving fraction of bacteria, on Oxoid nutrient agar containing no chloramphenicol, after exposure to a given dose of γ radiation. There was considerable scatter in the experimental points, particularly for thymine bacteria irradiated under oxygen. Difficulty was experienced in maintaining uniform radiation sensitivity over the 2-year period during which the experiments were made. These variations may reflect minor differences in the composition of the batches of sulphanilamide medium used as well as other uncontrolled factors which affect the radiation-sensitivity of bacteria grown in defined media. However the response of the bacteria to the post-irradiation treatments was reproducible, despite these variations in radiation-sensitivity. The lines which have been drawn through the points were fitted by least-square analysis according to the method of Moore & Edwards (1965). The calculated slopes of the lines are shown in Table 2. Despite the considerable scatter in the experimental points plotted in Figs. 2 and 3, the standard deviations of the calculated slopes are small, being less than 10% in each case, because a large number of observations were used to define the slopes of the lines, The extent of sensitization to γ radiation effected by 5-BU was measured by calculating the ratio of the slopes of the survival curves of the thymine and 5-BU bacteria. The oxygen enhancement ratios for 5-BU and thymine bacteria were calculated as the ratios of the slopes of the survival curves obtained after irradiation of the bacteria in the absence and presence of oxygen respectively. These ratios are also included in Table 2.

Table 2. *Calculated slopes of survival curves for Escherichia coli* B *exposed to γ-radiation in the presence or absence of oxygen*

The bacteria were grown in sulphanilamide medium + thymine (thymine bacteria) or in sulphanilamide medium + 5-BU (5-BU bacteria). The oxygen enhancement ratio is the ratio of the slope of the survival curve obtained after aerobic radiation to that of the survival curve obtained after anaerobic radiation. The errors shown are the standard deviations of the calculated slopes.

	Aerobic	Anaerobic	Oxygen enhancement ratio
5-BU bacteria	$0{\cdot}239 \pm 0{\cdot}0063$	$0{\cdot}0834 \pm 0{\cdot}0068$	$2{\cdot}86 \pm 0{\cdot}248$
Thymine bacteria	$0{\cdot}145 \pm 0{\cdot}0071$	$0{\cdot}0402 \pm 0{\cdot}00095$	$3{\cdot}60 \pm 0{\cdot}195$

Extent of sensitization of Escherichia coli B *by 5-BU to γ-radiation,* Sensitization by 5-BU was measured as the ratio of the slope of the survival curve for 5-BU bacteria to that of the survival curve for thymine bacteria.
Condition of γ-radiation: aerobic, $1{\cdot}65 \pm 0{\cdot}091$; anaerobic, $2{\cdot}07 \pm 0{\cdot}178$.

The sensitivity of 5-BU and thymine bacteria to u.v. radiation is illustrated in Fig. 4, the points being derived from data obtained from a series of separate experiments. The inconsistency in the sensitivity of the thymine bacteria precludes an accurate measure of the sensitization effected by 5-BU, but it appears to be by a factor of at least 2·3.

The response of 5-BU bacteria and thymine bacteria to treatment with chloramphenicol

After γ-irradiation under aerobic conditions. The effect of incubating 5-BU or thymine bacteria of *Escherichia coli* B on nutrient medium containing

chloramphenicol for various intervals after aerobic γ-irradiation is shown in Fig. 5. At the times indicated on the abscissa the bacteria on their cellophan carriers were transferred to nutrient medium containing no chloramphenicol. In this particular experiment 5-BU bacteria and thymine bacteria were decreased to approximately the same survival values, before treatment with chloramphenicol, by exposing them to 7·1 krad, and 14·2 krad. of γ-radiation respectively, The surviving fraction of thymine bacteria was little affected by incubation with chloramphenicol, whereas treatment of the 5-BU bacteria led to a fivefold increase in the numbers of bacteria

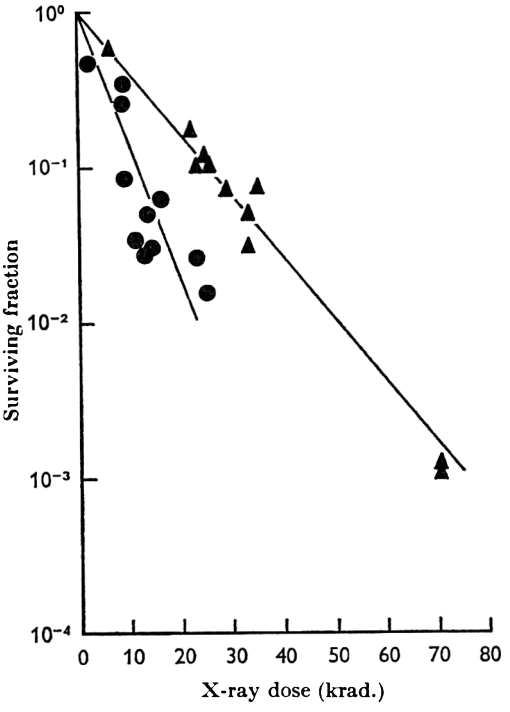

Fig. 3

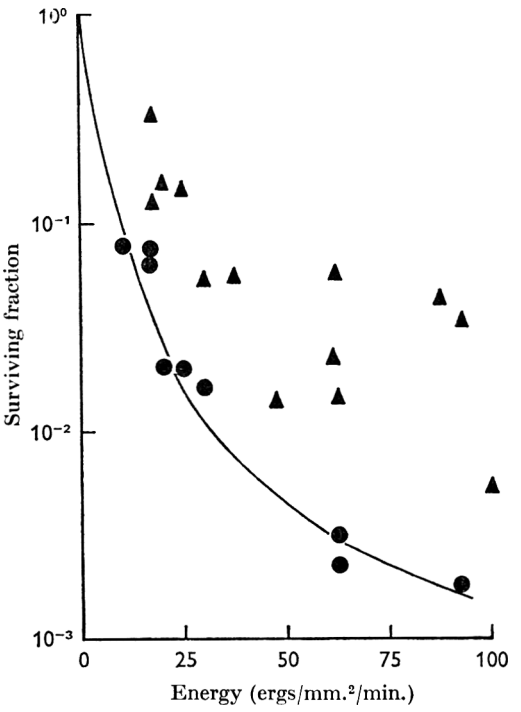

Fig. 4

Fig. 3. Survival of *Escherichia coli* strain B after exposure to γ-radiation under anaerobic conditions. Bacteria were grown before irradiation in: ●, sulphanilamide medium + 5-BU; ▲, sulphanilamide medium + thymine.

Fig. 4. Survival of *Escherichia coli* strain B after exposure to ultraviolet radiation. Bacteria were grown before irradiation in: ●, sulphanilamide medium + 5-BU; ▲, sulphanilamide medium + thymine.

able to originate colonies, although even then the survival was not as great as that of the untreated thymine bacteria; this is illustrated in Fig. 6. In this case, the two bacterial populations were each exposed to equal doses of 9 krad. of γ-radiation under oxygen. The extent of sensitization by 5-BU is evidenced by the much lower survival of the 5-BU bacteria when plated immediately after irradiation on nutrient medium containing no chloramphenicol. As before, incubation with chloramphenicol afforded a large increase in the survival of the 5-BU bacteria and little or no increase in the survival of the thymine bacteria, but the surviving fraction of 5-BU bacteria

after this 'rescuing' treatment still fell short of that of the thymine bacteria. Thus, the restoration brought about by chloramphenicol was relatively more effective in 5-BU bacteria than in thymine bacteria after aerobic γ-irradiation. However, in some experiments significant rescue of the thymine bacteria did occur, amounting to a two-/threefold increase in the surviving fraction. In these instances the rescue of 5-BU bacteria treated in parallel was even more extensive.

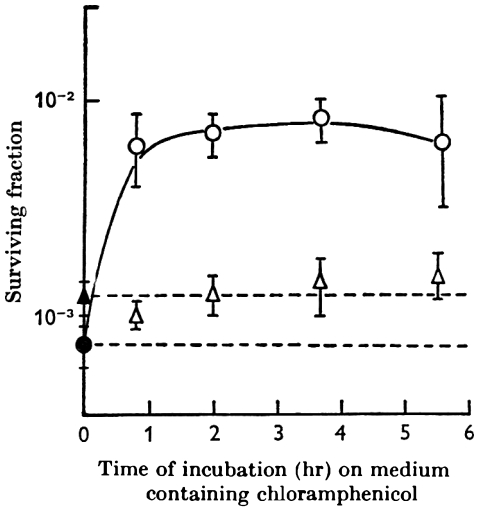

Fig. 5

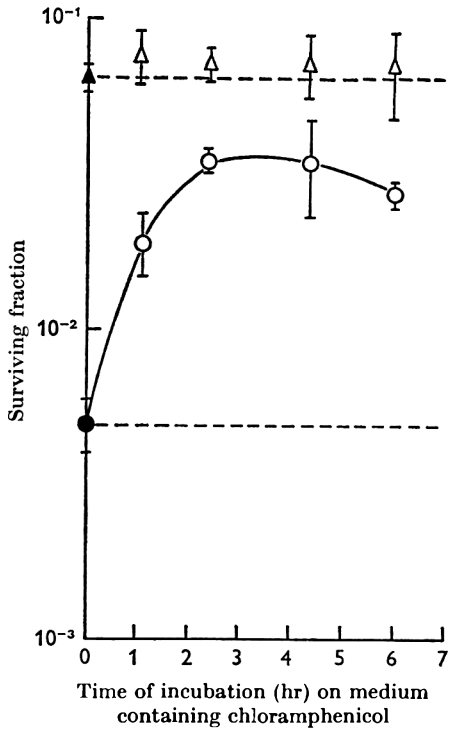

Fig. 6

Fig. 5. Survival of *Escherichia coli* strain B after exposure to γ-radiation under aerobic conditions, ● – – –, grown in sulphanilamide medium + 5-BU before irradiation; incubated throughout on Oxoid nutrient agar after exposure to 7·1 krad. γ-radiation. ○, Grown in sulphanilamide medium + 5-BU before irradiation; incubated on Oxoid nutrient agar + chloramphenicol (5 μg./ml.) after exposure to 7·1 krad. of γ-radiation, for the intervals indicated on the abscissa and then transferred to Oxoid nutrient agar. ▲ – – –, Grown in sulphanilamide medium + thymine before irradiation; incubated throughout on Oxoid nutrient agar after exposure to 14·2 krad. of γ-radiation. △, Grown in sulphanilamide medium + thymine before irradiation; incubated on Oxoid nutrient agar + chloramphenicol (5 μg./ml.), after exposure to 14·2 krad. of γ-radiation, for the intervals indicated on the abscissa, and then transferred to Oxoid nutrient agar.

Fig. 6. Survival of *Escherichia coli* strain B after exposure to 9 krad. γ-radiation under aerobic conditions. ● – – –, Grown in sulphanilamide medium + 5-BU before irradiation; incubated throughout on Oxoid nutrient agar, after irradiation. ○, Grown in sulphanilamide medium + 5-BU before irradiation; incubated on Oxoid nutrient agar + chloramphenicol after irradiation for the intervals indicated and then transferred to Oxoid nutrient agar. ▲ – — , Grown in sulphanilamide medium + thymine before irradiation; incubated throughout on Oxoid nutrient agar after irradiation. △, Grown in sulphanilamide medium + thymine before irradiation; incubated on Oxoid nutrient agar + chloramphenicol after irradiation for the intervals indicated and then transferred to Oxoid nutrient agar.

To provide a measure of the effect of treatment with chloramphenicol, a value, which we have called the Rescue Index, was calculated. This is the maximum number of bacteria which were rescued, expressed as % of the bacteria which would not form colonies if not incubated with chloramphenicol. For example, when a certain dose of γ-radiation initially decreased the surviving fraction of bacteria to 0·01, and incubation with chloramphenicol increased the surviving fraction to a maximum value of 0·06, then the Rescue Index is $[(0{\cdot}06-0{\cdot}01)/(1{\cdot}00-0{\cdot}01)] \times 100 = 5{\cdot}05$. Thus, the more extensive the restoration, the larger is the value of

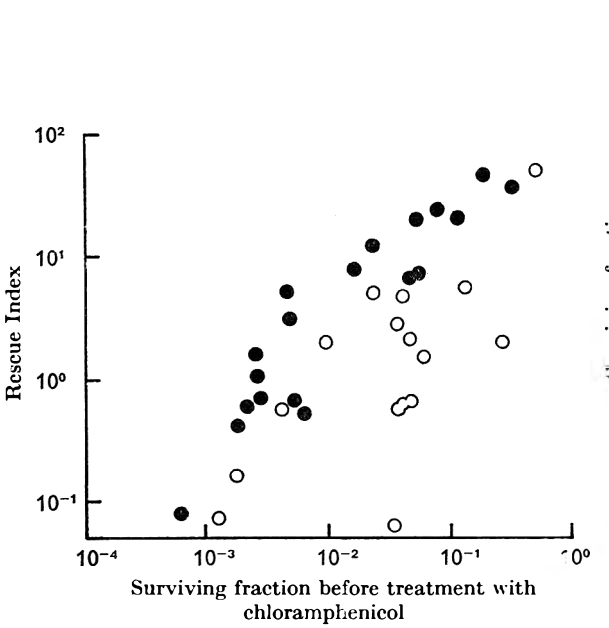

Fig. 7

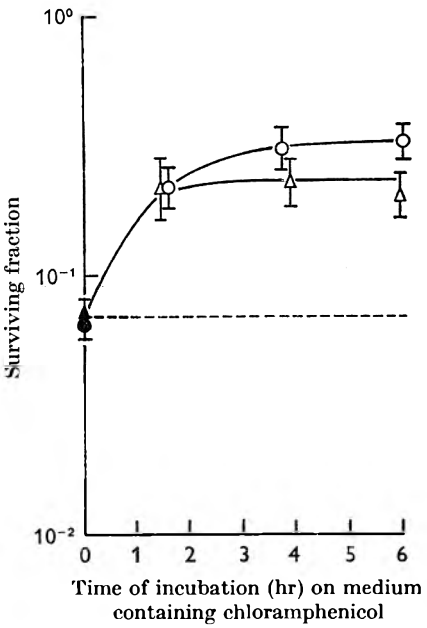

Fig. 8

Fig. 7. Rescue Indices for *Escherichia coli* strain B plotted against the surviving fractions obtained when the bacteria had not been incubated with chloramphenicol after aerobic γ-irradiation. The definition and the method of calculating the Rescue Index are given in the text. ●, Containing 5-BU; ○, containing thymine.

Fig. 8. Survival of *Escherichia coli* strain B after exposure to γ-radiation under anaerobic conditions. ● – – –, Grown in sulphanilamide medium + 5-BU before irradiation; incubated throughout on Oxoid nutrient agar after exposure to 16·1 krad. of γ-radiation. ○, Grown in sulphanilamide medium + 5-BU before irradiation, incubated on Oxoid nutrient agar + chloramphenicol, after exposure to 16·1 krad. of γ-radiation, for the intervals indicated and then transferred to Oxoid nutrient agar. ▲ – – –, Grown in sulphanilamide medium + thymine before irradiation; incubated throughout on Oxoid nutrient agar after exposure to 34·8 krad. of γ-radiation. △, Grown in sulphanilamide medium + thymine before irradiation; incubated on Oxoid nutrient agar + chloramphenicol, after exposure to 34·8 krad. of γ-radiation, for the intervals indicated, and then transferred to Oxoid nutrient agar.

the Rescue Index. The data obtained from a series of experiments have been summarized in Fig. 7 by plotting the calculated Rescue Indices against the survival observed when the bacteria were not treated with chloramphenicol. Both parameters have been plotted on a logarithmic scale to give the results conveniently in one

figure. Because of variations in the radiation-sensitivity of the bacteria observed in the experiments it was decided to plot the Rescue Indices against survival without restoration treatment, rather than against the dose of radiation used, as a means of obtaining a more valid comparison of the two populations of bacteria.

Since the surviving fraction of colony formers decreased with increasing exposure to radiation, the % of killed 5-BU and thymine bacteria which can be restored also decreased; this is indicated by the decrease in Rescue Index as the surviving fraction of the unrescued bacteria decreased. Although there is a large scatter in the points,

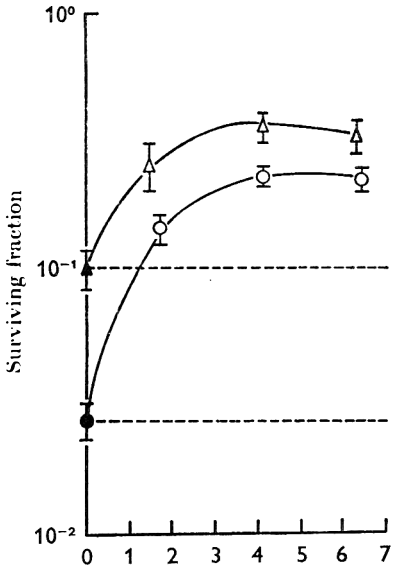

Fig. 9

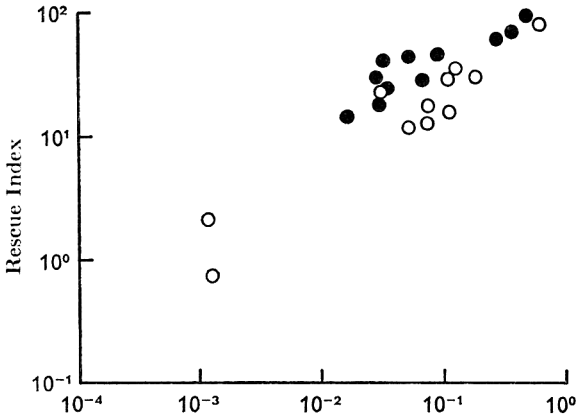

Fig. 10

Fig. 9. Survival of *Escherichia coli* strain B after exposure to 23·2 krad. of γ-radiation under anaerobic conditions. ● – – –, grown in sulphanilamide medium + 5-BU before irradiation; incubated throughout on Oxoid nutrient agar after irradiation. ○, Grown in sulphanilamide medium + 5-BU before irradiation; incubated on Oxoid nutrient agar + chloramphenicol after irradiation for the intervals indicated and then transferred to Oxoid nutrient agar. ▲ – – –, Grown in sulphanilamide medium + thymine before irradiation; incubated throughout on Oxoid nutrient agar after irradiation. △, Grown in sulphanilamide medium + thymine before irradiation; incubated on Oxoid nutrient agar + chloramphenicol for the intervals indicated and then transferred to Oxoid nutrient agar.

Fig. 10. Rescue Indices for *Escherichia coli* strain B plotted against the surviving fractions obtained when the bacteria had not been incubated with chloramphenicol after anaerobic γ-irradiation. ●, Containing 5-BU; ○, containing thymine.

particularly in those relating to thymine bacteria, the majority of the Rescue Indices for the 5-BU bacteria are larger than those for the thymine cells, indicating relatively greater restoration of the 5-BU bacteria. On two occasions treatment of irradiated thymine bacteria with chloramphenicol caused a decrease in survival and therefore no Rescue Index could be calculated in these cases.

After γ-irradiation under anaerobic conditions. The results from a typical experiment are illustrated in Fig. 8, in which is plotted the survival of thymine and 5-BU

bacteria against the time of incubation on nutrient medium containing chloramphenicol. The thymine bacteria were exposed to 34·8 krad. and the 5-BU bacteria to 16·1 krad. γ-radiation under anoxia to decrease them to the same degree of survival. Considerable restoration of both thymine and 5-BU bacteria occurred, but the extent of rescue of the 5-BU bacteria was just significantly greater. Figure 9 shows that for bacteria exposed to the same dose of γ-radiation under anoxia, the maximum degree of survival of thymine bacteria after treatment with chloramphenicol was higher than that of the 5-BU bacteria, even when the relative extent of restoration of the 5-BU bacteria was greater.

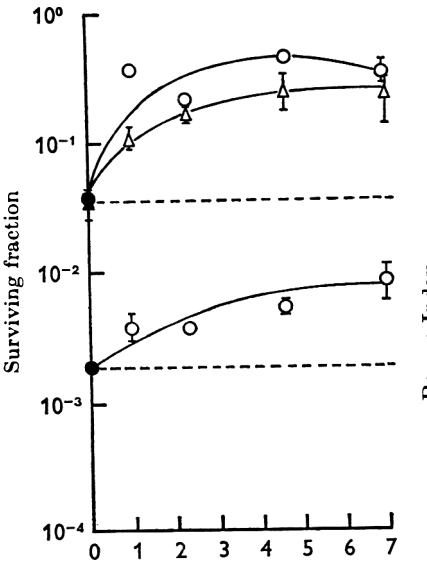

Fig. 11

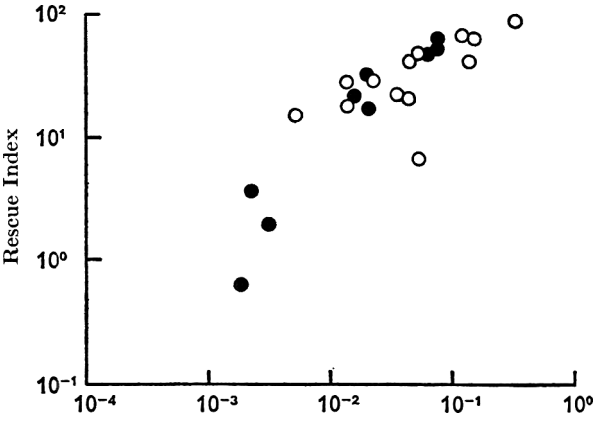

Fig. 12

Fig. 11. Survival of *Escherichia coli* strain B after exposure to u.v. radiation. ● – – –, Grown in sulphanilamide medium + 5-BU before irradiation; incubated throughout on Oxoid nutrient agar after exposure to 18 ergs/mm². (top curve) or to 95 ergs/mm². (bottom curve) of u.v. radiation. ○, Grown in sulphanilamide medium + 5-BU before u.v. irradiation; incubated on Oxoid nutrient agar + chloramphenicol after exposure to 18 ergs/mm.² (top curve) or to 95 ergs/mm.² (bottom curve) of u.v. radiation. ▲ – – –, Grown in sulphanilamide medium + thymine before irradiation; incubated throughout on Oxoid nutrient agar after exposure to 95 ergs/mm.² of u.v. radiation. △, Grown in sulphanilamide medium + thymine before irradiation; incubated on Oxoid nutrient agar + chloramphenicol, after exposure to 95 ergs/mm.² of u.v. radiation, for the intervals indicated, and then transferred to Oxoid nutrient agar.

Fig. 12. Rescue Indices for *Escherichia coli* strain B plotted against the surviving fractions obtained when the bacteria had not been incubated with chloramphenicol after u.v. irradiation. ●, Containing 5-BU; ○, containing thymine.

After u.v. irradiation. The data presented in Fig. 11, which are taken from one experiment, indicate that extensive rescue of thymine bacteria and of 5-BU bacteria occurred after treatment with chloramphenicol on a nutrient medium. The two lower curves in Fig. 11 show that when thymine and 5-BU bacteria were exposed to

the same dose of u.v. radiation (95 ergs./mm.^{2}), the surviving fractions were $3{\cdot}66 \times 10^{-2}$ and $1{\cdot}84 \times 10^{-3}$, respectively, when the cells were not treated with chloramphenicol. After incubation with chloramphenicol for 7 hr the survival of the thymine bacteria increased to $2{\cdot}48 \times 10^{-1}$ and that of the 5-BU bacteria to $8{\cdot}13 \times 10^{-3}$. Therefore, the survival of thymine bacteria increased to a greater extent. However, when comparison is made between the effect of chloramphenicol on the thymine bacteria and the 5-BU bacteria decreased to the same survival with 18 ergs./mm.^{2} of u.v. radiation, as shown in the upper curves of Fig. 11, the rescue of the 5-BU bacteria was significantly greater. When the Rescue Indices calculated from the data of several different experiments are plotted against survival of unrescued bacteria (Fig. 12) it appears that the restoration by chloramphenicol occurred to about the same extent in both thymine and 5-BU bacteria.

DISCUSSION

The extent of sensitization to γ-radiation due to the incorporation of 5-BU into *Escherichia coli* B was greater when the irradiation was performed under anoxic than under aerobic conditions. Despite the variations encountered in the sensitivity of the bacteria over a long time-interval this is clearly seen in the data presented in Table 2. These confirm the observation made by Alper & Moore (1964), who worked with the same strain of *E. coli* B. An examination of the data presented by Kaplan, Zavarine & Earle (1962) suggest likewise that greater sensitization to X radiation was effected by 5-BU in a thymine-deficient strain of *E. coli* B when it was irradiated in the absence of oxygen than in its presence, the sensitivity ratios being, respectively, 3·03 and 2·07.

It is difficult to estimate the increase in sensitivity of the *Escherichia coli* B 5-BU bacteria to u.v. radiation because of the variation in sensitivity of the thymine bacteria. However, when measured at the 10% survival value, the sensitization factor is at least 2·3. Therefore, the sensitization caused by 5-BU is most effective after u.v. radiation and least effective after γ-radiation in the presence of oxygen.

Treatment of *Escherichia coli* B with chloramphenicol for an interval after exposure to γ-radiation or u.v. radiation caused an increase to take place in the colony-forming ability of both 5-BU bacteria and thymine bacteria. It was difficult to obtain an exact measure of the effectiveness of chloramphenicol in restoring the irradiated bacteria, but an estimate of the Rescue Index at 10% survival was made from the data presented in Figs. 7, 10 and 12. These values are presented in Table 3 and can be used to compare the extent of restoration by chloramphenicol of damage caused under the three conditions of irradiation. Two features are apparent. First, restoration was greatest after u.v. radiation and least after γ-radiation in the presence of oxygen in both 5-BU bacteria and thymine bacteria. Secondly, after γ-radiation the restoration of 5-BU bacteria was relatively greater than that of thymine bacteria. Thus the radiation conditions under which most sensitization by 5-BU occurred were also those after which most restoration with chloramphenicol took place. Therefore it may be that the same type of damage is enhanced by the one agent and restored by the other. Although no direct examination was made of the location of the 5-BU in the *E. coli* B used here it seems clear from observations

by other workers (Greer, 1960; Kaplan, Smith & Tomlin, 1962) that radiation-sensitization only occurs when 5-BU replaces some of the thymine in the DNA. Thus the location of this lesion can be defined and it seems reasonable to suppose that treatment with chloramphenicol decreases the expression of some of this damage in DNA.

Table 3. *The extent of restoration of Escherichia coli* B *containing thymine (thymine bacteria) or 5-BU (5-BU bacteria) by incubation on a nutrient medium containing chloramphenicol, after different radiation treatments*

Estimated Rescue Index (R.I.) at 10% survival level

$$\text{Rescue Index} = \frac{\text{Maximum number of irradiated bacteria restored by chloramphenicol treatment}}{\text{Number of irradiated bacteria which survive without chloramphenicol treatment}} \times 100$$

Irradiation	5-BU bacteria	Thymine bacteria	Ratio of $\frac{\text{R. I. (5-BU bacteria)}}{\text{R. I. (thymine bacteria)}}$
γ-radiation, aerobic	22	7	3·14
γ-radiation, anaerobic	43	20	2·15
u.v. radiation	52	52	1·00

The dependence of the extent of sensitization by 5-BU and of the amount of restoration effected by chloramphenicol on the conditions under which *Escherichia coli* B was irradiated can be explained by a model proposed by Alper (1962, 1963). She concluded from a study of the dependence of the magnitude of the oxygen effect on post-irradiation cultural conditions in *E. coli* B (Alper & Gillies, 1958; Alper, 1961) that radiation damage in this strain can be divided into at least two classes, either of which can be lethal to the bacteria; these she called type N and type O. Type N damage is characterized by being more readily decreased after irradiation by treatment with inhibitors of protein synthesis or by poor growth conditions, for example, The sensitization of the lesion to ionizing radiation by oxygen is small. Type O damage, on the other hand, is not modified by post-irradiation treatments and has a much larger oxygen enhancement ratio. Whether a bacterium suffers lethal type O or type N damage will depend on the conditions at the time of irradiation, and also on the nature of the post-irradiation growth conditions. Thus a larger proportion of *E. coli* B will be inactivated as a result of type-O lesions when they are exposed to ionizing radiation in the presence of oxygen than when irradiated in its absence. Also the proportion of bacteria which suffer lethal type N damage will be decreased when they are placed in conditions after irradiation which decrease the expression of type-N lesions, In u.v. irradiated bacteria, the damage will be largely type N in nature, because no oxygen effect is associated with exposure to u.v. radiation and therefore no type-O damage will occur. Since u.v. radiation is absorbed principally in the nucleic acids, Alper suggested that type-N damage is located in the nucleic acids. Therefore the presence of 5-BU in DNA would be expected to increase the expression of damage caused by type-N lesions. It would follow that greater sensitization by 5-BU would occur under conditions in which more type N damage is likely to occur. This is borne out by the findings that sensitization of *E. coli* B by 5-BU was greatest with u.v. radiation, less with anoxic γ-

radiation when some of the lethal damage is also due to type-O lesions and least with aerobic γ-radiation when the proportion of type-O lesions is further increased. If treatment with chloramphenicol effects restoration by causing a decrease in the amount of type-N damage but not of type O damage which is expressed, then the effectiveness of the chloramphenicol will be greatest after conditions of radiation in which the proportion of lethal type-N damage is largest, i.e. after u.v. irradiation of 5-BU or thymine bacteria. Less restoration of 5-BU bacteria will be observed after anoxic γ radiation than after u.v. radiation because a proportion of the lethal damage will be caused by type-O lesions against which chloramphenicol is ineffective, but the extent of 'rescue' will be more than that seen in anoxically γ-irradiated thymine bacteria in which the fraction of lethal type-N damage is further decreased. In the same way it can be argued that resoration of aerobically γ-irradiated bacteria will be even less, but that the 5-BU bacteria will be restored to a greater extent than the thymine bacteria. The data shown in Table 3 bear out these predictions.

It is interesting that the u.v. irradiated 5-BU bacteria can be restored to such a marked degree by treatment with chloramphenicol, because reports by other authors have indicated that photo-reactivation of bacteria or bacteriophage which contain 5-BU or 5-bromodeoxyuridine (5-BUDR) is prevented or markedly decreased (Greer, 1960; Lorkiewitz & Szybalski, 1960; Stahl *et al.* 1961). Host cell reactivation of phage T1 which contained 5-BUDR is also much diminished (Sauerbier, 1961; Howard-Flanders, Boyce & Theriot, 1962). Shugar (1965) suggested that reactivation of 5-BU-containing micro-organisms might be relatively ineffective because repair enzymes (Setlow & Carrier, 1964; Boyce & Howard-Flanders, 1964) which appear to mediate the removal of thymine dimers induced in the DNA by u.v. radiation probably are unable to split the dimer formed between thymine and 5-BU. The present data suggest that restoration by chloramphenicol may not involve repair enzymes.

So far no completely satisfactory explanation has been put forward to account for the radiation sensitization by halogenated pyrimidines incorporated in cells. It has been suggested that the presence of 5-BU in DNA may prevent endogenous recovery processes from operating after irradiation, thereby producing an apparent sensitizing effect. Evidence for this in bacteria and bacteriophages was reviewed by Howard-Flanders (1961); recent work by Lett, Parkins, Alexander & Ormerod (1964) led these authors to conclude that 5-BUDR may interfere with recovery processes in mammalian cells. The present findings indicate that at least in *Escherichia coli* B, although spontaneous recovery mechanisms may be blocked by 5-BU, nevertheless restoration can be induced to take place under conditions which temporarily inhibit protein synthesis after irradiation.

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A Generalized Transducing Phage for a Female *Escherichia coli* 04

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SUMMARY

The major characteristics of a temperate phage specific, as far as is known, for one strain of *Escherichia coli* serotype 04 have been described. It is inactivated by ultraviolet light at a rate characteristic of temperate phages, and it is serologically unrelated to the P1, P22 and T phages. This phage (designated Φ 04–CF) is short-tailed, contains DNA, is about 55 mμ, needs no supplemental Ca^{2+} or Mg^{2+} for adsorption, shows a latent period of 46 min. and a burst size of 166, and is ultraviolet light inducible. Phage 04–CF can transduce markers to the susceptible indicator strain of *E. coli* (CF 2004–6). The variety of markers transduced indicates that this is another generalized transducing system for *E. coli*. The frequencies of transduction lie between 10^{-6} and 10^{-8} for different markers, but vary with phage multiplicities. This strain of *E. coli* also acts as recipient in matings with *E. coli* K12 strains.

INTRODUCTION

A temperate phage was isolated from a mixture of *Escherichia coli* serotypes 04 and 0127 in a continuous flow culture apparatus (Spaulding, Zubrzycki & Lanphear, 1964). This phage was subsequently shown to be capable of transducing characters to a strain of *E. coli* serotype 04. In this communication, we describe some characteristics of this phage and of the transduction system in the female strain of *E. coli* 04.

METHODS

Media. Penassay broth (Antibiotic Medium no. 3, Difco) and Trypticase Soy agar and broth (Baltimore Biological Laboratories) were used for routine cultivation of the organisms. Nutrient (Difco) or Penassay broths were used in the adsorption experiments. Semi-solid agar consisted of 0·7% agar in Trypticase Soy broth. The Davis minimal medium was prepared as described by Lederberg (1950) except that it was solidified with a final concentration of 1·8% (w/v) Noble agar (Difco), and sterile solutions of sugars were added to a final concentration of 0·5%. This minimal medium was supplemented, when required, with sterile solutions of amino acids to a final concentration of 30 μg./ml. The buffer for ultraviolet irradiation was the minimal medium without glucose. The phosphate buffer for the adsorption experiment was a solution of the phosphate salts of the minimal medium.

Bacterial strains. Escherichia coli strain CF 2004–6, isolated during a continuous flow (CF) experiment (Spaulding *et al.* 1964), was used for propagating the phage

04–CF. Its serotype was 04: H 5. The K composition remains undetermined since it reacts with K 12, K 13 and K 53 antisera on slide agglutination but does not do so in the confirming titration (W. H. Ewing, Communicable Disease Center, personal communication). Mutants of strain CF 2004–6 were obtained by ultraviolet irradiation or by ethyl methane sulfonate combined with the penicillin selection technique (Lederberg, 1950)

The *Escherichia coli* CF 2004–6 strain was identified as a female by using the W 1895 (Hfr Cavalli) strain of *E. coli* K 12.

Phage techniques. The procedures used for the one-step growth curves and for determining the adsorption and neutralization constants were those described by Adams (1959). Chloroform was employed in these experiments to inactivate bacteria and release any intracellular phage. High titre (10^{11}/ml.) phage stocks were prepared by a modification of a procedure described by Postic & Finland (1961). Tubes of melted semi-solid agar after seeding with 1000–5000 phage particles and the indicator strain of bacterium were poured onto Petri plates previously layered with 3–4 ml. of unseeded semi-solid agar. These plate cultures were incubated at 37° overnight after which time there was confluent lysis. They were frozen at −20° overnight, slowly thawed at room temperature and the fluid which had separated from the agar was pipetted off. The phage lysates were clarified by low speed centrifugation and stored at 4° over chloroform.

Spot test for neutralization. Immune sera for neutralization tests were obtained by injecting rabbits with Φ 04–CF in Freund's complete adjuvant. The phage had been filtered to remove bacterial debris, and the subsequent titre was 10^{10}/ml. Six subcutaneous injections of 5 ml. each, 3–4 days apart, were given to the rabbits which were bled 6 days after the last injection. The sera obtained from two rabbits were inactivated at 56° for 30 min. and showed K values of 288 and 158 min.$^{-1}$. The rabbit antisera for phages T1, T3, T6, P22 and P1 were obtained from Dr N. Yamamoto (Fels Research Institute, Temple University School of Medicine). Semi-solid agar containing appropriate indicator bacteria and enough phage to produce confluent lysis was poured onto a layer of hard agar. Ca^{2+} to a final concentration of 2×10^{-2} M was added to the agar for the P1 phage system. Loopfuls of antisera diluted in broth were then spotted on the semi-solid agar soon after hardening. The plates were incubated at 37° overnight. Bacterial growth at the spot where antiserum was placed indicated that neutralization of the phage had occurred.

Ultraviolet irradiation. A 15 W. G.E. germicidal lamp was used as the u.v. source. The lamp was turned on 15 min. before use to help stabilize the output. All ultraviolet experiments were performed under reduced room lighting in order to minimize photo reversion. One-tenth ml. broth dilution of bacteria or phage was added to 10 ml. of the clear buffer in a 10 cm. Petri plate which was hand-rotated during irradiation. T2 phage was used as the reference for the ultraviolet intensity under these conditions and at distances of 70 and 50 cm. from the lamp it took 18 and 8 sec. respectively to produce 99% inactivation.

Transduction techniques. Phage 04–CF was added to 24 hr cultures of CF 2004–6 mutants at phage multiplicities of 1 to 4 (unless otherwise indicated). The mixtures were incubated 15 min. at 37°. It had been determined that under these conditions 85 to 99% of the phage was adsorbed and 50% or more of the bacteria survived. One-tenth ml. samples were plated on appropriate selective media. After 48 hr

at 37° the transductants were scored and the few revertants on the control plates subtracted.

Nucleic acid determination. Phage stocks were filtered, treated with 10 μg./ml. each of DNAse and RNAse at 37° for 1 hr and clarified by low-speed centrifugation. The phage was then centrifuged at 30,000 rev./min. for 2 hr in a model L Spinco, resuspended in buffer and clarified by low-speed centrifugation. The nucleic acid was extracted by the hot phenol method (Guthrie & Sinsheimer, 1963). The diphenylamine and orcinol reactions (Schneider, 1957) were used to detect DNA and RNA respectively.

Conjugation technique. A log phase culture of W 1895 was mixed with a stationary culture of CF 2004–6 at a ratio of 1:20. Mating was facilitated by filtering the mixture onto Millipore filters according to the technique of Matney & Achenbach (1962). After 1 hr incubation the mixture was vigorously agitated in cold saline to break the mating pairs and then plated. Recombinants were scored after 48 hr.

RESULTS

Morphology of the DNA phage. The plaques of Φ 04–CF on *Escherichia coli* CF 2004–6 were about 2 mm. in diameter and contained lysogenic survivors. The phage on electron-micrographs appeared to have a very short tail, and was essentially spherical with a diameter estimated to be about 55 mμ.

The nucleic acid preparation of Φ 04–CF was negative for RNA by the orcinol reaction when examined with a Spectronic 20 at 660 mμ wavelength, but it gave a visibly positive diphenylamine reaction for DNA.

Phage specificity. The bacteria to be checked for susceptibility to phage were inoculated into semi-solid agar which was then poured on to a bottom layer of hard agar (1·5 %). A drop of phage stock containing 10^{10} or more phage per ml. was spotted onto the lawn of bacteria. The bacteria tested are listed in Table 1. These include strains susceptible to phages P1, P22, T1 through T7, a variety of colicins, and *Escherichia coli* K 12 strains lysogenic and non-lysogenic for lambda (λ), as well as donor and recipient strains. Only *E. coli* strains of serotype 04 were susceptible to Φ 04–CF by our spotting method, and this susceptibility was evidenced as an area of inhibition similar to that seen when a colicin acts upon a susceptible indicator strain. Phage 04–CF would only plaque and propagate on one strain, CF 2004–6.

Adsorption rates. Log growth cells of bacteria were used to adsorb Φ 04–CF. These bacteria were also resuspended in nutrient broth, with and without the addition of Ca^{2+} or Mg^{2+}, and in phosphate buffer. The results, summarized in Table 2, indicate an average K value of 2×10^{-9} ml. min.$^{-1}$. The results also show that the addition of 2×10^{-2} M-Ca^{2+} or Mg^{2+} does not increase the adsorption rates. In fact, the K value in phosphate buffer was comparable to those in nutrient broth with or without the addition of the cations. Phage 04–CF does not adsorb to the following strains: *Escherichia coli* 0127, Hfr donor K 12 ($\lambda -$), Hfr donor K 12 (λ^+), F^- recipient K 12 (λ^-).

Neutralization. Neutralization tests were carried out as described in the Methods section. The results shown in Table 3 indicate that Φ 04–CF is not serologically related to the other phages tested.

One-step growth curve. Figure 1 is a typical one-step growth curve. The results of

three such experiments indicate a minimal latent period about 46 minutes and an average burst size of 166 with a range of 145 to 182.

Ultraviolet inactivation. Figure 2 is the semilog plot of survival after u.v. irradiation. The slope of inactivation relative to T2 phage is about 0·05 and to T1 phage is about 0·4. This rate of inactivation is similar to that of other temperate phages (Bertani, 1958).

Induction by ultraviolet light. Phage 04–CF can lysogenize *Escherichia coli* CF 2004–6. This was demonstrated in the following way: CF 2004–6 isolated from the

Table 1. *Bacterial strains resistant to phage Φ 04–CF by the spot-testing method*

	No. tested		No. tested
Escherichia coli serotypes	123	*Serratia marcescens* SM6	1
Strain K12	22	*Bacillus subtilis* 168	1
E. coli B and B/r	3	*Salmonella typhimurium* LT2	3
E. freundii	3	*Shigella sonnei*	3
E. coli C	1	*S. flexneri*	1
		S. dysenteriae 16	1

Table 2. *Adsorption rates in various media*

Media	Bacterial counts ($\times 10^7$/ml.)	Phage-bacterium ratio	Percentage adsorption and K values (ml. min.$^{-1}$) 5 min.	15 min.
Nutrient broth	3·6	1·5	52 % $4{\cdot}1 \times 10^{-9}$	94 % $5{\cdot}1 \times 10^{-9}$
Nutrient broth + 2×10^{-2} M-Ca^{2+}	3·6	1·5	56 % $4{\cdot}5 \times 10^{-9}$	87 % $3{\cdot}7 \times 10^{-9}$
Nutrient broth + 2×10^{-2} M-Mg^{2+}	3·6	1·5	52 % $4{\cdot}1 \times 10^{-9}$	95 % $5{\cdot}6 \times 10^{-9}$
Phosphate buffer	5	0·72	67 % $4{\cdot}4 \times 10^{-9}$	92 % $3{\cdot}3 \times 10^{-9}$
Penassay broth*	7–13	0·01–0·09	53 %–74 % 2×10^{-9}	.

* The K value in Penassay broth is an average of five determinations, with the ranges of count, phage multiplicities and percentages adsorbed shown in the table.

Table 3. *Results of neutralization by the spot-testing method*

	Phages					
Antisera*	T1	T3	T6	P22	P1	Φ 04-CF
T1	+	–	–	.	.	–
T3	–	+	–	.	.	–
T6	–	–	+	.	.	–
P22	.	.	.	+	.	–
P1	.	.	.	.	+	–
Φ 04-CF	–	–	–	–	–	+

* A plus sign indicates neutralization.

centre of phage plaques was repeatedly subcultured in broth containing Φ 04–CF antiserum to neutralize free phage, and then re-isolated on agar. Such strains still harbored Φ 04–CF and were now resistant to the same phage. These lysogenic derivatives of CF 2004–6 could be induced by ultraviolet irradiation to produce Φ 04–CF. Log phase cultures were centrifuged, washed, resuspended in broth, diluted 1/100 in buffer to contain approximately 5×10^7 bacteria/ml., and then irradiated. Appropriate dilutions (determined by trial runs) were made in warm broth for the first and second growth tubes to determine the burst sizes and latent periods.

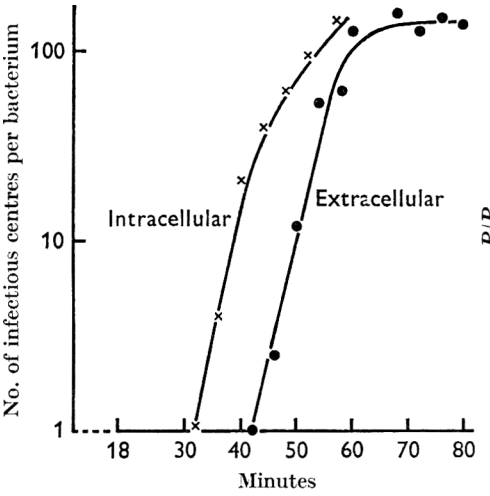

Fig. 1

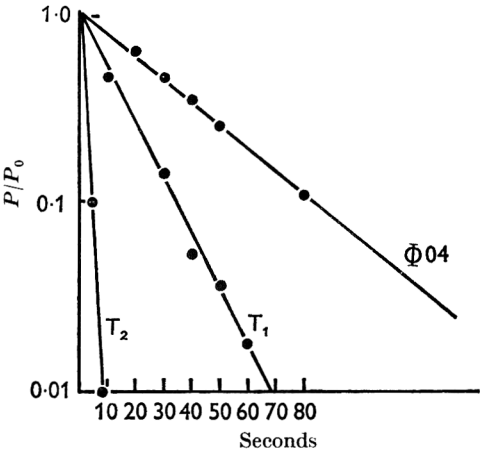

Fig. 2

Fig. 1. A typical one-step growth curve at 37° showing the intra- and extra-cellular appearance of Φ 04-CF.

Fig. 2. Survival curves after ultraviolet irradiation at 50 cm. from the lamp.

Table 4. *Induction of lysogenic E. coli strain* CF *2004–6 upon irradiation with ultraviolet light at 70 cm distance*

(The results in parentheses are from a repeat experiment.)

Ultraviolet dose	Approximate latent period (min.)	Average burst size	Percent induced bacteria
20 sec	82–88	152	27
40 sec	88–94	103–(130)	25–(30)
60 sec	88–94	112–(102)	26–(30)
80 sec	94–100	60–(89)	21–(24)

When a lysogenic broth culture was plated with a phage-susceptible indicator strain, 2–15 % of the bacteria were found eventually to produce phage as evidenced by plaque formation. Since phage was released at widely variable times after plating, the plaques differed markedly in size and were difficult to count. After chloroform treating the lysogenic culture to kill the bacteria, there was an average of about one free phage particle/100 bacteria when determined by plaque count. After ultraviolet irradiation with the doses reported in Table 4, 21–30 % of the bacteria show

up as plaques. These were large, and uniform in size, presumably because induction had occurred at about the same time. Though the induction percentages were quite constant during the period between 20 min. after irradiation and the end of the latent period, the bacterial colony counts decreased until there were usually fewer than 1 % survivors. With increasing doses of irradiation the minimal latent periods increased and the burst sizes decreased. Weigle & Delbruck (1951) reported similar shifts in the one-step growth curves and erratic bacterial counts after u.v. induction of *Escherichia coli* lysogenic for λ.

Table 5. *Comparison of transduction frequencies at various phage multiplicities*

Overnight bacterial cultures of a double auxotroph were used and selection was made for methionine transductants on minimal medium containing tryptophan.

Phage multiplicities	Frequency of transductants per phage adsorbed	Number of transductants per ml.
0·1	$7·2 \times 10^{-7}$	145
1·0	$2·2 \times 10^{-7}$	361
3·0	$4·9 \times 10^{-8}$	240
10·0	$3·2 \times 10^{-8}$	346

Table 6. *Transduction of mutant strains of E. coli* CF *2004–6*

All transductions were run with overnight cultures. The last six were concentrated 10 ×.

Mutant strains	Approximate phage multiplicity	Maker transduced	Frequency per phage adsorbed
tryp⁻ pro⁻	3	*pro*	$2·0 \times 10^{-7}$
tryp⁻	3	*tryp*	$4·4 \times 10^{-7}$
tryp⁻ leuc⁻	1	*leuc*	$2·2 \times 10^{-7}$
tryp⁻ isol⁻	1	*isol*	$1·3 \times 10^{-6}$
tryp⁻ cys⁻	1	*cys*	$2·8 \times 10^{-7}$
(Conc. 10 ×)			
tryp⁻ leuc arg⁻	3	*arg*	$2·3 \times 10^{-8}$
phe⁻	3	*phe*	$3·0 \times 10^{-8}$
gal⁻	4	*gal*	$3·3 \times 10^{-9}$
lac⁻	2	*lac*	$2·9 \times 10^{-8}$
xyl⁻	3	*xyl*	$2·0 \times 10^{-8}$
malt⁻	4	*malt*	$1·7 \times 10^{-8}$

The transduction system. Log and stationary cultures are equally suitable for transduction experiments, but the former must be concentrated in order to obtain a moderate number of transductants per plate. For convenience, therefore, stationary cultures were used. As seen in Table 5, the number of transductants was approximately the same in the phage multiplicity range of 1–10, but it decreased when the multiplicity was reduced to 0·1. In unpublished experiments, using cultures concentrated tenfold so that the effects of phage multiplicities between 3·0 and 0·01 could be studied, the number of transductants was approximately the same except for a marked decrease when the multiplicity was reduced from 0·1 to 0·01 (Green, 1965).

These results differ from those reported for the P22 transduction system. Zinder (1953) reported that the number of *Salmonella typhimurium* transductants rises

linearly with phage P22 multiplicities up to 10. It has been reported for the phage P1 transduction system that the frequency of transduction is a linear function of the P1 titre (Lennox, 1955). The data in Table 5 shows that this is not the case with the Φ 04–CF transduction system. In fact, the frequency of transduction is higher at the lower phage multiplicities (or titres). This increase is attributable to the fewer phage particles adsorbed per transductant.

Like phages P22 and P1, Φ 04–CF can transduce a variety of genetic markers. The list of markers transduced is shown in Table 6.

In order to determine whether transduction was always accompanied by lysogenization, transduction experiments were run using a double auxotroph (*tryp*$^-$, *met*$^-$) at a multiplicity of 0·1. This low multiplicity was chosen to lessen the chance of carrying over excess phage when checking for lysogenization. After the adsorption period, the phage-bacterium mixture was incubated with phage antiserum sufficient to inactivate greater than 99% of unadsorbed phage, and then centrifuged at 5° to spin down the bacteria. After a series of such treatments the cells were resuspended in saline and plated on media selective for methionine transductants. The transductants were then picked, grown in broth, chloroform treated, and spotted on a soft agar seeded with bacteria susceptible to Φ 04–CF. A strain of *Escherichia coli* serotype 04 (labelled z20), which adsorbs Φ 04–CF but can neither lyse nor become lysogenic, was also used in these experiments. As seen in Table 7, all 150 transductants tested were lysogenic. The fact that the z20 transductants were negative indicates that there was no extensive mechanical carry over of phage into the broth. However, the possibility of secondary lysogenization of the phage-susceptible auxotroph during one of the procedures subsequent to transduction cannot be excluded.

Table 7. *Test for lysogenization of transductants at a 0·1 phage multiplicity*

The recipients were concentrated 10× overnight.

Mutant strains	Number of transductants tested	Number of broth cultures containing phage
CF 2004–6 *tryp*$^-$ *met*$^-$	150	150
z20 *met*$^-$	50	0

Strain CF 2004–6, a female. We were curious to see whether CF 2004–6 was sexually competent with K 12 strains of *Escherichia coli*. If so, it would be possible to study genetic homology between these two distinct strains of *E. coli* using the 04–CF or P1 transducing phages. Similar studies are being conducted with salmonella and *E. coli* using P22 phage (see Discussion).

In mating experiments with donor strains of K 12, CF 2004–6 behaves as a recipient. This was proved by an experiment patterned after that described by Hayes (1952) to establish sexual polarity among K 12 strains. The results are recorded in Table 8. Plating the mating mixture on agar containing streptomycin eliminated the recombinants when the CF 2004–6 strain was susceptible to the antibiotic. Of 50 recombinants isolated at random, all were susceptible to Φ 04–CF, and 44 were agglutinated by anti-04 serum. The six failures could be due to an inadequate heating procedure used to remove the envelope (K) antigens which interfere with

O agglutination. In unpublished experiments a clear plaque (virulent) mutant of Φ 04–CF was substituted for the streptomycin, and it similarly eliminated the recombinants. These results prove that the recombinants on the selective media were CF 2004–6.

Several attempts have been made to obtain recombinants in mating experiments between CF 2004–6 and F⁻ strains of *Escherichia coli* K 12. None has been found. Brinton (1965) reported a good correlation between the presence in bacteria of the F episome and susceptibility to male specific phage. Phage f2, a male specific phage (Loeb & Zinder, 1961), does not grow on or adsorb to CF 2004–6. These negative findings support the view that CF 2004–6 is strictly a female strain, devoid of the F episome and donor ability.

Table 8. *Recombinants per 0·3 ml. resulting from crosses between streptomycin susceptible (Str-s) and resistant (Str-r) strains of E. coli w 1895 lac⁺ met⁻, and CF 2004–6 lac⁺ met⁺, as selected on minimal lactose agar (ML)*

		ML	ML + streptomycin
w 1895 *Str-r* × CF 2004–6 *Str-s*		$2{\cdot}3 \times 10^4$	0
w 1895 *Str-s* × CF 2004–6 *Str-r*		$4{\cdot}8 \times 10^4$	$5{\cdot}1 \times 10^4$
w 1895 *Str-s* × CF 2004–6 *Str-s*		$3{\cdot}4 \times 10^4$	0
Revertants in 0·2 ml.	w 1895 *Str-r*	0	0
	w 1895 *Str-s*	0	0
	CF 2004–6 *Str-r*	1	0
	CF 2004–6 *Str-s*	2	0

In preliminary experiments it has been shown that there is a gradient of transmission of multiple markers from w 1895 and other Hfr donors to polyauxotrophic mutants of CF 2004–6. This conjugation system is currently being investigated.

DISCUSSION

Characterization of phage 04–CF was of interest because it had first been detected in a continuous flow culture mixture of *Escherichia coli* 04 and *E. coli* 0127 only after the 21st day (Spaulding *et al.* 1964). The indicator strain (CF 2004–6) for Φ 04–CF was also isolated from a sample of the same CF culture which yielded the phage. This phage has been characterized and shown to be specific for *E. coli* serotype 04. Neither *E. coli* 0127 nor 04 seemed at the time to be lysogenic for this phage. However, after incubating *E. coli* 04 in an Erlenmeyer flask culture for 3 days, a phage producing a tiny plaque on CF 2004–6 was isolated. This phage (labelled Φ X1) is serologically related to Φ 04–CF by the spot testing method (unpublished data). The exquisite specificity of Φ 04–CF, and the isolation of a serologically related phage from *E. coli* 04, suggests that *E. coli* 04 is the source of Φ 04–CF. The characteristics of Φ X1 will eventually be compared with Φ 04–CF since it may be that ΦX1 is a mutant of Φ 04–CF or vice versa.

Early in these investigations it was discovered that Φ 04–CF is another generalized transducing phage. It does not require additional Ca^{2+} for adsorption as does the other *Escherichia coli* transducing phage P1 (Lennox, 1955), nor does it seem to have a long tail like P1 (Bertani, 1958). In addition, the results of host range and neutralization studies show it to be distinct from P1 and P22, the generalized transducing phage of *Salmonella typhimurium*.

It resembles the specialized transducing phage lambda, in being readily inducible by ultraviolet irradiation. The 25 % induction of lysogenic CF 2004–6 to produce infective centres is not so great as the 95 % induction reported for lambda (Weigle & Delbruck, 1951). However, Φ 04–CF is more inducible than P22 as reported by Levine (1961) who used mitomycin C which mimics the inducing action of ultraviolet light. He observed an induction of up to 91 % of the lysogenic *Salmonella typhimurium* cells surviving exposure to mitomycin C. On this basis, lysogenic CF 2004–6 is more than 99 % inducible. Another specialized transducing phage, Φ 80 (Matsushiro, 1961), has also been shown to be readily inducible by ultraviolet light.

The transduction system of Φ 04–CF appears to be similar to the generalized transducing systems mediated by phages P1 (Lennox, 1955) and P22 (Zinder & Lederberg, 1952) in that the frequencies of transduction for a variety of markers ranged from 10^{-6} to 10^{-8}. However, these transduction frequencies and the absolute number of transductants appearing per plate do not appear to be linearly related to the phage multiplicities used. The reason for these results is not clear.

Occasional transductants mediated by phage P22 have been found to be non-lysogenic (Stocker, Zinder & Lederberg, 1953). Adams & Luria (1958) reported that transductions by P1 at phage multiplicities of less than one are generally not accompanied by lysogenization. It is to be noted that these conclusive results with phage P1 were obtained by using selective medium lacking Ca^{2+} which P1 needs for adsorption. Non-lysogenic transductants mediated by Φ 04–CF have not been found, but it is difficult to eliminate secondary lysogenization.

Dettori, Maccacaro & Piccinin (1961) and Hakura, Otsuji & Hirota (1964) reported the isolation of phages specific for F^- strains of *E. coli* K 12, but no mention was made of the transducing ability of these phages. Though Φ 04–CF is a transducing phage for a female strain of *E. coli* 04, this sexual characteristic is not necessary for transduction. In fact, Φ 04–CF can transduce markers into a recently isolated donor strain of CF 2004–6 (unpublished). In this respect phage 04–CF is analogous to P22 which is a transducing phage for *Salmonella typhimurium* regardless whether it is a recipient in matings with *E. coli* K 12 strains, or not. The interest in CF 2004–6 being a female strain is that transduction by phage can now be used to check the extent of genetic homology within *E. coli* strains (Zubrzycki & Spaulding, 1965) as was done for *E. coli* and salmonella (Zinder, 1960; Demerec & Ohta, 1964) using phage P22.

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With 1 *plate*
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Electron Microscope Observations of *Helminthosporium victoriae*

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SUMMARY

In this investigation of *Helminthosporium victoriae* routine techniques of electron microscopy were used, except for a slightly modified potassium permanganate fixative. The cell wall consisted of two parts: an outer thin electron-dense layer and an inner broader electron-lucid fibrillar layer. A thin plasma membrane was contiguous with the inner layer of the cell wall. The sparse endoplasmic reticulum ramified throughout the cytoplasm in a sinuous fashion. Mitochondria displayed classical structure and were ubiquitous in distribution. Glycogen and lipids were constantly occurring inclusions. Simple septal pores were present. Large vacuoles with tonoplasts and also numerous smaller vesicles were seen. Hyphae were often multi-nucleate.

INTRODUCTION

A transmissible disease of the fungus *Helminthosporium victoriae* M & M was described by Lindberg (1959) from the cultures obtained from oats with Victoria blight. Light microscope investigations of the normal and the diseased fungus showed that the latter could be distinguished by stunted growth and lysed aerial mycelium (Psarros & Lindberg, 1962). Investigations are now underway to study the pathology of the diseased fungus, and possible identification of the causal agent, by means of electron microscopy. The present paper is concerned with the fine structure of the normal fungus.

METHODS

Vigorous young colonies of *Helminthosporium victoriae* were sectioned into 3 mm. cubes of mycelial mats on agar and transferred to fresh culture plates of potato glucose agar. Small sections were harvested from the corners of the subculture 24–36 hr after plating. These sections contained a small piece of mycelial mat and projecting hyphae 0·5–1 cm. in length. The hyphae were fixed in a 1·5% (w/v) solution of potassium permanganate and a detergent (Werner, Wright & Baker, 1964). Hyphae were rinsed in distilled water, dehydrated in a graded series of ethanol + water mixtures, and embedded in Maraglas (Freeman & Spurlock, 1962). Sections were cut with a diamond knife in a Porter-Blum MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn., U.S.A.). All sections were post-stained with lead citrate as described by Reynolds (1963). Photographs were taken with an HU-11A Hitachi electron microscope.

DISCUSSION

The hyphae of *Helminthosporium victoriae* (Pl. 1, figs. 3, 4) were characteristically elongate filamentous structures. The cell walls of *H. victoriae* were considerably different from those of *Rhizopus homothallicus* (Hawker & Abbott, 1963) and *Neurospora crassa* (Shatkin & Tatum, 1959), and thus merit comparisons. The cell wall of *R. homothallicus* consisted of tangentially arranged elongate elements which gave the wall a striate appearance. In *N. crassa* the wall was divisible into two areas. The outer surface consisted of a dense fibriform meshwork. The fibres of this area had a tendency to aggregate and slough-off; consequently this portion of the wall appeared frayed. The inner portion of the wall was a broader zone of low density in which only occasionally could one observe the internal structure which consisted of a network of fine and difficultly discernible fibres. In *H. victoriae* the cell wall (Pl. 1, fig. 1) consisted of an outer thin uniformly electron-dense layer. The frayed appearance of the outer wall, as reported in *N. crassa*, was not evident in *H. victoriae*. The inner wall of *H. victoriae* was invariably broader, slightly fibrillar and quite electron-lucid. The plasma membrane was visible as a thin electron-dense delimiting membrane touching the inner surface of the cell wall (Pl. 1, fig. 1).

Particulate clumped areas in the cytoplasm of *Helminthosporium victoriae* with an electron density greater than that of the cytoplasm were glycogen deposits (Pl. 1, fig. 1). Their size, density, and general appearance agreed with the description of glycogen (Revel, Napolitano & Fawcett, 1960). Lipid inclusions (Pl. 1, fig. 2) were present in most hyphae. Evidently, the lipids in *H. victoriae* contained few unsaturated fatty acids and have reacted to a limited degree, thus leaving the characteristically clear space in the inclusions.

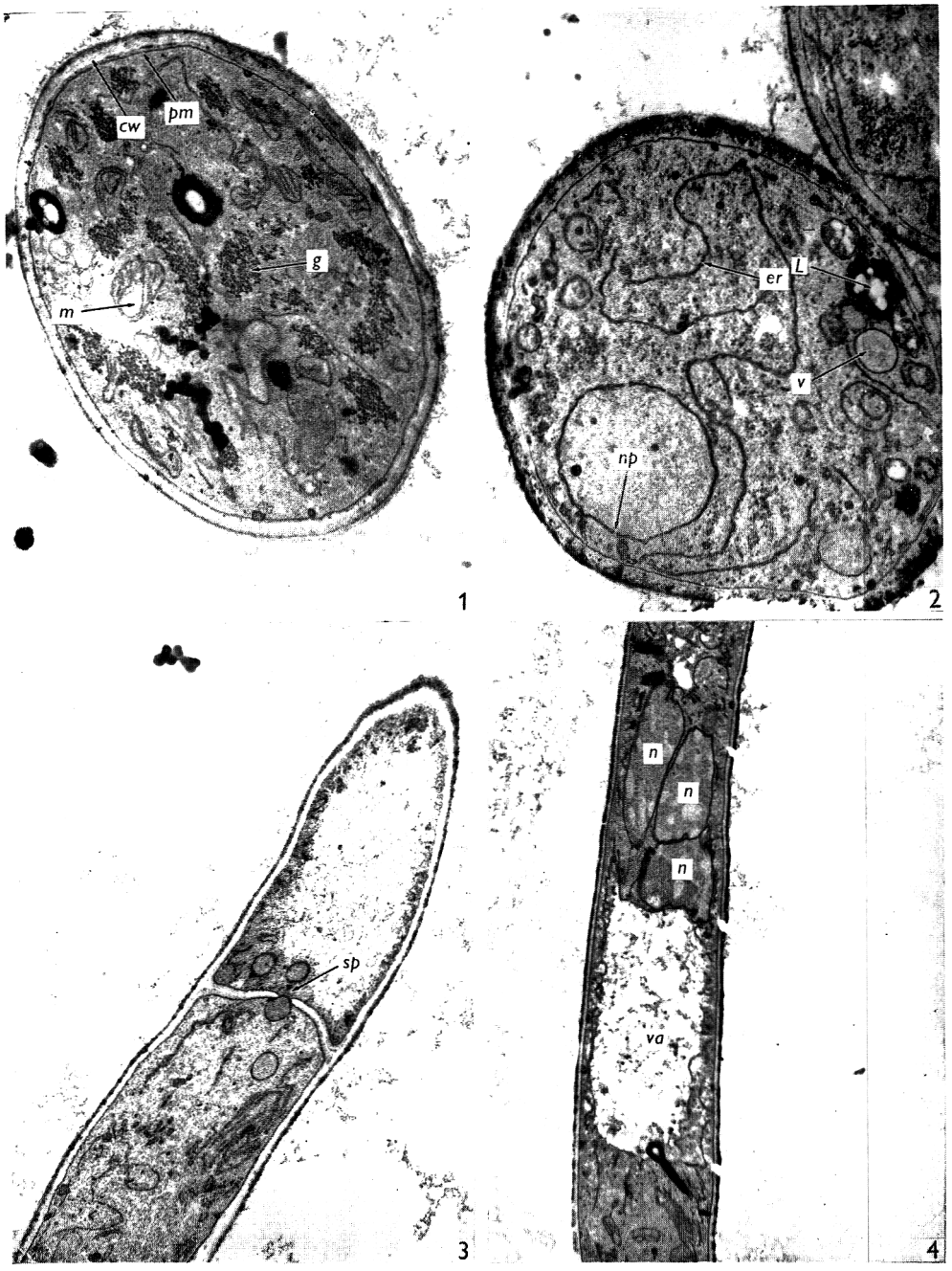
The endoplasmic reticulum (Pl. 1, fig. 2) showed no distinctive pattern, instead ramified throughout the entire cell. This system seemed to occur mostly in a sheet-like form. Serial sections indicated that the endoplasmic reticulum was a relatively sparse organelle.

The hyphae of *H. victoriae* were commonly multinucleated structures. The nuclei had typical double envelopes with intervening pores (Pl. 1, fig. 2). Ordinarily, the nucleoplasm displayed uniform density; however, periodically a considerable amount of electron-density variation was encountered (Pl. 1, fig. 4).

A large vacuole with flocculent contents enclosed by a single-membrane, electron-dense tonoplast was noted (Pl. 1, fig. 4).

Smaller vesicles (Pl. 1, figs. 2, 3) with single-membrane systems and contents with an electron-density similar to that of the cytoplasm were frequently encountered. The vesicle (Pl. 1, fig. 3) might be interpreted either as a plug or as a vesicle about to begin migration through the septal pore. Migration of material and the role of pores were described (Bracker & Butler, 1963; Giesy & Day, 1965).

This investigation was supported by a National Institutes of Health (U.S.A.) grant, no. EF AI 00564-01.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EXPLANATION OF PLATE

Helminthosporium victoriae electron micrographs: *cw*, cell wall; *pm*, plasma membrane; *g*, glycogen; *m*, mitochondria; *np*, nuclear pores; *er*, endoplasmic reticulum; *L*, lipid inclusions; *v*, small vesicles; *sp*, septal pore; *n*, nucleus; *va*, large vacuole with tonoplast.

Fig. 1. Cross-section of a vegetative hypha. The cell wall (*cw*) consists of an outer thin electron-dense area and an inner, broader, slightly fibrillar area. The plasma membrane (*pm*) is the single thin electron-dense delimiting membrane. Glycogen (*g*) has a tendency to clump in areas rather than being spread throughout the cytoplasm. Mitochondria (*m*) present the usual picture with double membranes and cristae. Potassium permanganate fixation. × 25,000.

Fig. 2. Cross-section of a hypha. The nuclear membrane is double with typical nuclear pores (*np*). The endoplasmic reticulum (*er*) ramifies irregularly through the cell. Lipid inclusions (*L*) are seen as electron-dense areas and frequently vacuolated. Vesicles (*v*) with single membranes are frequently encountered. Potassium permanganate fixation. × 30,000.

Fig. 3. Longitudinal section through a hypha shows a simple septal pore (*sp*). Vesicle may be a plug or about to begin migration. Potassium permanganate fixation. × 12,500.

Fig. 4. Longitudinal section through a hypha showing the frequent multinucleated condition (*n*). A large vacuole (*va*) surrounded with its tonoplast and enclosed flocculent material is evident. Potassium permanganate fixation. × 15,000.

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Observations on the Protoplasts of *Fusarium culmorum* and on their Fusion

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SUMMARY

Some characteristics of protoplast extrusion from hyphae of *Fusarium culmorum* are described. One, two or more protoplasts might be released from one mycelial compartment. The release was stimulated by controlled dilution of the stabilizing solution. NH_4Cl and mannitol were the best stabilizers of those tested. Connecting threads between protoplasts were observed; their meaning is discussed. Changes in the growth medium did not affect mycelium sensitivity for protoplast formation. Various stages of a protoplast fusion process were followed. Protoplast fusion usually began by an attraction of two bodies followed by junction and fusion to give a single large body. There was no evidence that these fusions represent a sexual process. Fusion of protoplasts in Fusarium is infrequent.

INTRODUCTION

The formation and development of spherical forms called protoplasts have been described for a few filamentous fungi: *Neurospora crassa* (Emerson & Emerson, 1958; Bachman & Bonner, 1959), *Fusarium culmorum* (Rodriguez Aguirre & Villanueva, 1963; Rodriguez Aguirre, García Acha & Villanueva, 1964; García Acha, Lopez-Belmonte & Villanueva, 1966) and *Polystictus versicolor* (Strunk, 1964). Transformation of mycelial cells into protoplasts under the influence of a cell-wall lysing enzyme called strepzyme RA was obtained with a large number of other fungi (Rodriguez Aguirre, García Acha & Villanueva, 1963). Striking differences in the rate and the proportion of protoplast formation among different species were observed. The purpose of the present paper is to report a more detailed description of the formation and subsequent development of the spherical protoplast bodies from *F. culmorum*, including the phenomenon of protoplast fusion.

METHODS

Organisms and cultural conditions. The organism chiefly used in this investigation was *Fusarium culmorum* CECT no. 2148, but other fusarium species were also used. Maintenance of the stock cultures and growth conditions were as described by Rodriguez Aguirre *et al.* (1964).

Source of the lytic enzymes and estimation of activity. The preparation of cell-wall lysing enzyme from *Streptomyces* RA and the estimation of activity were as previously reported (Rodriguez Aguirre *et al.* 1964).

Preparation of protoplasts. Young mycelium was harvested by centrifugation,

washed with distilled water and stored as a thick suspension at 4°. Usually fresh mycelium was employed. For the preparation of protoplasts, intact mycelium (equiv. 5 mg. dry wt./ml.) was suspended in a solution (usually 0·8 M-mannitol), and incubated for two or more hours with a lytic enzyme preparation from *Streptomyces* RA (strepzyme RA; Rodriguez Aguirre *et al.* 1964) with gentle agitation. Samples were taken from time to time for observation in the phase-contrast microscope. Protoplasts were liberated in 20–30 min. depending on the activity and concentration of the lytic enzyme preparation. Protoplasts sometimes appeared earlier when NH_4Cl was used as stabilizer. This particular system contained only 0·8 M-NH_4Cl solution, enzyme and mycelium. Its main advantage was the greater yield of protoplasts, being nearly double that obtained with mannitol or other stabilizers. Protoplast forms appeared as high contrast structures in phase-contrast microscopy; they were stable for more than 24 hr under the conditions of preparation, but at 5° lasted for several weeks.

RESULTS

The release of protoplasts

Before the release of protoplasts the hypha swells at certain points, presumably of greatest sensitivity to the enzymic attack. Then the protoplast emerges, at first as a small round bud-like structure at one side or at one end of a broken hypha. This bud increases in size relatively rapidly at the expense of the mycelium contents, which simultaneously decrease in size. The protoplasmic content of the hyphal compartment is eventually incorporated into one large body. Nothing, or only a small portion of the protoplasmic contents, remains inside the mycelium. Less frequently, the extrusion of one spherical body is followed by another of the same size connected to the first one by a thin thread. Two pores can be formed in the cell wall of the same hyphal compartment, but as a rule only one is formed. The length of time required for one or two spherical bodies to be formed varies with the concentration of the enzyme and the incubation conditions. In general, protoplasts were not released slowly and gradually, but suddenly and in steps. Physical forces may help this liberation. When the incubation mixture was diluted with water just at the moment when protoplasts started to form, the liberation of protoplasts took place much more quickly. Changes in osmotic conditions apparently helped in the extrusion of the protoplasts.

The evolution of protoplast liberation from a single compartment in the mycelium was followed with the microscope in hanging drop preparations. Figure 1 shows diagrammatically the stages of protoplast formation when originating from one compartment between two septa. The contents pass out through one pore formed in the hyphal cell wall. On several occasions we watched the cytoplasmic contents pass through the septal pore. It is easy to see the thin thread connecting the contents of two neighbouring cells (Pl. 1, fig. 1). We have also seen these threads in the branching of the mycelium. The thread was not homogenous but sometimes included small spherical enlargements. It was possible sometimes to see two of these little structures, one on each side of the septum. These globules may be small structures of the cytoplasm, retained inside the extended membranes.

Not all hyphae were equally sensitive to lytic enzyme attack. Age may have been

one reason for this, but probably not the only one, since we were normally using very young mycelium (18 hr). However, after incubation for 2 days with enzyme the whole mycelium was degraded to small pieces.

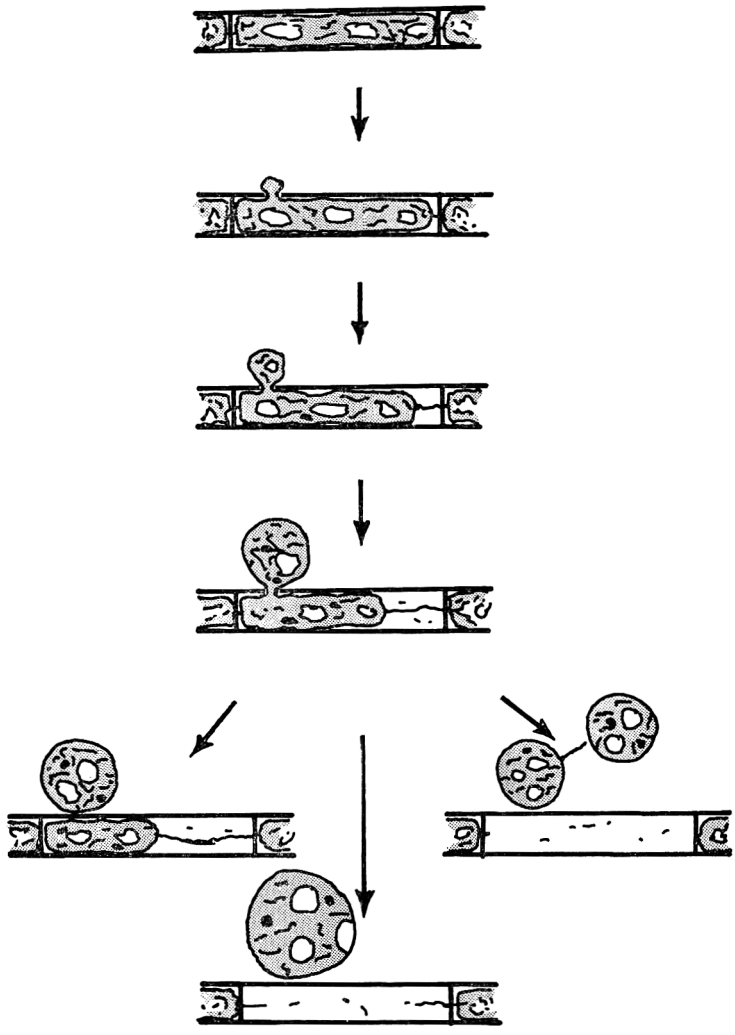

Fig. 1

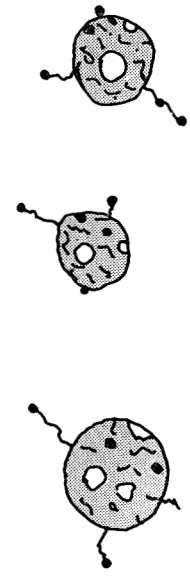

Fig. 2

Fig. 1. Diagrammatic representation of the various stages of protoplast release in *Fusarium culmorum*.

Fig. 2. Diagram of protoplasts with adhering 'satellites'.

Phase-contrast appearance of the protoplasts and residues

The protoplasts were not uniform; they were of different sizes and appearances. Some of them were refractile and others were quite dark, very grey but more transparent and appeared to be more fragile (Pl. 1, fig. 2). However, there were no noticeable differences to osmotic shock.

The contents of the protoplasts were also not uniform. The number of vacuoles

and other internal structures varied greatly and was largely influenced by the conditions used for the growth of the fungus. Some of the protoplasts had one very large vacuole or several vacuoles, whereas others had none or they were very small (Pl. 1, fig. 3). The external aspect of the protoplasts also varied, in good agreement with the very different appearances of the hyphae. It was observed that gentle heating caused the protoplasts to burst the containing membrane, releasing the protoplasmic contents; a vacuole would remain more or less intact and unaltered (Pl. 1, fig. 4). Observations by using specific nuclear stains and fluorescent microscopy suggested that most of the protoplasts contained only one nucleus, although some may have had two or more.

Some of the protoplasts showed very clearly what we call 'satellites', that is, very small, generally dark, spheres attached to the protoplasts by thin threads of different sizes (Fig. 2). They might be the remains of threads connecting protoplasts which had broken; but some protoplasts had three or more of these satellites. We also observed in several cases that after the liberation of protoplasts, the respective hypha was not completely empty, as might have been the case once the cellular contents had been extruded, but there remained inside the digested cell wall small spheres similar in size and appearance to the so-called satellites (Pl. 2, fig. 5).

In a hypotonic medium the protoplasts might increase in volume up to three times their original size; their appearance also changed. The membrane became almost invisible under the phase-contrast microscope, the cytoplasmic content was less refractile, and the internal structures were more evident. After incubation for several days in the medium where the protoplasts were formed, a considerable number of them are not digested by the lytic enzyme present. In some cases, however, the protoplasts were crenated and lost their spherical shape.

After long incubation, the hyphae were extensively digested and showed big pores or holes all along the wall. The septa, however, were much less attacked by the lytic enzyme, and could be seen free in the medium or united to the remains of the attacked hyphal walls (Pl. 2, fig. 6).

Effect of the stabilizing substances

Several osmotic stabilizers were used, all at 0·6 M concentration, for the formation of protoplasts from fusarium mycelium. Xylose, sucrose and maltose allowed the formation of only a small number of protoplasts; fructose and sorbose were intermediate; the best results were obtained with NH_4Cl, mannitol, NaCl or rhamnose. Negative results were obtained with Na_2CO_3, NaH_2PO_4 and $(NH_4)_2SO_4$, HN_4NO_3 and KNO_3 gave better results and KCl gave the same yield of protoplasts as mannitol. The NH_4Cl, however, gave much better results, and in a shorter time. The pH values may have been responsible for some of these effects. The stability of the protoplasts was markedly affected by the reaction of the suspending medium; the optimum for stability was pH 6–8.

The effect of stabilizers at several concentrations was studied. As a rule protoplasts burst at 0·1 M concentration, but with KNO_3, NaCl and $MgCl_2$ complete breakage was obtained at 0·05 M. When glycine or urea was used as stabilizer, the protoplasts are well stabilized at 1·5 M concentration, but at 1·0 M nearly all the protoplasts lysed. The phenonemon in this case may have been due to a direct effect on the membrane rather than to osmotic pressure. At the critical osmotic point the

protoplasts burst slowly, liberating the internal contents. At this stage the membrane was broken at one or more places and sometimes several internal structures remained within. Often the vacuoles remained visible in the medium for a long time before they burst, under these osmotic conditions.

The stability of the protoplasts was affected not only by the stabilizer used but also by the concentration of the medium in which the fungus was grown (Villanueva, 1966).

Effect of alterations in the composition of the growth medium

Cultural conditions may affect the sensitivity of cell walls to lytic enzymes, and changes in the composition of the growth medium may affect the composition of the cell walls. Dunwell, Ahmad & Rose (1961) suggested that growth conditions markedly affected the polysaccharide composition of fungal walls. Holter & Ottolenghi (1960) reported that the substitution of melibiose for glucose in the growth medium of a saccharomyces markedly affected its susceptibility to the action of lytic enzyme from snail. This may be explained by the building of a different type of polysaccharide in the cell wall of the yeast.

The effect of the carbon source in the growth medium of *Fusarium culmorum* on the susceptibility of the mycelium to the lytic enzyme was examined. No differences in the ability to release protoplasts were found when the glucose of the medium was replaced by fructose, galactose, mannose or sorbose at the same concentration.

Susceptibility of different fusarium species to the strepzyme RA

After preliminary trials with *Fusarium culmorum* other species of the same genus were tested for their susceptibility to strepzyme RA for obtaining protoplasts. No significant differences from those described above were found with: *F. oxysporum*, *F. avenaceum*, *F. equisetibulletum*, *F. scirpi*, *F. moniliforme*, *F. pomae*, *F. sporotrichiodes*, *F. tumidum*, *F. solani*, *F. cubense*, *F. roseum*, *F. semitectum* var. *majus*, *F. sambucinum*, *F. poae*.

Fusion of protoplasts

Protoplasts of *Fusarium culmorum* prepared by the digestion of the cell wall with the lytic enzyme preparation strepzyme RA (Rodriguez Aguirre *et al.* 1964) were observed to fuse. The frequency of this was very low and the whole process took less than 2 min. Direct continuous observations leave no doubt that fusion does occur. Plate 2, fig. 7 illustrates various stages of the fusing process. Owing to difficulties of resolution the details of union have not been seen within living fusarium protoplasts. The various stages of the process of fusion of protoplasts leave no doubt that the larger protoplasts thereby derived are complex forms. The principal conclusion at present is that fusion of fusarium protoplasts can take place and that the resulting form remains viable. Although the actual process of fusion may vary somewhat from case to case, each has in common the union of two spherical protoplasts. Protoplast fusion in *F. culmorum* usually occurs between two bodies which seem to be connected by a thin nearly invisible filament. Occasionally the filaments had some enlargements in the form of granules (Pl. 2, fig. 8). Their relative positions probably represented sister protoplasts from one mycelial compartment, although the possibility cannot be excluded that fusion may occur between bodies from

different compartments which become connected after release from the mycelium. Sometimes several protoplasts were connected by one thread (Pl. 2, fig. 9).

Practically nothing is known of the behaviour of the cell contents during or after protoplast fusion which results in the formation of a larger body. The spherical forms produced in this way have not been seen to develop further.

With the phase-contrast microscope it was observed that the vacuoles of the protoplasts after fusion remained unaltered and spherical, and some of the cytoplasmic elements appeared as dark as in the individual units. In general the resolution of structures within each of the fusing protoplasts was difficult. Although we earlier described the presence of cytoplasmic membrane enveloping each protoplast (Rodriguez Aguirre *et al.* 1964), phase-contrast microscopy of the fused protoplasts showed protuberances on the cell surface, mainly corresponding to the vacuoles. This is clear in the micrographs of the last stages of the fusing process (Pl. 2, fig. 7). The possibility cannot be neglected that fusion of free masses of protoplasm previously released into the suspending medium may take place. However, our experience with protoplasts, together with the fact that attempts to produce the fusing bodies by plasmolysis of the protoplasts has produced only negative results, speak in favour of fusion of true protoplasts.

The fusion of protoplasts of *Fusarium culmorum* was not very common under our experimental conditions. The physical properties of the environment may play a role in the fusion. We do not know whether the large bodies are able to develop by division or rupture. Some of these fused bodies do not develop and may remain apparently unchanged during a 6 hr period of observation. Some fused bodies undergo lysis and this may be preceded by increase in size as a consequence of water penetration. The lysis occurs suddenly and leaves only traces of cell body. Attempts to isolate a fused body with the help of the micromanipulator and to cultivate it have thus far been unsuccessful.

DISCUSSION

Much doubt has been expressed in the past in our laboratory about the fusion phenomenon with protoplasts. Strunk (1966) reported that protoplasts of *Polystictus versicolor* could fuse under appropriate conditions, two coalescing to form a larger sphere; the whole process was shown micro-cinematographically. As far as we are aware, no other reports of fusion of fungal protoplasts have appeared. The fusion of two protoplasts to form a larger body may be a consequence of the union between two spherical entities by visible filaments. It remains to be seen whether fusion can be produced between protoplasts arising from different hyphal compartments and lacking any connecting filaments. Fusion between protoplasts of *P. versicolor* seems chiefly to be a consequence of the attraction between spherical bodies joined by threads (Strunk, 1966). In this organism time-lapse cinematography showed a high frequency of fusion and the phenomenon was chiefly attributed to the presence of the connecting filaments. In the present work with *Fusarium culmorum*, however, no fused bodies were seen to form when protoplasts were suspended in stabilizers other than mannitol solution.

Large body formation with fusarium protoplasts appears to be primarily a physical phenomenon. No evidence of what happens to the enveloping membranes of the individual protoplasts is available. Whether the formation of a large body may

be interpreted as a sexual process is also unknown. Our impression is that the phenomenon is the result of some physical attraction, but it is difficult to tell whether the membrane exists as an independent entity in the mycelial cell. Evidence in other systems suggests that elasticity and versatility of the plasma membranes, mainly in the plant cells, exist (Dr D. H. Northcote, private communication). Whether the fused bodies are capable of reversion to form new mycelium, as already shown with isolated protoplasts of *Fusarium culmorum* (García Acha, Lopez-Belmonte & Villaneuva, to be published), remains to be seen. It has not been observed microscopically and direct observation of such reversion is essential. None of the results obtained in the present work yielded sufficient information to postulate a significance for the fused forms. Strunk (1966) considered the formation of fused bodies with *Polystictus versicolor* to be a natural phenomenon occurring during the process of protoplast formation.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Phase-contrast micrograph showing thread connecting the cytoplasmic contents of the hyphae (× 6000).

Fig. 2. Purified suspension of fusarium protoplasts; extent of contaminating mycelium showing the great variability in sizes and appearance (× 6000).

Fig. 3. Protoplasts of *Fusarium culmorum* showing vacuoles of different sizes (× 6000 and × 8000).

Fig. 4. Burst protoplasts of *F. culmorum* releasing the vacuoles (× 6000).

PLATE 2

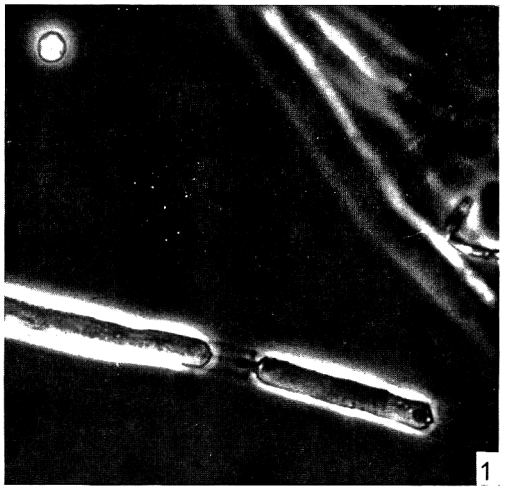
Fig. 5. Micrograph showing empty hypha with remaining granular materials (× 6000).

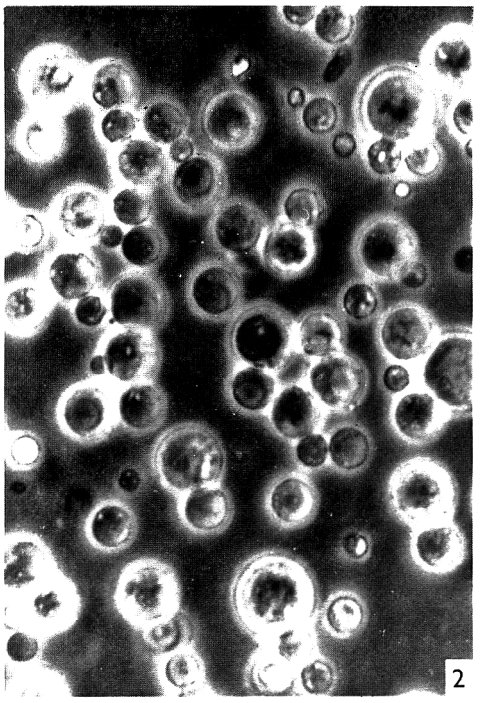
Fig. 6. Remnants of fusarium mycelium after protoplasts have been released. Note the intact septa. (× 6000).

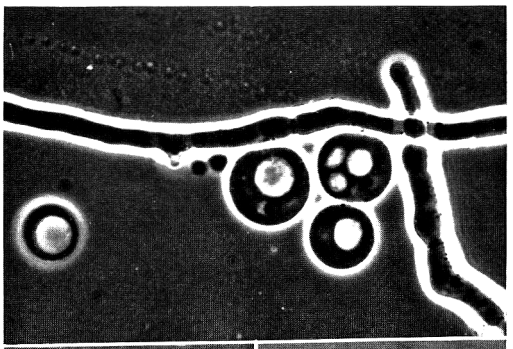
Fig. 7. Phase contrast micrograph of various stages of the fusing process of *Fusarium culmorum* protoplasts (× 6000).

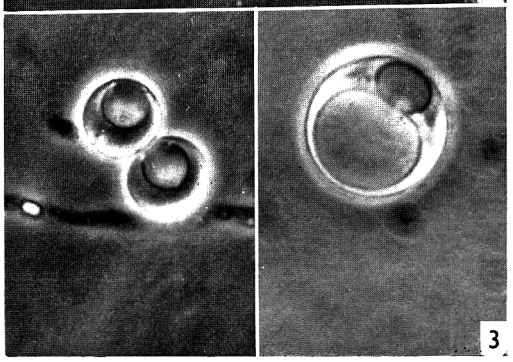
Fig. 8. Micrograph showing filaments connecting protoplasts (× 6000).

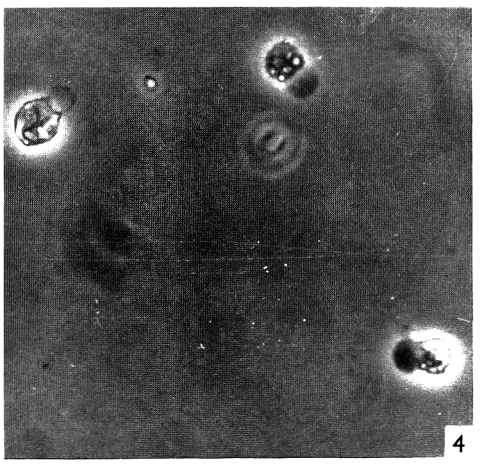
Fig. 9. Various protoplasts of *F. culmorum* connected by threads (× 4000).











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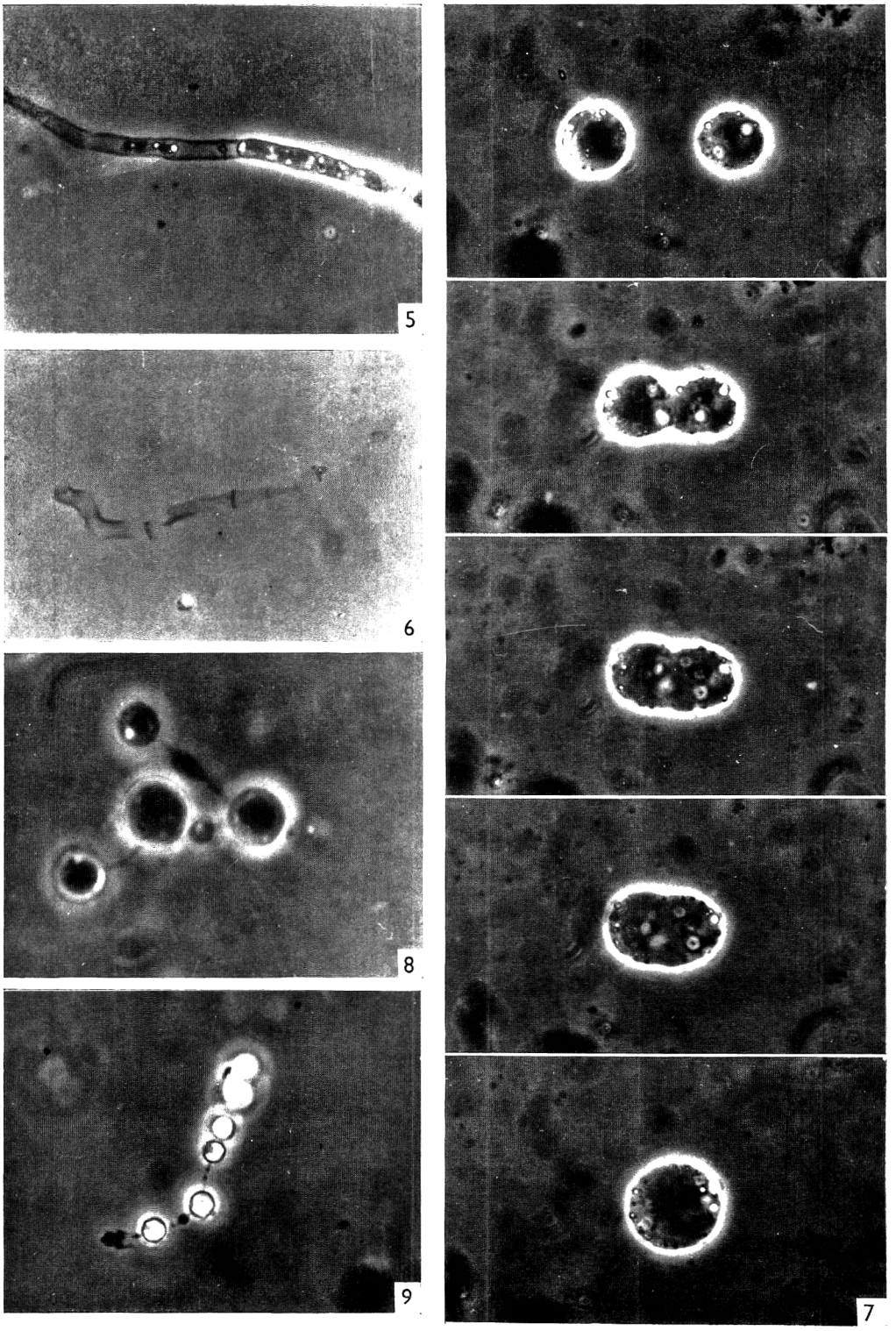

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The Interrelationship between Potassium, Magnesium and Phosphorus in Potassium-limited Chemostat Cultures of *Aerobacter aerogenes*

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(*Received* 3 *March* 1966)

SUMMARY

The growth of *Aerobacter aerogenes* cultures in a chemostat under conditions of K^+-limitation was investigated. At a fixed dilution rate there was a linear relationship between bacterial concentration and the K^+ concentration in the culture. The extrapolated plot did not pass through the origin, however; this indicated the presence in the medium of substance(s) supporting some growth in the absence of K^+. The growth yield varied markedly with the dilution rate; bacterial concentration decreased and the cellular K^+, Mg^{2+}, RNA and phosphorus contents increased as the 'steady-state' growth rate was increased. The yield variation was similar to that observed when either Mg^{2+} or PO_4^{3-} was the limiting component of the medium. Analysis of K^+-limited organisms revealed a molar stoichiometry between cellular Mg^{2+}, K^+ and P (close to 1:4:8, respectively) that was almost independent of growth rate. It is suggested that a precise intracellular K^+:Mg^{2+} ratio may be of importance for maintaining ribosomal structures in a suitable functional configuration or degree of aggregation, and it is for this purpose that high concentrations of K^+ are present in growing bacteria. K^+-limited *A. aerogenes* cultures oxidized glycerol rapidly, as did washed supensions of these organisms in phosphate buffer (pH 6·5). Glycerol (10 mM) accelerated the death-rate of K^+-limited bacteria; potassium (15 mM) and magnesium (15 mM) each decreased this glycerol toxicity and, when added together, abolished it. The death-rate of washed K^+-limited *A. aerogenes* suspensions in phosphate saline (pH 6·5) was a function of their 'steady-state' growth rate.

INTRODUCTION

Although most, if not all, bacteria have a requirement for potassium for growth (Lester, 1958), and the potassium content of organisms may be considerable (Rouf, 1964), the physiological basis of this requirement is not fully understood. Lubin & Kessel (1960) isolated a mutant of *Escherichia coli* which had a decreased ability to concentrate potassium from the environment and found that the growth rate of this mutant, in a defined medium, was proportional to its intracellular K^+ concentration. Furthermore, with this mutant Ennis & Lubin (1961, 1965) showed that when the intracellular K^+ concentration was decreased to a low value the organisms were unable to synthesize protein, though synthesis of RNA proceeded at a linear rate for some hours. They suggested that these conditions produced a specific impairment of protein synthesis similar to that imparted by chloramphenicol. Eddy & Hinshelwood (1951) concluded that potassium '. . . is associated with the actual functioning

of the cell, and is not, at any rate in the full amount, among the permanent structural requisites'. These authors suggested that intracellular potassium was associated with certain intermediates involved in the degradation of carbon substrates (as did Roberts, Roberts & Cowie, 1949) and that the specificity for this ion resulted from it being of a size suitable for incorporation into a temporary structure involving phosphorylated intermediates and enzyme surfaces. They further suggested that binding afforded protection to the bacteria by excluding hydrogen ions which would have a deleterious effect when present intracellularly in high concentration. Neither the above observations, nor the fact that potassium is an activator of certain enzyme systems (Dixon & Webb, 1958) seem to explain fully the need for the very high concentrations of potassium found in rapidly growing bacteria.

Since microbial growth can be made dependent on potassium, the concentration of this element in the medium can be adjusted to limit the growth of organisms in a chemostat culture. In such a K^+-limited environment the organisms must contain the minimum concentration of potassium necessary to satisfy the structural and functional needs for growth at the imposed rate. Comparison of these organisms with those from chemostat cultures in which other components of the medium limited growth, for example Mg^{2+}-limited organisms (Tempest, Hunter & Sykes, 1965), and a systematic analysis of the effect of changes in growth rate on the physiology of the organisms in the culture may thus provide an approach to the problem of identifying the structural and functional sites which require the high concentrations of potassium found in the growing organism. The present paper describes the changes in bacterial RNA, K^+, Mg^{2+} and PO_4^{3-} contents which followed changes in the growth rate of K^+-limited *Aerobacter aerogenes* cultures. The significance of these findings is discussed in relation to the control of ribosome structure and function for which, it is suggested, precise K^+:Mg^{2+} ratios may be of fundamental importance. A preliminary report on our findings has been published (Dicks & Tempest, 1966).

METHODS

Organism. Aerobacter aerogenes (NCTC 418) was maintained, and subcultured monthly on tryptic meat digest agar slopes.

Growth conditions. Cultures were grown in 0·5–1 chemostats of the design described by Herbert, Phipps & Tempest (1965). Temperature was controlled automatically at 35°, and the pH value at $6·5 \pm 0·1$. Foaming of the culture was suppressed by the regular addition of small volumes (0·1–0·2 ml.) of Polyglycol P-2000 antifoam (Dow Chemical Co., Midland, Michigan, U.S.A.).

The culture medium (K^+-limited) contained: Na_2HPO_4, $5·0 \times 10^{-3}$M; $(NH_4)H_2PO_4$, $4·5 \times 10^{-2}$M; $(NH_4)_2SO_4$, $2·5 \times 10^{-2}$M; citric acid, $1·0 \times 10^{-3}$M; $MgCl_2$, $1·25 \times 10^{-3}$M; $CaCl_2$ and $FeCl_3$, each $1·0 \times 10^{-4}$M; Mn^{2+}, $2·5 \times 10^{-5}$M; Cu^{2+} and Na_2MoO_4, each $5·0 \times 10^{-6}$M. Glycerol was added to a final concentration of 30 mg./ml. and K_2SO_4 to $5·0 \times 10^{-4}$M. Medium was prepared in 20 l. volumes with distilled water which had been passed through a mixed-bed ion-exchange resin column. The final pH value after autoclaving (121°, 30 min.) was about 5·3; the medium was adjusted to the pH value required for the growth of the organism in the culture vessel.

Analytical procedures. Bacterial concentration (mg. dry weight organism/ml. culture), and their RNA, DNA, carbohydrate and protein contents were determined

by previously described methods (Tempest *et al.* 1965). Magnesium contents of organisms were determined by the method of Gardner (1946) on 0·5 N perchloric acid extracts of organisms. Traces of Polyglycol P-2000 present in these extracts interfered with the assay and were removed by treatment with light petroleum (b.p. 60°–80°) (Tempest & Strange, 1966). Total phosphorus was determined by the method of King (1932) after digestion of the organisms with 60% (w/v) perchloric acid.

Determination of bacterial potassium content. Difficulty was experienced in obtaining a reliable estimate of the K^+ content of growing organisms because the separation of the bacteria from the culture fluid and subsequent washing procedures generally resulted in the loss of some potassium from the organisms. Thus, more than 15% was lost when the organisms were washed once in saline, and over 40% when they were washed once in distilled water. It was found that no loss of K^+ occurred when organisms were washed in 0·1 M-$MgCl_2$ solution (pH 6·0–6·5). It was also found that the extracellular K^+ concentration in K^+-limited cultures of *Aerobacter aerogenes* was exceedingly small; this simplified the problem by eliminating the necessity for washing these bacteria before determining their potassium content. A further difficulty due to the presence of interfering substances (principally phosphate) in the culture supernatant fluid and in bacterial extracts was overcome by adopting the following procedure for determining the distribution of K^+ in the cultures. Two 5 ml. samples of culture were collected, one of which was heated at 100° for 5 min. to release the K^+ from the organisms. Both samples were centrifuged (3000 *g*. 5 min.) and the clear supernatant fluids collected. These were analysed for potassium by using an E.E.L. Flame Photometer (mark II) and standard solutions of K^+ (10–50 μg./ml.) in 0·05 M-Na_2HPO_4 (i.e. the concentration of PO_4^{3-} in the medium). With this method recovery of added K^+ was better than 98% and estimates of total K^+ in the culture were always within 3% of the amount initially added to the medium (taking into account dilution by NaOH additions necessary for pH control).

Determination of culture Q_{O_2} *and* Q_{CO_2} *values.* These were calculated from the rates of air flow through the culture, the oxygen and carbon dioxide contents of the effluent gas (determined with a Beckman Model E-2 oxygen analyser and a Mines Safety Appliances Co. 'Lira' Infra-red CO_2 Analyser), the culture volume and the bacterial concentration. The Q_{O_2} values of washed suspension of organisms were determined by standard techniques with a Warburg manometer.

Viability. Measurements were made by the slide culture technique described by Postgate, Crumpton & Hunter (1961).

Storage. 0·8–5·0 ml. volumes of culture were diluted with K^+-free phosphate saline (6·7 mM-phosphate in 0·85% (w/v) NaCl; pH 6·4) to contain the equivalent of 1 mg. dry wt. organism/ml. The suspension (5 ml.) was centrifuged, and the deposited bacteria washed in K^+-free phosphate saline, and resuspended in this buffer to a concentration equivalent to 20 μg. dry wt. organism/ml. The suspensions were incubated at 37°, with aeration, and viabilities determined at regular time intervals.

RESULTS

Influence of K^+ concentration and dilution rate on the 'steady-state' bacterial concentration

Figure 1 shows the relationship between bacterial concentration and potassium content of *Aerobacter aerogenes* cultures grown at a dilution rate of 0·4 hr^{-1}. The linear nature of this relationship indicates that growth was being limited by the availability of potassium, though it is apparent from the fact that the plot does not pass through the origin that some other substance (or substances) in the medium was supporting a small amount of growth of *A. aerogenes*, and could do so in the

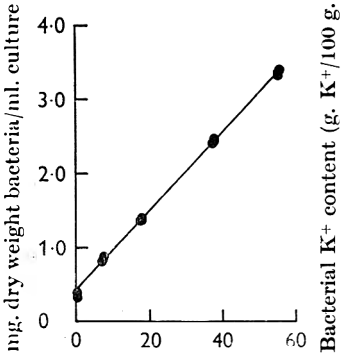

Fig. 1

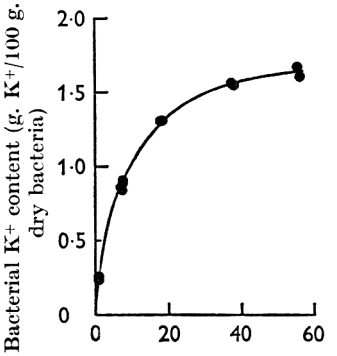

Fig. 2

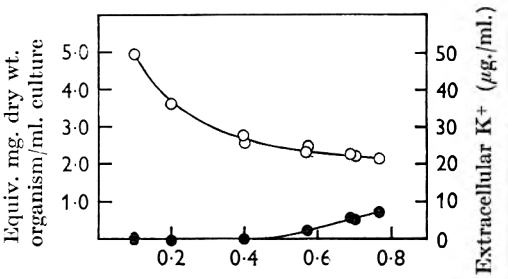

Fig. 3

Fig. 1. Plot of 'steady-state' concentration of *Aerobacter aerogenes* against culture K^+-concentration. Dilution rate fixed at 0·4 hr^{-1}; temperature 35°; pH 6·5. Values determined over a period of 2–3 days, after initial equilibration of the culture for 2 days at each K^+ input concentration.

Fig. 2. Plot of 'steady-state' potassium content of *Aerobacter aerogenes* against culture K^+-concentration. Dilution rate fixed at 0·4 hr^{-1}; temperature 35°; pH 6·5. Values determined over a period of 2–3 days, after initial equilibration of the culture for 2 days at each K^+ concentration.

Fig. 3. Plot of 'steady-state' concentrations of *Aerobacter aerogenes* and extracellular K^+ as a function of dilution rate. Values determined over a 2–3 day period at each dilution rate, and after at least 2 days equilibration. ○, mg. dry wt. bacteria/ml. culture; ●, μg. extracellular K^+/ml. culture.

virtual absence of potassium. Since all the culture K^+ was present in the organisms, it follows from Fig. 1 that the bacterial K^+ content varied with the culture K^+ concentration. This is shown in Fig. 2. The probable explanation of this finding is that the sum of the K^+ and 'K^+ replacement factor' contents of the bacteria was constant, but the relative proportion of each depended on their concentration in the culture. Since the culture concentration of 'replacement factor' was constant, increasing the medium K^+ concentration resulted in progressive increases in bacterial K^+ content. The latter tended to a maximum value (about 1·5% of the bacterial dry weight) at concentrations above 40 μg. K^+/ml. culture, where, presumably, the growth contribution from 'replacement factor' was relatively insignificant. All subsequent experiments were carried out on organisms growing in the presence

of about 40 μg. K^+/ml. culture (i.e. 1 mM-K^+). With such cultures the bacterial concentration varied markedly with the dilution rate (Fig. 3). This variation in yield (g. bacteria/g. K^+) was similar to that observed by Tempest *et al.* (1965) with Mg^{2+}-limited cultures of this organism and by Dr D. Herbert (personal communication) with PO_4^{3-}-limited cultures. A similar pattern of change has been reported

Table 1. *Gross composition of K^+-limited Aerobacter aerogenes organisms grown in a chemostat at different dilution rates*

The figures quoted in this table are average values from at least three samples collected, processed and analysed on separate days. At each dilution rate (D) the culture 'steady-state' viability was greater than 90 %.

D (vol./hr)	Protein	Acid sol. material (as RNA)	RNA	DNA	Carbohydrate
	g. component/100 g. lyophilized bacteria				
0·1	72·9	1·2	7·3	2·2	2·5
0·2	73·3	1·4	11·7	1·8	2·8
0·4	72·5	1·3	15·0	2·4	3·6
0·6	73·8	1·2	17·4	2·6	4·0
0·8	67·6	1·6	17·6	2·1	4·7

Table 2. *Potassium, magnesium and phosphorus contents of Aerobacter aerogenes organisms grown in a chemostat at different dilution rates (D)*

D (vol./hr.)	Component		
	K^+	Mg^{2+}	P
	g. component/100 g. dry bacteria		
0·09	0·81	0·124	—
0·10	0·80	0·122	1·31
0·19	1·07	0·156	1·69
0·21	1·09	0·163	—
0·37	1·38	0·189	—
0·38	1·45	0·188	—
0·40	1·40	0·198	2·02
0·40	1·49	0·200	2·32
0·56	1·59	0·211	—
0·57	1·55	0·227	2·38
0·58	1·47	0·208	2·23
0·69	1·45	0·231	2·44
0·70	1·51	0·224	2·39
0·76	1·68	—	—
0·77	1·46	0·212	2·56

with NH_4^+-limited cultures (Holme, 1957; Herbert, 1961) which resulted from a gross variation in the polysaccharide content of the organisms. The changes in polysaccharide content of K^+-limited *A. aerogenes* with dilution rate (Table 1) could not account for the above yield variation.

Variation in bacterial macromolecular composition with changes in growth rate

The protein, RNA, DNA and carbohydrate contents of K^+-limited *Aerobacter aerogenes*, grown at five different dilution rates, are detailed in Table 1. Whereas irregular patterns of change were apparent in bacterial DNA and protein contents, both the RNA and carbohydrate contents increased progressively with dilution rate. The variation in RNA content of these organisms was similar in amount to those observed with other types of growth limitation (Tempest *et al.* 1965) and with other organisms (Herbert, 1961; Neidhardt, 1963), and were most probably due to variations in the ribosome content of organisms (Ecker & Schaechter, 1963). The changes in bacterial polysaccharide content were similar to those observed with Mg^{2+}-limited cultures (Tempest *et al.* 1965) and opposite to those with NH_4^+-limited organisms (Herbert, 1958).

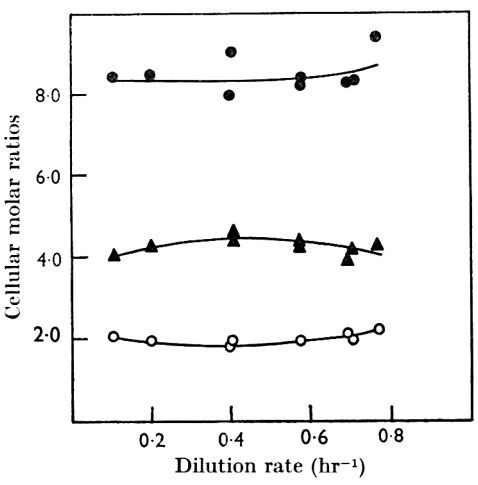

Fig. 4

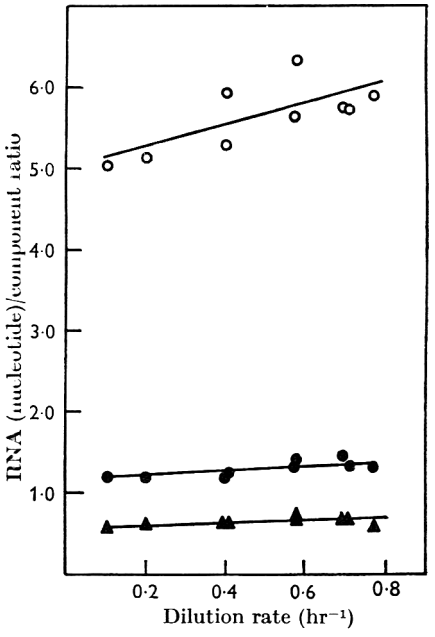

Fig. 5

Fig. 4. Stoichiometry between intracellular potassium, magnesium and phosphorus in chemostat cultures of *Aerobacter aerogenes* grown at different dilution rates. Molar ratios of: ○, phosphorus:potassium; ●, phosphorus:magnesium; ▲, potassium:magnesium.

Fig. 5. Stoichiometry between cellular RNA and potassium, magnesium and phosphorus in chemostat cultures of *Aerobacter aerogenes* grown at different dilution rates. Molar ratios of: ○, RNA (nucleotide):magnesium; ●, RNA (nucleotide):potassium; ▲, RNA (nucleotide):phosphorus.

Variations in bacterial potassium, magnesium and phosphorus contents with changes in growth rate

The changes in bacterial growth yield with dilution rate (Fig. 3) indicated a correlation between the K^+ content of K^+-limited *Aerobacter aerogenes* organisms and their rate of growth. As similar correlations have been found with the Mg^{2+} content of *A. aerogenes* (Tempest *et al.* 1965; Tempest & Strange, 1966) and the

phosphorus content of *Escherichia coli* (Wade, 1952), the K^+, Mg^{2+} and P contents of K^+-limited *A. aerogenes* organisms were determined at several 'steady-state' growth rates between 0·09 hr^{-1} and 0·77 hr^{-1} (Table 2). The corresponding molar K^+:Mg^{2+}, P:Mg^{2+} and P:K^+ ratios (Fig. 4) show that the intracellular concentrations of all three components were similarly influenced by growth rate and the molar ratios (approximately 1:4:8, for Mg^{2+}:K^+:P) were almost independent of growth rate. Furthermore these changes paralleled changes in bacterial RNA content (Table 1) as is evident from the molar RNA (nucleotide):Mg^{2+} and RNA:K^+ ratios, shown in Fig. 5.

Changes in the respiratory activity of K^+-limited Aerobacter aerogenes cultures with growth rate

Herbert (1958) reported the Q_{O_2} and Q_{CO_2} values of glycerol-limited *Aerobacter aerogenes* cultures varied from 150 to 485 and 115 to 400, respectively, as the dilution rate was increased from 0·1 to 0·6 hr^{-1}; comparative figures for K^+-limited cultures of this organism are shown in Table 3. In carbon-limited cultures the rate of oxygen uptake must reflect the minimum rate of substrate oxidation necessary to support the growth of organisms at the imposed rate. The values reported in Table 3 are clearly much larger than these estimated minima (i.e. Q_{O_2} of 150 at D = 0·1 hr^{-1} and 485 at D = 0·6 hr^{-1}), suggesting that the growth of K^+-limited *A. aerogenes* was not restricted by its ability to oxidize the carbon source (glycerol) at the necessary rate. This conclusion was supported by the observation that washed suspensions of K^+-limited organisms (grown at a dilution rate of 0·2 hr^{-1}), which contained substantially less potassium than that present during growth because they had been washed in water, oxidized glycerol at a rate which was not influenced by the presence of 10 mM K^+ in the suspending medium (Q_{O_2} = 201). These organisms also oxidized glucose, without delay, at a rate which was only slightly increased by 10 mM K^+ (Q_{O_2} values of 266 and 273, respectively).

Table 3. *Influence of dilution rate on the respiratory activity of K^+-limited Aerobacter aerogenes cultures grown in a chemostat*

Average 'steady-state' values measured over 2–4 days.

Dilution rate (hr^{-1})	Oxygen uptake (µl./equiv. mg. dry wt. bacteria/hr)	CO_2 output (µl./equiv. mg. dry wt. bacteria/hr)
0·1	289	315
0·2	375	436
0·4	574	634
0·6	721	720

Effect of storage, in the presence and absence of glycerol, on the viability of washed K^+-limited Aerobacter aerogenes

Routine viability measurements on K^+-limited *Aerobacter aerogenes* cultures, grown at several dilution rates, showed the presence of more non-viable organisms (i.e. up to 10% of the population at some dilution rates) than had been encountered with corresponding glycerol-limited or Mg^{2+}-limited cultures (Tempest *et al.* 1965). Experiments were done, therefore, to determine the death-rate of K^+-limited *A. aerogenes* in the presence and absence of a carbon + energy source (glycerol).

Figure 6 shows the changes in viability with time of saline-phosphate-washed bacteria from K^+-limited *Aerobacter aerogenes* cultures, grown at several different dilution rates and incubated at 37° in aerated phosphate saline (pH 6.5). The death-rate correlates with the dilution rate; the higher the dilution rate at which organisms were grown, the longer they survived in the non-nutrient environment (cf. Postgate & Hunter, 1962). K^+ (15 mM) did not significantly influence the death-rate of these starved K^+-limited bacteria, but glycerol was markedly toxic (Fig. 7). The toxicity of glycerol for washed K^+-limited *A. aerogenes* was reduced by the addition of either 15 mM K^+ or 15 mM Mg^{2+} to the suspension fluid; mixtures of these ions totally prevented glycerol-accelerated death of these populations (Fig. 7).

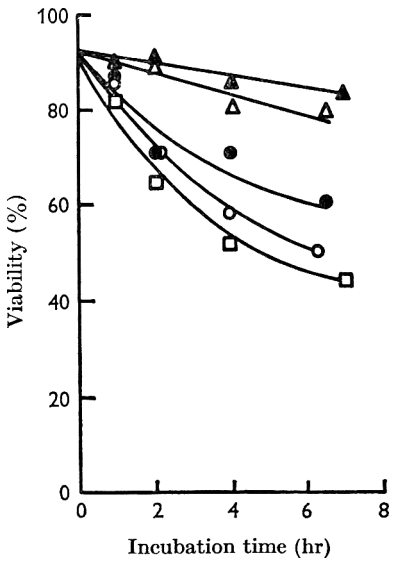

Fig. 6

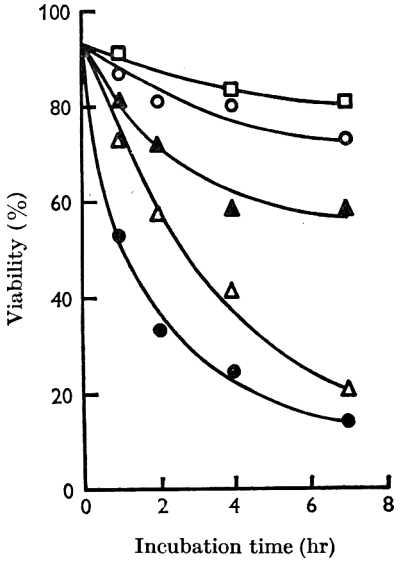

Fig. 7

Fig. 6. Effect of storage on the death of K^+-limited *Aerobacter aerogenes* grown in a chemostat at different dilution rates. Organisms were washed, suspended in phosphate saline (pH 6·5), aerated and incubated at 37°. Viabilities were determined by slide culture. Organisms were grown at dilution rates of: □, 0·1 hr^{-1}; ○, 0·2 hr^{-1}; ●, 0·4 hr^{-1}; △, 0·6 hr^{-1}; ▲, 0·8 hr^{-1}.

Fig. 7. Effect of potassium and/or magnesium on glycerol-accelerated death of K^+-limited *Aerobacter aerogenes*. Organisms were grown at a dilution rate of 0·6 hr^{-1} (temperature 35°; pH 6·5), washed and suspended in phosphate saline (pH 6·5). Suspensions were aerated and incubated at 37° in the presence of: ●, 10 mM-glycerol; △, 10 mM-glycerol + 15 mM K^+; ▲, 10 mM glycerol + 15 mM Mg^{2+}; □, 10 mM glycerol + 15 mM K^+ + 15 mM Mg^{2+}. No additions were made to the control suspension (○).

DISCUSSION

Although the 'steady-state' concentration of *Aerobacter aerogenes* at a fixed dilution rate was a function of the culture K^+-concentration, the extrapolated plot did not pass through the origin, thus suggesting the organisms did not have an absolute potassium requirement for growth. It has been reported that rubidium can replace potassium for the growth of bacteria (Eddy & Hinshelwood, 1950;

Lester, 1958) but this element was not intentionally added to the medium and, as a contaminant of the salts used in compounding the medium, would have to be present in a concentration of 0·15–0·2 mM (assuming the molar-growth yield to be similar to that for potassium) to account for the apparent degree of growth in the complete absence of potassium. Although the most obvious explanation for the observed growth of *A. aerogenes* in the absence of potassium is the presence of a substance, such as rubidium, which can substitute, functionally, for potassium, other explanations are possible. For example, errors in the determination of either culture bacterial dry weight or K^+ concentration could produce the result observed. However, duplicate determinations of bacterial dry weights never varied by more than 5%, and day-to-day variations in the culture dry weights were also small (Fig. 1); lysis of organisms was not observed. The potassium assay was thoroughly checked; determinations of potassium present in the culture always agreed closely with that calculated to be present, amounts of potassium added to the culture were quantitatively recovered and hot water extracts of K^+-limited bacteria contained amounts of potassium equal to that detected in the whole culture (little potassium being present in the extracellular fluid of cultures growing at a dilution rate of 0·4 hr^{-1}).

The limited amount of growth observed in the absence of added potassium has theoretical implications. Thus, the yield of organisms (g. bacteria/g. K^+) varied with culture K^+ concentration and tended to infinity as the culture K^+ concentration approached zero. Bacterial growth yields are generally supposed to be independent of the growth-limiting substrate input concentration (Monod, 1950; Herbert, Elsworth & Telling, 1956; Powell, 1965); clearly this is only true when the organisms have an absolute requirement for the growth-limiting component of the medium. Although in our experiments the growth yield varied with culture K^+ concentration, the 'incremental yield' (increase in dry weight/unit increase in culture K^+ concentration) was constant. The incremental yield and growth yield (defined above) are equal when the organisms have a specific, and absolute, requirement for the growth-limiting component of the medium; that is, when the plot of culture bacterial concentration against limiting component concentration passes through the origin.

The marked variation in culture bacterial concentration with dilution rate was almost identical to that observed with Mg^{2+}-limited and PO_4^{3-}-limited cultures of *Aerobacter aerogenes*; this suggested that the bacterial K^+, Mg^{2+} and phosphorus contents were each functions of the growth rate. This was clearly so with K^+-limited organisms (Table 2) and the observed growth-rate-independent stoichiometry between these three components (Fig. 3) implied a functional interrelationship. The only major macromolecular component of bacteria the concentration of which is invariably a function of growth rate (at a fixed temperature) is the RNA (Herbert, 1961; Neidhardt, 1963, Rosset, Monier & Julien, 1964), or more specifically the bacterial ribosome content (Ecker & Schaechter, 1963). The stoichiometry between RNA, K^+, Mg^{2+} and phosphorus contents of *A. aerogenes* (Fig. 4) is strongly indicative of K^+ and Mg^{2+} involvement in RNA (presumably ribosome) synthesis and function. Magnesium is a structural component of ribosomes (Tissières & Watson, 1958; Rodgers, 1964) and the magnesium content of bacteria varies with their ribosome content (Tempest & Hunter, 1965; Tempest & Strange, 1966). The structural integrity and functional ability of ribosomes may, however, require other

cations beside Mg^{2+}. The degree of aggregation, and the stability, of ribosomes *in vitro* depends not only on the concentration of Mg^{2+} in the environment, but on the monovalent cation:Mg^{2+} ratio (Petermann, 1964; Cammack & Wade, 1965). The close correlation between the K^+ and Mg^{2+} contents of growing *A. aerogenes* suggests, therefore, that the high intracellular K^+ concentration observed may be required to maintain ribosomal structures in a suitable functional configuration or state of aggregation.

A large proportion of the potassium of growing *Aerobacter aerogenes* organisms was leached from the organisms by suspension in distilled water, without significant effect on their oxidative metabolism and viability. However, these organisms did not synthesize protein when suspended in a K^+-free, but otherwise complete medium (unpublished observation). This agreed with the conclusions of Ennis & Lubin (1961) and of Anderson (1966) that potassium depletion impaired the ability of organisms to synthesize protein. On the other hand, Strange & Shon (1964) observed that heat-shocked *A. aerogenes* rapidly lost RNA and viability when suspended and aerated in high concentrations of K^+. They interpreted this result as indicating an increased uptake of K^+ by the organisms and breakdown of ribosomal structures because of K^+:Mg^{2+} imbalance. These observations reinforce the view that a critical intracellular K^+:Mg^{2+} ratio may be essential for protein synthesis to occur at the maximum rate; excretion of K^+, such as occurs when bacterial growth ceases (Eddy, Carroll, Danby & Hinshelwood, 1951), may be the mechanism whereby protein synthesis is initially halted—particularly when growth ceases through the depletion of some nutrient other than the carbon and nitrogen sources.

Because potassium is rapidly lost from organisms which cease to grow and, in K^+-limited cultures, would be immediately taken up by the growing organisms, changes in the yield and bacterial K^+ content of such cultures with dilution rate might be caused merely by a large variation in culture viability. Viabilities were, on the whole, lower in K^+-limited *Aerobacter aerogenes* cultures than in corresponding glycerol-limited or Mg^{2+}-limited cultures (Tempest *et al.* 1965), but nevertheless they were always more than 90% and therefore do not undermine the significance of the observed quantitative relationships between RNA, K^+, Mg^{2+} and phosphorus in these organisms. Variations in culture viability with dilution rate did occur, but were slight; viabilities were marginally higher in the faster-growing cultures which correlated with their decreased susceptibility to death by starvation (Fig. 5). The death-rate of washed K^+-limited *A. aerogenes* was accelerated by glycerol (Fig. 6); thus, the presence of excess glycerol in K^+-limited cultures may have had an effect on viability. Magnesium ion prevented glycerol-accelerated death of other organisms (Postgate & Hunter, 1964; Strange & Dark, 1965) and were present in excess of requirement in the K^+-limited *A. aerogenes* cultures. However, Mg^{2+} did not abolish the toxicity of glycerol for K^+-limited organisms, except when added in the presence of K^+ (Fig. 6).

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Host Influence on the Ultrastructure of Root Nodules of *Lupinus luteus* and *Ornithopus sativus*

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SUMMARY

The ultrastructure of *Lupinus luteus* L. and *Ornithopus sativus* Brot. root nodules derived from infection by *Rhizobium lupini* strain D25 was examined. In both cases the associations produced completely effective nodules but their gross morphology was markedly different. Bacteria within the *O. sativus* nodules were enclosed in groups by a membranous envelope, and the bacteria had electron translucent regions. Bacteria within the *L. luteus* nodules were enclosed singly and did not exhibit similar electron translucent regions. Starch granules were observed within infected cells of *L. luteus* but not within uninfected cells; the reverse was observed in *O. sativus* cells.

INTRODUCTION

Electron microscope investigations of structural relationships between the bacteria and host cell in legume root nodules were first undertaken by Bergersen & Briggs (1958). Extensive studies in this field have subsequently appeared in the literature (Dart & Mercer, 1963*a–c*, 1964; Mosse, 1964; Jordan, Grinyer & Coulter, 1963; Dixon, 1964). A common feature of these investigations has been the demonstration of a membrane surrounding one or more bacteroids at the bacteroid stage of nodule development. The origin of this membrane, following the release of bacteria from the infection thread, appeared to differ in different legume species (Dixon, 1964; Jordan *et al.* 1963); this was not investigated in the work.

Starch deposits within infected host cells of Trifolium nodules have been noted by Dart & Mercer (1964), but the identity of storage substrate within the bacteria of fully effective nodules has not generally been well defined. Mosse (1964) tentatively identified electron-translucent regions within bacteria in Trifolium nodules as carbohydrate; however carbohydrate inclusions are often associated with ineffectiveness (Bergersen, 1957; Nutman, 1959; Golebiowska & Sypniewska, 1962). In an examination of laboratory-grown rhizobia Vincent, Humphrey & North (1962) identified electron-translucent regions within the cells as granules of poly-β-hydroxybutyric acid. This polymer has also been detected in nodules of *Mimosa* and *Pueraria* species (Forsyth, Hayward & Roberts, 1958); however, Golebiowska & Sypniewska (1962) reported the absence of lipid substances in *Rhizobium lupini* within nodules of *Lupinus* species.

The present study forms part of a research programme aimed at elucidating some of the host-bacteria relationships at the biochemical level. Strain D 25 of *Rhizobium lupini* offers a unique approach to this study since it forms effective symbiotic associations with *Lupinus luteus* and with *Ornithopus sativus*. Thus differences in nodule ultrastructure between the two species may be attributed to host influences. Properties such as viability and morphology, which would enable classification of these nodule bacteria as either the normal rod or bacteroidal type, have been insufficiently examined to yield a definite separation according to the criteria adopted by Jordan (1962). Previous estimates (Spicher, 1954) indicate that the normal rod types may predominate in nodules of *L. luteus* and *O. sativus*. For these reasons, the term bacteroid has not been used in the present study.

METHODS

Rhizobium lupini strain D 25, used for seed inoculation, was grown on a yeast-mannitol agar medium. Surface-sterilized seeds were inoculated and glasshouse grown on a sand + vermiculite medium (Bergersen, 1958). Nodules were harvested at 40 days and material selected from the central regions of the smallest and largest nodules of *Lupinus luteus* and from two regions, distal and proximal to the root respectively, of single *Ornithopus sativus* nodules. In terms of nodule development these selections were not exactly analogous since the nodules of *L. luteus*, unlike those of *O. sativus*, do not possess a well-localized meristematic region. However, the two selections from each plant may be regarded as approximately comparable in terms of the age of the tissues selected. Leg haemoglobin, indicating nitrogen-fixing activity, was present in all samples.

The embedding agent used was: Epon 812 (Shell Chemical Co., Sydney, New South Wales), 15 g.; Epikote 826, 8 g.; Epikote 871 (Indalab Pty. Ltd., Granville, New South Wales) 2·5 g.; CIBA Hardener HY 964, 21·5 g.; CIBA Hardener HY 906 (CIBA Co. Pty. Ltd., Lane Cove, New South Wales), 6·25 g.. To 35 g. of this, 0·66 g. of the catalyst DMP-30 (CIBA Accelerator Dy 064, CIBA Co. Pty. Ltd., Lane Cove, New South Wales) was added for polymerization.

Samples were fixed in 2·5% (v/v) glutaraldehyde in 0·1 M- phosphate buffer (pH 7·0) for 12 hr at 4°, washed in buffer alone with four changes over 2 hr and then fixed with 2·0% (w/v) osmium tetroxide in 0·1 M-phosphate buffer at 4° for 2 hr and washed as for pre-fixation. Dehydration was done in 25, 50, 70 and 100% (v/v) acetone in water in 30 min. steps, except for an overnight period in 70% acetone. The embedding polymer catalyst (DMP-30) was introduced in a 0·5% (w/w) solution in acetone for 30 min. and followed by 2 × 15 min. washings with acetone. A 50% (w/w) solution of the embedding monomer (Epon) was introduced for 2 hr and followed by a 24 hr incubation at 45° in Epon alone. Tissue samples were then placed in gelatin capsules, Epon (0·6%, w/w, DMP-30) was added and polymerization allowed to proceed for 3 days at 60°.

An LKB Ultratome with glass knives was used for sectioning. Sections were stained with saturated uranyl acetate in 50% (v/v) ethanol in water followed by a lead hydroxide stain (Karnovsky, 1961).

RESULTS

Gross morphology and development

The different nodule habits of these plants are illustrated in Pl. 1, figs. 1, 2. The 'single' nodule encircling the root of *Lupinus luteus* resulted from several separate sites of infection which had developed as discrete nodules and then merged together (material for electron microscopy was sampled just before this merging of separate infection sites). When sectioned, the apparently single nodule revealed discrete regions of infected tissue corresponding to the initial infection sites. Merging together of nodules was not exhibited by *Ornithopus sativus*, this was apparently due to the relatively localized meristematic region at the nodule tip. Unlike *L. luteus* nodules, which in healthy plants do not exhibit senescence until near the flowering stage, *O. sativus* nodules exhibit partial senescence, as evidenced by loss of leg haemoglobin, in the older region of the nodule at about 30 to 45 days after nodulation. This region occupies an increasingly larger proportion of the nodule until, at flowering, nearly all of the first formed nodules are devoid of leg-haemoglobin.

Electron microscopy

The relationship of the bacteria to the enclosing membrane in the younger tissue in each host is illustrated in Pl. 2, figs. 3, 4. Membranes enclosed only one bacterium in *Lupinus luteus* while enclosed groups were common in *Ornithopus sativus*. In the older tissues (Pl. 3, figs. 5, 6), the region enclosed by each membrane was expanded and in *O. sativus* most membranes contained groups of bacteria, while in *L. luteus* only single bactera were enclosed. The latter observation is supported by the recent work of Jordan & Grinyer (1965).

The presence of large electron-translucent regions in *Ornithopus sativus* nodule bacteria and their absence from *Lupinus luteus* is illustrated in Pl. 4, figs. 7, 8. The presence of starch grains within infected cells of *L. luteus* and in uninfected cells adjacent to infected cells in *O. sativus* is also illustrated in Pl. 4, figs. 7, 8. Mitochondria within infected host cells were similarly distributed in each species, being largely confined to the cell-wall regions, and in particular, adjacent to intercellular spaces. The numbers of mitochondria in infected cells appeared to be much greater than in uninfected cells.

DISCUSSION

The differences observed here in nodule ultrastructure suggest host control of (i) division of the membrane envelopes; (ii) certain bacterial cell inclusions; (iii) the site of deposition of starch within infected tissue. The enclosure of a group of bacteria by a single membrane envelope in *Ornithopus sativus* resembles the situation in soybean (Bergersen & Briggs, 1958). The reason for this is unknown, but it is apparent that bacterial division, at least in the final one, two or three divisions, was not accompanied by division of the enclosing membrane which nevertheless continued to grow in size. Observations of ultrastructure in *O. sativus* nodules 50–60 days old showed that membrane expansion continued until very little of the host cell volume was external to the membrane envelopes. Thus the region enclosed by the membranes, external to the bacteria, became a very large proportion of the total volume of the plant cell. From the point of view of spatial relationships, this region

suggests itself as a site of some importance in the metabolic interactions of bacteria and host.

The electron-translucent inclusions in *Ornithopus sativus* nodule bacteria closely resemble those observed in soybean which have been identified as granules of poly-β-hydroxybutyric acid (Dr F. J. Bergersen, personal communications). However, chemical analysis indicates the absence of poly-β-hydroxybutyric acid from bacteria of either host species, although a large proportion of chloroform-extractable material, as yet unidentified, has been found. A sharp peak at 284 mμ was obtained for this substance by the poly-β-hydroxybutyric acid assay method of Law & Slepecky (1961). The host influence responsible for this difference in cellular inclusions is possibly one involving the nutritional environment but at present no essential biochemical differences have been found. The differences in the distribution of starch are not readily explicable and must await further biochemical elucidation. Both types of starch distribution have been described from light microscope studies of root nodules (Fred, Baldwin & McCoy, 1932). The distribution of mitochondria suggests localization of certain metabolic processes and the reason for this localization might be explained in terms of an oxygen requirement not provided by the essentially anaerobic interior of an infected plant cell. Host influences on the nodule, including nodule size, absence or short-lived leg haemoglobin, glycogen inclusions within the bacteria, failure to develop the characteristic bacteriodal morphology and premature nodule senescence are well known in ineffective associations (Nutman, 1959). It is also evident that even in completely effective symbioses, the host plant may not only cause marked differences in the gross morphology of the nodule but also in ultrastructure within the infected cells, including the bacterial cell.

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EXPLANATION OF PLATES

Key to symbols. *B*, bacterium; *St*, starch; *M*, mitochondrion; *Et*, electron translucent areas in bacteria; *Cw*, cell wall of host cell; *Em*, enclosing membrane.

Electron micrographs are from tissue of nitrogen-fixing nodules fixed in 2·5% (v/v) glutaraldehyde followed by 2% (w/v) osmium tetroxide. The tissue pieces were embedded in Epon and sections were stained with uranyl acetate and lead hydroxide.

PLATE 1

Fig. 1. *Lupinus luteus* plant with nodule encircling the root.

Fig. 2. *Ornithopus sativus* plant with many nodules developed on the root system.

PLATE 2

Fig. 3. Young *Lupinus luteus* nodule tissue showing bacteria enclosed singly by a membrane.

Fig. 4. Young *Ornithopus sativus* nodule tissue showing one or more bacteria enclosed by a single membrane.

PLATE 3

Fig. 5. Old *Lupinus luteus* nodule tissue showing bacteria enclosed singly by a membrane with an enlarged electron transparent region between the membrane and the bacterium.

Fig. 6. Old *Ornithopus sativus* nodule tissue showing up to six bacteria enclosed by a single membrane and an enlarged electron transparent region between electron translucent areas within the bacteria.

PLATE 4

Fig. 7. *Lupinus luteus* nodule tissue showing the presence of starch grains within cells infected with bacteria.

Fig. 8. *Ornithopus sativus* nodule tissue showing the presence of starch grains in a cell adjacent to an infected cell, and electron translucent areas within the bacteria.

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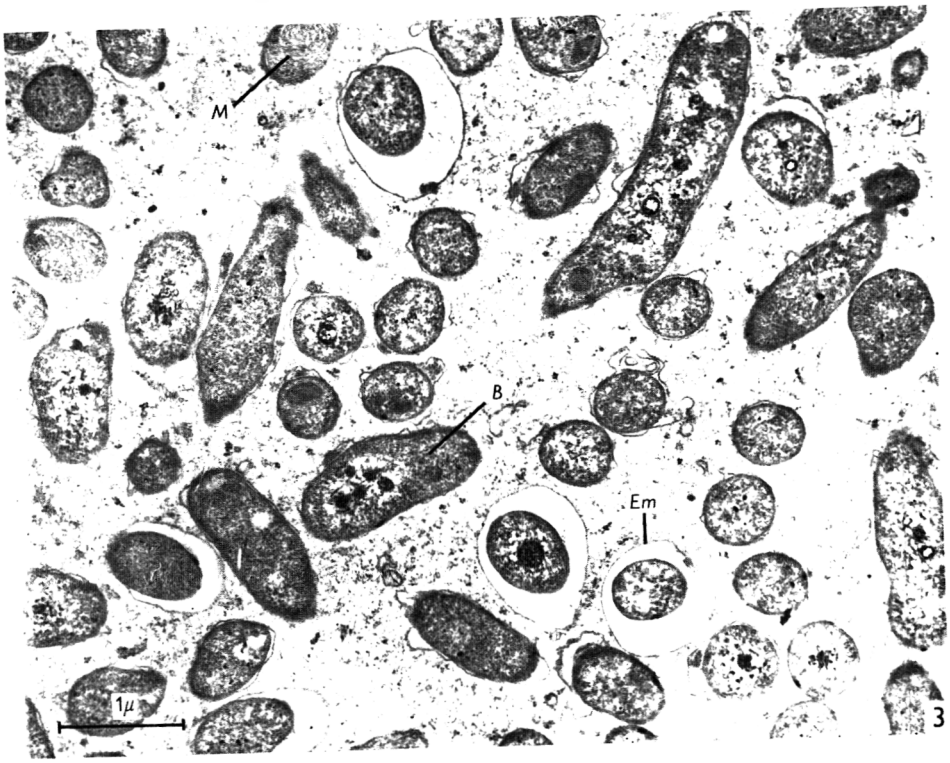
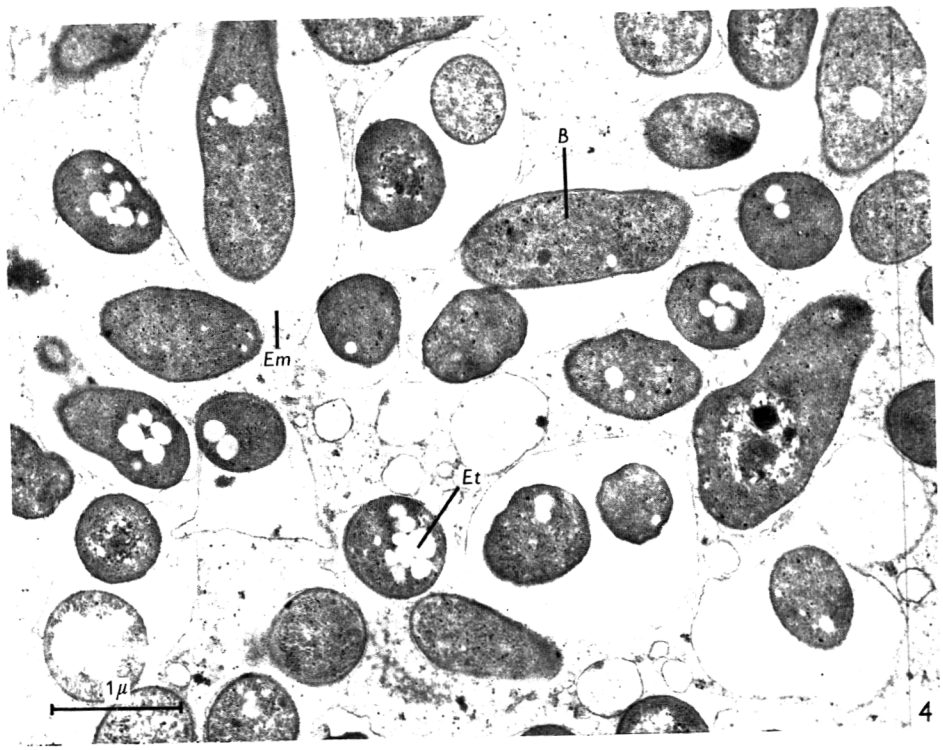
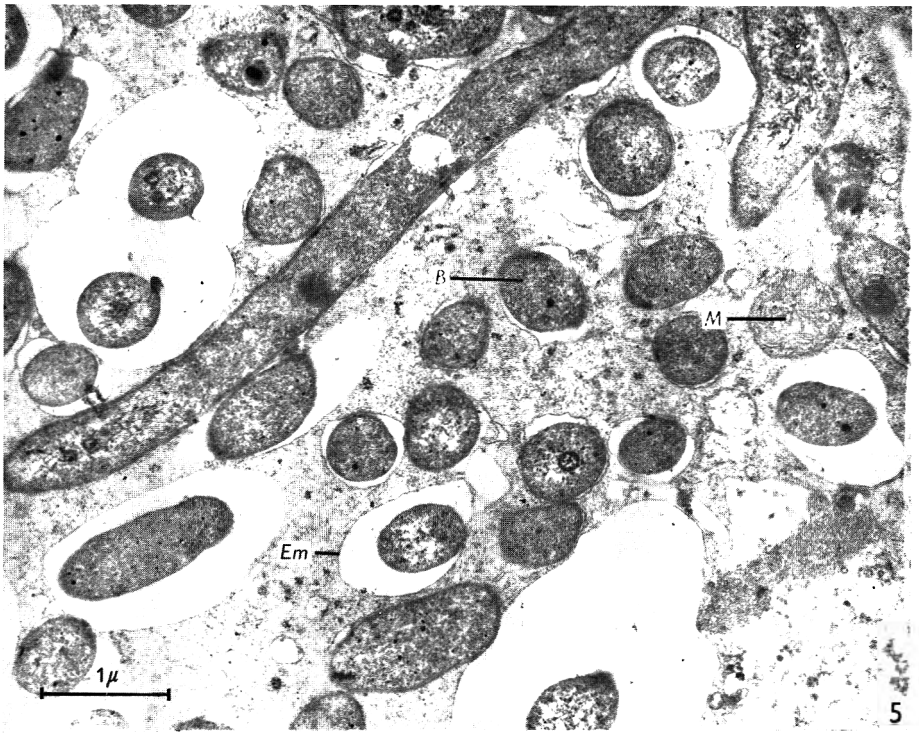




D. K. KIDBY AND D. J. GOODCHILD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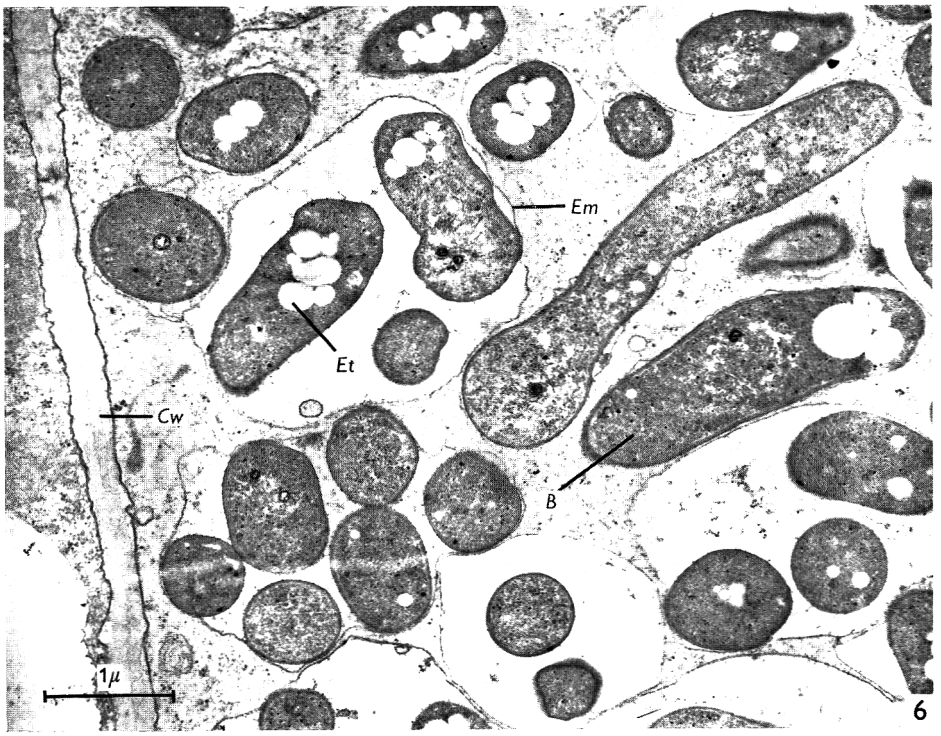
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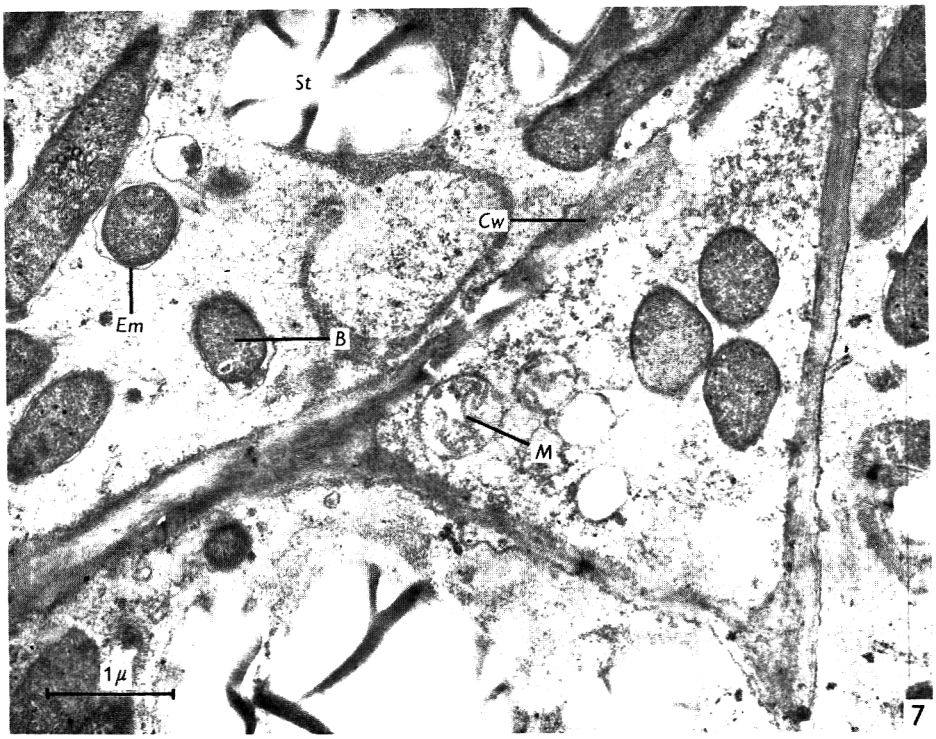
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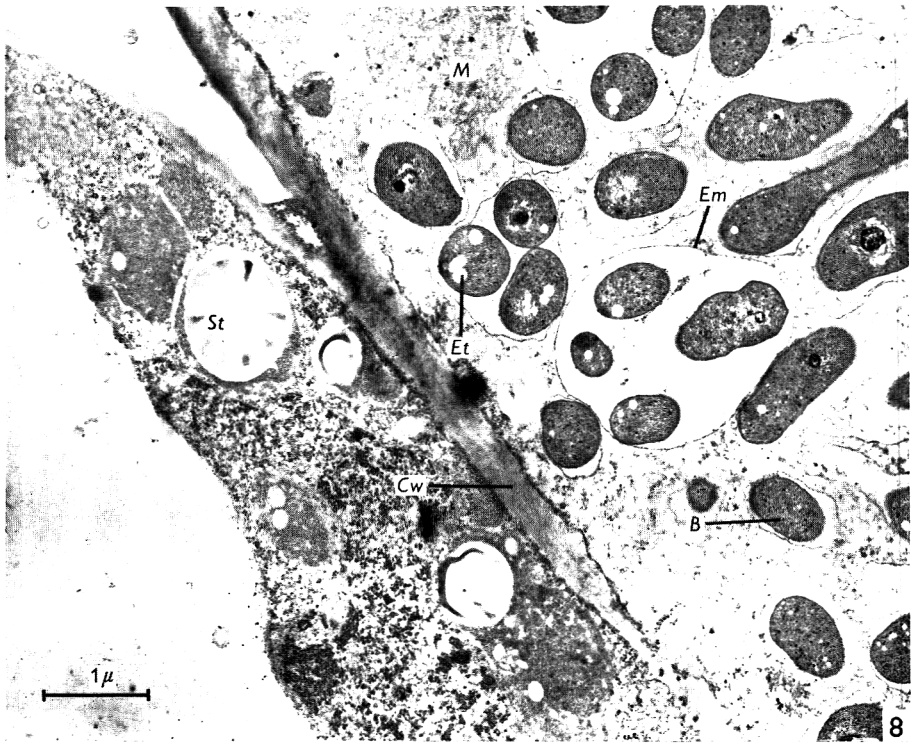
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Studies on the Intracellular Haemagglutinin Component of Fowl Plague Virus and other Myxoviruses

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SUMMARY

In contrast to other members of the influenza virus group, a strain of fowl plague virus produced large amounts of intracellular haemagglutinin in the chorioallantoic membranes of infected chick embryos. The titres of intracellular haemagglutinin obtained with the fowl plague virus were 400–2000-fold higher than those of the human influenza viruses tested. The fowl plague virus also differed from the human influenza viruses in being capable of growing through the chorioallantoic membrane when inoculated by the chorionic route. The only other myxovirus so far discovered that elicited a high intracellular haemagglutinin titre was Newcastle disease virus, although this type of virus is neither serologically nor morphologically related to the influenza viruses.

Electron microscopic investigations of membrane specimens from embryos infected with fowl plague virus showed the presence of a large number of structures, ranging in size from 300 Å to more than 7000 Å, with a surface configuration indistinguishable from that of the envelope component of typical particles. Similar structures were found very rarely in specimens of membranes infected with the human influenza viruses.

INTRODUCTION

During a study of the rate at which different factors appear in the course of virus multiplication, high titres of viral haemagglutinin were found in chorioallantoic membranes of chick embryos infected with fowl plague virus. The present report describes comparative investigations with other myxoviruses and efforts, by electron microscopy, to characterize the fowl plague haemagglutinin present in the chorioallantoic membrane.

METHODS

Virus strains used were the 'Dutch' strain of fowl plague virus, the PR 8 strain of influenza virus A, the B/England/939/59 strain of influenza virus B, and an undesignated, but characterized, strain of Newcastle disease virus kept at the National Institute for Medical Research. The strains were propagated by allantoic infection of 10-day-old chick embryos incubated at 37°, or other chosen temperatures. In addition, the fowl plague virus was inoculated by the chorionic route.

Infectivity assays were made by plaque counts in monolayers of chick embryo fibroblasts (Dulbecco, 1952).

Haemagglutination (HA) titrations. Each infected membrane was washed in three changes of saline, suspended in 1 ml. saline/membrane, frozen and thawed once, ground in a glass homogenizer and centrifuged for 10 min. at 1000 rev./min. Supernatant fluids and infective allantoic fluids were titrated in plastic trays in accordance with the technique described by the WHO Expert Committee on Influenza (1953). Haemagglutination inhibition tests with immune sera were also done as recommended by the Committee.

Electron microscopy. Specimens were prepared from infected allantoic fluids and membranes. For specimens from allantoic fluid, 5 ml. of fluid was cleared of debris by centrifuging at 2000 rev./min. for 10 min. The supernatant fluid was spun at 144,000*g* for half an hour and the pellet suspended in 0·1 ml. distilled water. A drop of this suspension was first negatively stained by mixing with an equal quantity of 3% phosphotungstic acid previously adjusted to pH 6 (Brenner & Horne, 1959), and then placed on a carbon-formvar-coated grid. Excess fluid was withdrawn from the grid with filter paper and the specimen immediately examined with a Philips 200 Electron Microscope. Specimens from chorioallantoic membrane, since it was desired to view the cell constituents of the membrane with as little alteration as possible, were examined in the electron microscope after a minimum of preparatory procedure. Small portions of freshly harvested membrane were rinsed in phosphate-buffered saline (PBS) and then transferred to the well of a hollow ground slide. After adding 0·2 ml. distilled water, the fluid was agitated with a Pasteur pipette. This served to disrupt the cells and a suspension of cell fragments was obtained. This suspension was then negatively stained in the same manner as the suspension of the allantoic fluid pellet.

RESULTS

Haemagglutinin

Twenty-four hr after inoculating chick embryos by the allantoic route with 10^3–10^4 plaque forming units (p.f.u.) of fowl plague virus, the viral haemagglutinin in the allantoic fluid had reached almost its peak titre. The intracellular haemagglutinin of membranes, however, continued to increase steeply to a very high maximum titre 48 hr after infection (Fig. 1). Further increase was not observed since this virus was lethal for the embryos. The infectivity titres of allantoic fluid and membrane extract were very similar. However, because of the discrepancy between the haemagglutination titres, the p.f.u : HA ratio of the membrane extract was approximately 1·5 log lower than that of the allantoic fluid. By contrast, the PR 8 strain of influenza A and strain B/England/939/59 viruses gave much lower titres of intracellular haemagglutinin at all periods up to 72 hr after virus inoculation (Table 1). In this respect Newcastle disease virus of fowls (NDV) resembles fowl plague virus, particularly when grown at 42°. NDV grown at 33° had a slightly lower titre than NDV grown at 42°.

The haemagglutinin that appears within cells infected by fowl plague virus is serologically specific, as is that produced by NDV. In fact, the intracellular haemagglutinin of NDV was inhibited by immune serum to a fourfold higher titre than the

haemagglutinin of the allantoic fluid, but was not inhibited by antiserum to fowl plague virus. The intracellular haemagglutinins of fowl plague and of NDV also showed a very rapid elution from chick red cells, so that within 1–2 hr of reading a pattern test at room temperature, the haemagglutination pattern had disappeared.

Further information on the production of intracellular fowl plague haemagglutinin was, following a suggestion by Dr A. Harboe, obtained by infecting the chorionic cells of the chorioallantoic membrane with the virus. In contrast to

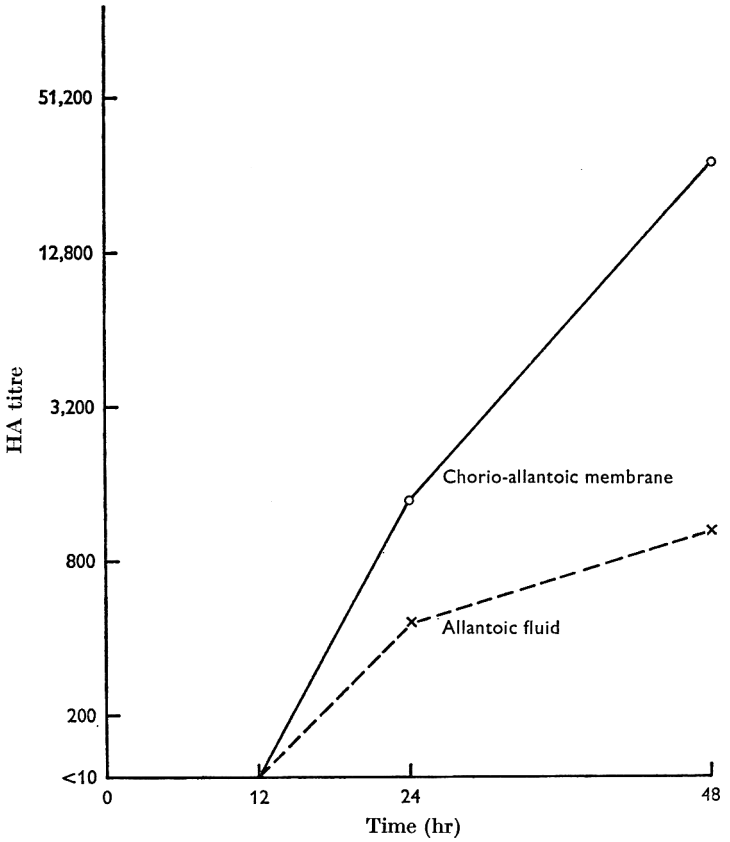

Fig. 1. Haemagglutinin titres of fowl plague virus in the chorioallantoic membrane and allantoic fluid of 10-day-old eggs infected allantoically. The ordinate shows the haemagglutinin titre with 0·5 % chick red cells in a pattern test. Membranes were suspended in 1 ml. saline, and the haemagglutinin was extracted by freezing and thawing once, followed by grinding in a glass homogenizer.

human influenza viruses, which show only a single cycle of multiplication when inoculated on the chorion (Fulton & Isaacs, 1953), fowl plague virus, after inoculation on the chorion spread to, and infected the allantoic cells. Forty-eight hr after infection, membranes yielded high haemagglutinin titres comparable with those obtained from allantoically inoculated eggs; in addition, haemagglutinin was present in the allantoic fluids (Table 2). The titres of haemagglutinin in the allantoic fluids of eggs inoculated on the chorion were variable, but nearly as high as those of eggs inoculated by the allantoic route.

Intracellular haemagglutinin extracted from membranes infected with fowl plague virus, and infective particles in allantoic fluid were treated for 30 sec. in an ultrasonic bath (Burndept Ltd.). The viral haemagglutinin in the allantoic fluid showed no change in titre. The intracellular haemagglutinin recovered from the deposit after centrifuging the membrane homogenate at 500 rev./min. for 10 min., however, showed a 4 to 6-fold increase in titre, while the haemagglutinating activity of the supernatant increased only by 50%.

Table 1. *Haemagglutinin titres of allantoic fluid and chorioallantoic membranes at different times after infection with myxoviruses*

Virus	Temperature (°)	Period of incubation (hr)	Haemagglutinin titres of: Allantoic fluid	Haemagglutinin titres of: Chorioallantoic membrane
PR8	37	19	8	—
		48	300	16
		72	320	20
B/Eng./939/59	37	19	2	—
		48	1,600	80
		72	1,280	2
NDV	33	48	600	1,200
	42	48	600	4,800
Fowl plague	37	48	1,000	32,000

Table 2. *Haemagglutinin titres of allantoic fluids of embryos inoculated with fowl plague virus by the allantoic or chorionic route*

Egg no.	Allantoic route	Egg no.	Chorionic route
1	1920	6	160
2	10	7	320
3	1920	8	480
4	1280	9	1280
5	960	10	120

Electron microscopy

The results of electron microscopic examination of specimens prepared from the allantoic fluid and the chorioallantoic membrane of chick embryos infected with fowl plague virus are illustrated in Pl. 1, figs. 1–2 and Pl. 2, figs. 3–5. The specimens originating from the allantoic fluid (fig. 1) contained large numbers of pleomorphic fowl plague virus particles, 700–1000 Å in diameter. In addition, filamentous forms were seen, similar to those found with other influenza viruses. In chorioallantoic membrane preparations, typical viral forms were few and, at times, difficult to find. All membrane specimens (figs. 2–5) contained, however, large numbers of structures exhibiting the fringed surface seen with negatively stained influenza viruses. These structures differed from typical particles in their greater variation of shape and the wider size range from 300 Å to more than 7000 Å. The smaller structures resembled rosettes (fig. 4) and bottle-brush forms (fig. 5). Within the size range of typical particles, many forms were seen which, because of their staining characteristics appeared to be merely viral envelopes. Instead of the amorphous, electron trans-

parent appearance usually presented by the central area of influenza virus particles (fig. 1), the central area of these forms was such that the end-on orientated surface projections were well resolved. A similar effect was also seen with the larger fringed structures (figs. 2 and 3).

In specimens prepared from chorioallantoic membranes infected with the PR8 strain of influenza virus A, and with the B/England/939/59 influenza virus strain a similarly small number of typical virus particles was present as for fowl plague, but the virus-associated structures were present in such small numbers that they could be found only with difficulty.

DISCUSSION

The comparative investigations on the presence of haemagglutinin in infected chorioallantoic membranes show a distinct difference between the behaviour of the human influenza viruses tested and that of the fowl plague virus and of NDV. Membranes infected with the fowl plague virus contained, for example, 400–2000 times more haemagglutinin than membranes infected with the human influenza viruses. The avian influenza virus and the NDV may have produced large amounts of haemagglutinin because they found in the cells of the chick embryo—their natural host—a more suitable milieu than did the influenza viruses of human origin. This is supported by the observation that when inoculated by the chorionic route, the fowl plague virus, in contrast to human influenza viruses (Fulton & Isaacs, 1953), was capable of growing through the chorioallantoic membrane.

The very high haemagglutination titres of the extracts of the chorioallantoic membrane infected with fowl virus plague are paralleled by the electron microscopical finding of large numbers of structures with a fringed surface. Many of these structures are morphologically similar to the incomplete fowl plague virus particles described and depicted by Waterson, Rott & Schäfer (1961). The small rosettes and bottle-brush forms present in our preparations are very similar to the forms seen by these authors and by Waterson (personal communication) in ether-treated preparations of standard and incomplete fowl plague virus recovered from infected allantoic fluids. Inasmuch as haemagglutinating properties have been ascribed to structural features of the viral envelope of many myxoviruses (cf. Hoyle, Horne & Waterson, 1961; Rott & Schäfer, 1961) it is likely that the fringed structures illustrated in figs. 2–5 bear the intracellular haemagglutinin. The results of the ultrasonic treatment of fractions of the homogenate of fowl plague-infected membrane makes it likely that the observed increase in haemagglutinating activity is primarily due to a fragmentation of the larger structures. The presence of the small rosettes and bottle-brush forms in our membrane specimens is of interest because, hitherto, such structures have been seen only in preparations of influenza viruses treated with a solvent, whereas in membranes infected with fowl plague virus they appear to occur naturally.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Group of typical fowl plague particles obtained from allantoic fluid. Both rounded and elongated forms can be seen. Negatively stained with PTA (× 210,000).

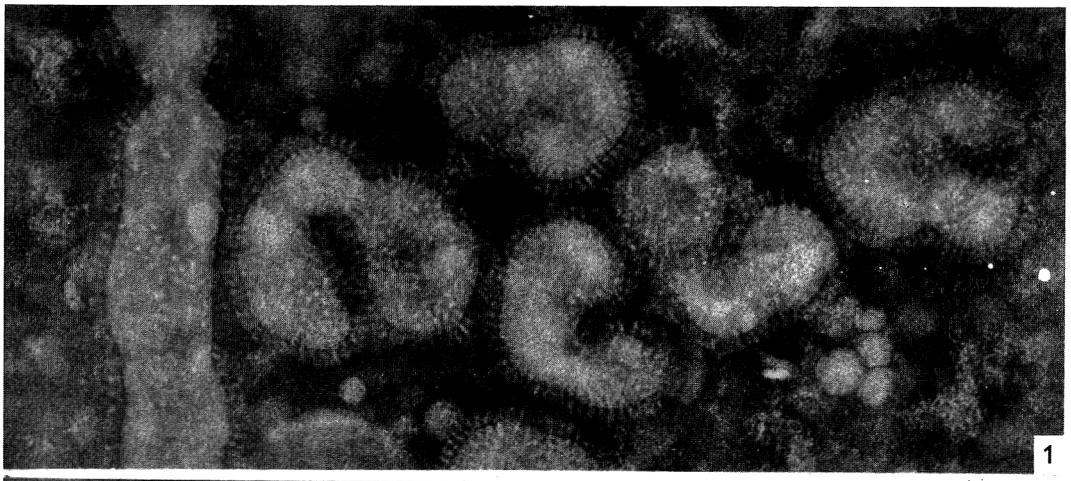
Fig. 2. Very large structure, probably membranous in nature, with fringed surface and dense, unstructured marginal areas. The vertically orientated projections in central parts of the surface are resolved. Cell spread preparation of chorioallantoic membrane infected with fowl plague virus. Negatively stained with PTA (× 210,000).

PLATE 2

Fig. 3. Large balloon-shaped structure with round or kidney shaped dense areas in the periphery. In the central area, the vertically orientated projections of the fringed surface component are resolved. Origin of cell spread preparation as described in legend to fig. 2. Negatively stained with PTA (× 450,000).

Fig. 4. Collection of rosettes in cell spread preparation of chorioallantoic membrane infected with fowl plague virus. Negatively stained with PTA (× 450,000).

Fig. 5. Several bottle-brush forms present in cell spread preparation of fowl plague-infected membrane. Negatively stained with PTA (× 450,000).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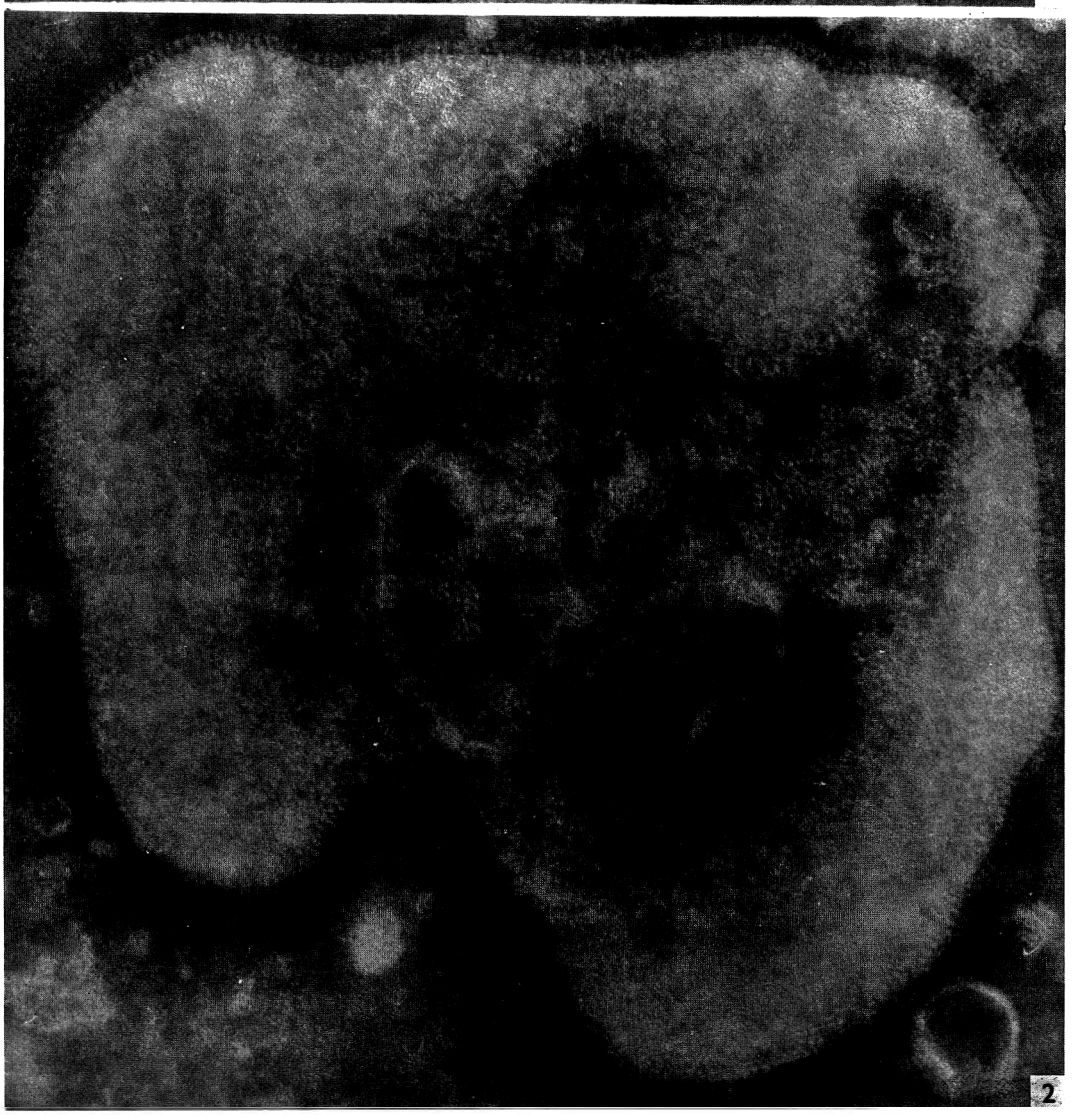
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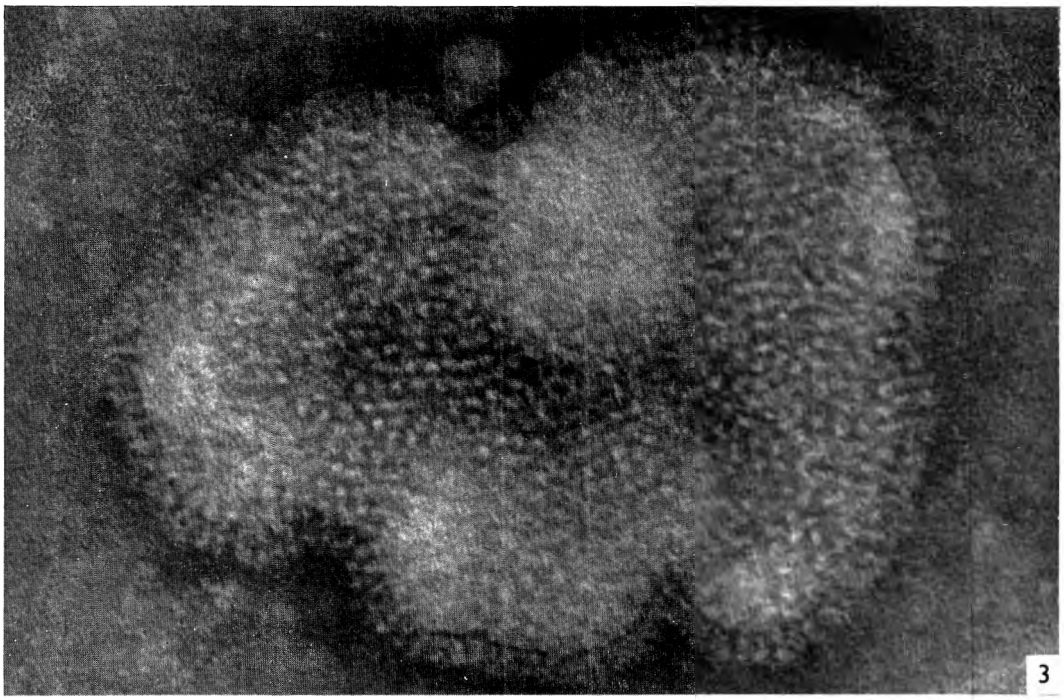
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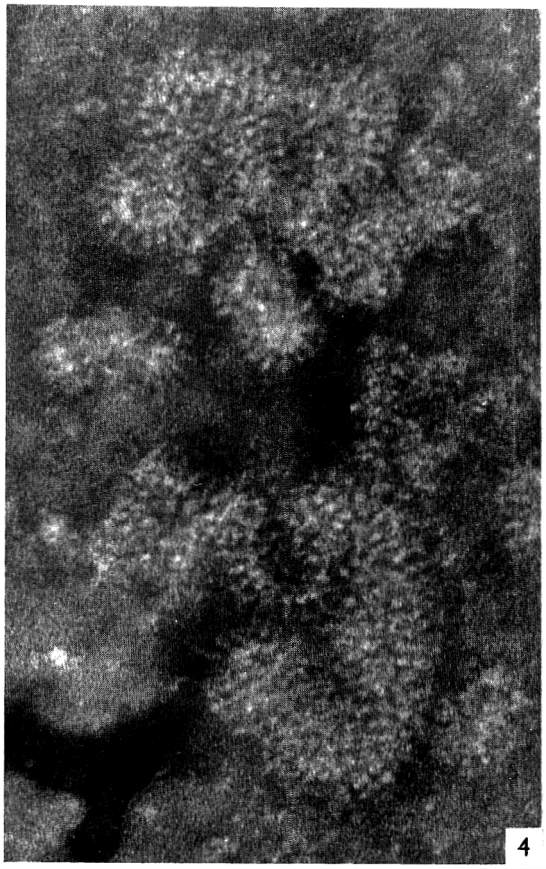
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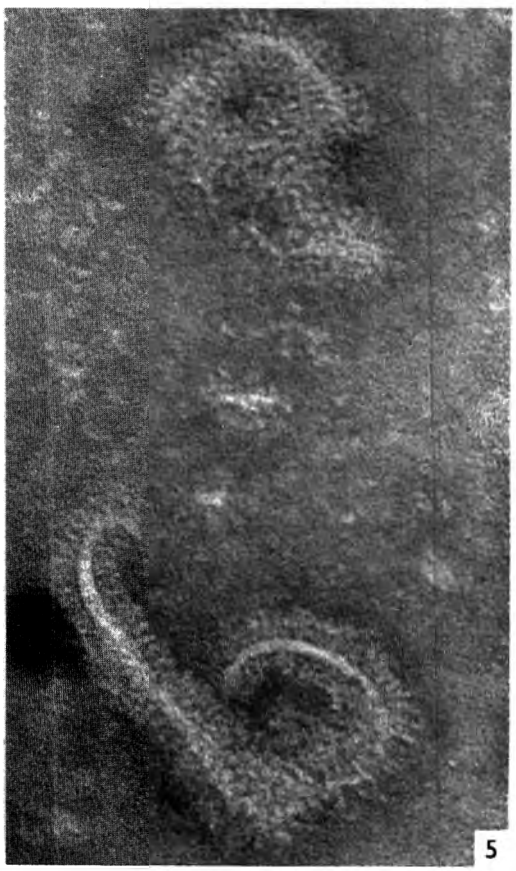
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