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THE JOURNAL OF GENERAL MICROBIOLOGY

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

THE PREPARATION OF PAPERS

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

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

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

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

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

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

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in English and should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*) Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) acknowledgements; (*g*) References.

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References at the end of the paper should be given in alphabetical order according to the name of the first author of each publication, and should include the title of the paper. Titles of journals should be abbreviated in accordance with the *World List of Scientific Periodicals*, 3rd edn. (1952). References to books and monographs should include year of publication, title, edition, town of publication and publisher, in that order. It is the *duty of the author to check his references* and see that the correct abbreviations are used.

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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: c. = circa or approximately; 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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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 references 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.

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JOHANNA WESTERDIJK

(Facing p. 1)

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Obituary Notice

JOHANNA WESTERDIJK, 1883–1961

Johanna Westerdijk died on 15 November 1961 at the age of 78 in her apartments in the Phytopathological Laboratory 'Willie Commelin Scholten' at Baarn, where she had lived and worked for over 40 years. Johanna Westerdijk was born on 4 January 1883 at Nieuwer-Amstel (now part of Amsterdam), where her father was a general practitioner. Her mother's family came from France. These French genes, she said, were responsible for her temperament, extraordinary vitality, and her fondness for a glass of good wine. As a young girl she knew quite well what she wanted. In the elementary school she refused to participate in plain needlework training; she read stories to the other girls instead. To her teacher she remarked that she would make sure to earn sufficient to have all domestic duties done for her.

She had a fine ear for music and originally intended to become a professional pianist; but when she was old enough to decide her own future a persistent neuritis in one arm made this impossible. As she also had a keen interest in botany she decided at the age of 17 to enter the Amsterdam University to follow the lectures of the famous botanist Hugo de Vries. During her studies she kept contact with her former friend of many plant-collecting excursions Dr C. J. J. van Hall, who was then assistant to Professor Ritzema Bos, the first director of the Phytopathological Laboratory 'Willie Commelin Scholten' at Amsterdam.

After finishing her biological studies at Amsterdam she spent some time in Munich working in the laboratory of Professor Goebel and later she studied in Zürich under Professor H. Schinz. Here she got her doctors' degree for a thesis, 'Die Regeneration der Laubmoose (The regeneration of mosses). Back in the Netherlands she was offered the directorship of the Phytopathological laboratory 'Willie Commelin Scholten' in 1906 when only 23 years old, and she took over from Ritzema Bos (who became director of the Government Institute for Phytopathology and the Netherlands Phytopathological Service in Wageningen).

In 1907 Dr F. A. F. C. Went, professor of general botany in the University of Utrecht, asked Johanna Westerdijk to care for a collection of pure cultures of fungi which had been established as a result of discussions at a meeting of the 'Association Internationale des Botanistes' at Leiden in 1903. The collection contained about 80 cultures, some of tropical origin. After some years Johanna Westerdijk changed the name of the collection to 'Centraal Bureau voor Schimmelcultures' (CBS) and under her active leadership this collection expanded to over 10,000 strains of more than 6000 different species of filamentous fungi, yeasts and actinomycetes. The aim of the Centraalbureau was, and still is, to keep a large variety of fungi in culture on artificial media for distribution to research workers all over the world; cultures can be sent to the CBS for identification.

Johanna Westerdijk studied many plant diseases. She wrote over 60 publications covering many aspects of plant pathology and mycology. Her main interest became parasitic diseases of trees and physiological diseases of plants, but in the first 10–15 years many other diseases were studied as well. Since plant pathology

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is an applied science Johanna Westerdijk not only studied the cause of a disease but she also tried to find means for effective control. In most of her Annual Reports trials with new chemicals were recorded. In 1918 she wrote about spraying potatoes against late blight. During the early years she worked alone or with students of the Amsterdam University. In 1910 a young practical potato selectionist with much interest in plant disease problems became her permanent assistant. This self-taught scholar later published the results of his investigations in three languages and in due course developed a phenomenal mycological knowledge. For his pioneer work on antagonistic soil fungi Mr A. van Luijk won an honorary doctor's degree from Utrecht University in 1946; during more than 25 years van Luijk was a tremendous help to Dr Westerdijk.

In 1913 Johanna Westerdijk went to the former Dutch East Indies to get acquainted with the diseases of tropical crops. She collected diseased parts of such plants and isolated the parasitic organisms in pure culture; these were later included in the CBS collection. Many excursions were made to plantations of tea, coffee, quinquina, cocoa, tobacco, rubber and sugar cane. Diseased parts of food crops (rice, potato, coconut) were also collected. Dr Westerdijk gave special attention to root parasites of rubber and tea such as *Fomes semitostus* Petch. In Sumatra she studied *Sclerotium rolfsii* Sacc. as a parasite of tobacco (1916*a*). Back in Java potato crops in the Tenger mountains were visited. There *Phytophthora infestans* appeared to cause late blight, but the disease spread more slowly and the necrotic spots were smaller and drier than in temperate climates (1916*b*). She found no tuber infection, but the varieties grown were not resistant since the same tubers grown in the Netherlands became heavily infected with the fungus.

Dr Westerdijk had the impression that plant diseases in the tropics were in general less serious than in the temperate zone (1915*a*). She suggested that the high temperature in the tropics might be responsible for this and that the high water content of the vessels of woody plants might be responsible for lack of parasitic fungi. No powdery mildews (Erisyphaceae) were found in the Malayan Archipelago; the mildew (*Oidium heveae*) of rubber trees did not cause any trouble then, but probably existed already since it occurred on many trees in Java in 1918. During her stay (October 1913 to June 1914) Dr Westerdijk not only became acquainted with many diseases of tropical crops, but also established valuable contacts which have remained even after Indonesia has become independent.

After her stay in Java Dr Westerdijk visited Japan. She has left a diary of this exciting period of her life and it is enchanting to read her impressions and experiences in this fascinating country. Her hosts showed her many diseased crops and made a mycological collecting excursion with her. She examined the cultivation of tobacco and the Tobacco Experiment Station at Hadano. As in Sumatra she found *Sclerotium rolfsii* on these plants. Several typical parasites of coniferous trees in the mountain area of Nikko were collected. Unfortunately all this material and her manuscripts were lost as a result of the outbreak of war. She continued her journey to the U.S.A. although the necessary funds could not reach her. She was able to earn her living by giving lectures at the various universities she visited. She joined an excursion, under the guidance of Dr W. A. Orton from the USDA in Washington who studied potato diseases, to many widely separated states and established valuable contacts everywhere. Many of Dr Westerdijk's pupils have

since been received not only with great hospitality but as old friends if only they could show an introductory letter from her. She enjoyed her stay there immensely. Several plant pathologists of the older generation expressed their great admiration for Dr Westerdijk when I met them some decades later. In her record of this trip in the Annual Report of her Institute (1913/14) Dr Westerdijk stated that for a plant pathologist a journey through the U.S.A. is extremely instructive; some parasitic diseases cause epidemics on a scale that is rarely met in Europe and she offered some possible explanations. She was struck by the quantities of arsenicals used, for example against the Colorado beetle; spraying with Bordeaux mixture was less common than in the Netherlands. In the U.S.A. she collected material of typical American plant diseases. In a letter to friends in Europe she commented on the peculiar position of a learned woman in the U.S.A. at that time. She attended a dinner party of scientific people, amongst whom there were only three women. 'When leaving I was only a few steps away from the other two women when I was told to leave the building by a back door. I went and fell on an unlighted staircase. I met my company again outside the front door. Why had the women to leave by the back door? Because people should not see women leave the building! Is the difference between front-door and back-door women not known in the U.S.A.?'

In 1917 Dr Westerdijk was appointed professor in plant pathology in the University of Utrecht. She was the first woman to be appointed a professor in the Netherlands, and she was also one of the first professors of plant pathology in a European university.

She now had to prepare weekly lectures to students in addition to her responsibilities as research worker and director of the plant pathology laboratory and the Central Bureau for Fungus Cultures. But she enjoyed excellent health and did not know what the word headache meant. Her practical courses in plant pathology became famous. They were at first given at the laboratory in Amsterdam; space soon became the limiting factor, but in 1920 she was able to move to more adequate quarters. A mansion in the nearby village of Baarn was bought; after alterations it was large enough to accommodate the rapidly extending culture collection, to house the many students who flocked there for practical training and to provide living quarters for Dr Westerdijk.

Attracted by Professor Westerdijk, more and more biologists took plant pathology as a second course and the number of post-graduates working for a doctor's degree at the Baarn laboratory steadily increased. When Dr Westerdijk retired from the chair no less than 56 plant pathologists had obtained their doctor's degree; they had all worked under her stimulating guidance. No wonder that Dr Westerdijk soon had little time left for her own research, which became quite impossible after she had accepted a second chair in plant pathology at the University of Amsterdam in 1930. Students from other Dutch Universities were also attracted by her personality and her practical courses at Baarn, and many foreign mycologists and plant pathologists came to the Baarn laboratory for shorter or longer periods.

Besides being fond of music she liked to read novels, not only in Dutch, but also in French, English, German and Spanish. Her command of these languages made her a highly appreciated president at international meetings. She was President of the International Federation of University Women, an office which brought her frequently to Crosby Hall in London. In 1937 she presided at the General Assembly

of this association in Krakau (Poland). The vote of thanks at the end of the congress read '...that she made the meetings so human and happy'. This can be said of all meetings at which she presided, such as those of the Netherlands Phytopathological Society of which she was President from 1945 to 1951.

Her outstanding leadership was recognized by the Dutch Government. She received the Royal Decoration of 'Ridder in de Nederlandse Leeuw' (Knight in the order of the Dutch Lion), was elected member of the Royal Netherlands Academy of Sciences and Fellow of the Linnean Society, was awarded honorary Doctor's degrees of the Universities of Uppsala (1957) and the Justus Liebig University, Giessen, Germany (1958); the Government of Portugal nominated her Knight of the Order of Santiago da Espada, and she was the first to receive the Otto Appel Medal (established for outstanding plant pathologists) at Heidelberg in 1953. She was also an honorary member of many Dutch and foreign societies.

Her work and publications will now be surveyed in short. The specimens in the Central Bureau for Fungus Cultures came from all parts of the world and entailed much correspondence. Great use was made of these fungi, yeasts and actinomycetes, at first mainly for biological and agricultural purposes and later, after the discovery of penicillin, by many industrial organizations and medical pathologists. This resulted in the establishment of a section for medical mycology.

The cultivation of fungi in pure culture is by no means a simple uniform technique. This was clearly explained by Johanna Westerdijk (1947) who told how the founder of the fungus collection, F. A. F. C. Went, had learned the cultivation of fungi in pure culture on nutrient gelatine and nutrient agar from A. de Bary, to whom he went in the 1880's. Her paper illustrates her keen observations about the nutritional demands of her beloved fungi.

In another article (1949) she introduced the concept of 'association' in mycology, following the example given by plant ecologists and in descriptions by field mycologists of toadstool combinations in certain terrains. Westerdijk defined 'association' as a specific microflora belonging to a special substrate and she gave many examples of such associations. As a striking example she mentioned the fungal association on mouldy oranges: *Penicillium italicum* and *P. olivaceum* were always found, independent of the type of the fruit and the country where they were grown.

In 1939 Dr Westerdijk lectured on antagonism between micro-organisms, a subject of special interest to her assistant van Luijk who had started his investigations in 1936, using some species of Pythium parasitic on the roots of grasses and lucern as pathogens and *Pullularia pullulans*, *Penicillium expansum* and other saprophytes or their sterile filtrates as antagonists. A selection of the best antagonistic fungi could be obtained by making suspensions of soil organisms from those spots in a heavily diseased lawn where the grass developed normally.

The financial basis of the Central Bureau of Fungus Cultures remained very difficult and uncertain for a long time, but Dr Westerdijk always succeeded in keeping the Bureau's activities going and the collections growing. Some private persons like the late Mr Odo van Vloten gave large sums of money to extend the laboratories and collections. The Royal Netherlands Academy of Sciences, The National Council for Agricultural Research TNO (the Central Organization for Applied Scientific Research in the Netherlands) and the Organization for the Health Research TNO gradually established a sound basis for this internationally

important institute. Altogether more than 70 publications have been written by Dr Westerdijk's collaborators of the C.B.S. on new fungus species, yeasts and on keratinophyle fungi as parasites of man and on taxonomic comparisons between related genera of moulds.

In 1955 Dr Westerdijk and I had the privilege of being present at the official opening ceremony of the new building of the famous Commonwealth Mycological Institute at Kew. There Dr Westerdijk expressed her sincere gratitude for the help she always got from Kew and for the general contribution to mycology made by this institute in the form of many important mycological papers and the *Review of Applied Mycology*. She ended her short speech with the words: 'Long live the precious mycology of Great Britain!'

From the Annual reports of the Phytopathological laboratory 'Willie Commelin Scholten' and from the contributions of this laboratory (at first all written in German) one gets an impression of the large variety of phytopathological subjects which Dr Westerdijk studied, at first alone, and later on with her assistant van Luijk and others. In this first period Sclerotium and Botrytis diseases of flower bulbs and Sclerotinia diseases of various crops had her special interest. She quite rightly disputed Sorauer's concept that Sclerotinia diseases had a conidial form which belongs to the genus Botrytis and attacks other parts of the plants. On several occasions she could prove this to be wrong. Extensive investigations were carried out with *Sclerotinia libertiana* (= *S. sclerotiorum*) on various crops. This fungus appeared to be very destructive on lettuce, less serious on beans, caraway and carrots, and unimportant on clover and mustard. Sclerotinia isolated from one host could easily attack another crop plant, unlike the Sclerotium and Botrytis of monocotylous bulb crops which are specific to one host plant only. So no physiological races restricted to a special host were detected. High atmospheric humidity appeared to be essential for infection; when grown for a long time on artificial media Sclerotinia did not lose its virulence. The fungus never produced a conidial form.

We are impressed by the amount of experimental work involved, the careful planning of her experiments, her keen observation of disease symptoms and the logical and clear discussion of the results. She frequently made cross-inoculations, using isolates from one crop to infect another. This technique was not restricted to fungi like Sclerotinia or Fusarium but was also used with stem eelworm isolates. The results of such cross-inoculations have a particular bearing on crop rotation. When studying a disease she always made anatomical investigations of the affected host tissues, a procedure which is unfortunately often overlooked by many modern plant pathologists because it is so time-consuming.

Other diseases studied by Dr Westerdijk in the first 10 years of the work of her phytopathological laboratory (1906–16) were various anthracnoses, e.g. caused by *Verticillium* sp., raspberry-cane blight caused by *Conyothyrium* sp., Phoma of sugar beets, *Rhizoctonia violacea* on potato, *Fusarium* sp. (now considered to be *F. nivale*) which caused serious losses of seedlings of winter wheat in 1906/7 in the northern provinces of the Netherlands. She was also able to prove that the wheat seed was already heavily infested with Fusarium before sowing. Seed disinfection trials with copper sulphate and formaldehyde were ineffective, and organic mercury and other strong seed disinfectants were still unknown at the time.

She could not isolate a possible pathogen from diseased oats on acid soils and

suggested that this disease had a non-parasitic cause; this was confirmed by others; it appeared to be a physiological disease due to magnesium deficiency.

In 1910 Dr Westerdijk published a paper on the mosaic disease of tomato, found in greenhouses. She concluded that the disease must be caused by a virus since no micro-organisms could be isolated which gave the same symptoms, whereas the sap of diseased leaves was highly infective to healthy plants. Young leaves were easily infected but the infective agent had no effect on full-grown leaves. Beside discoloration of a mosaic type, the virus caused monstrosities. Mosaic disease developed most clearly in full sunshine in greenhouses, and the diseased plants were very susceptible to *Cladosporium fulvum.* No symptoms were found in outdoor-grown tomatoes. The virus was destroyed between 50° and 100° and in dry form kept its virulence for a long time. Dr Westerdijk considered that, unlike the mosaic disease of tobacco, that of tomatoes is seed-transmitted. She also stated that the mosaic disease of tomato and of tobacco are caused by different viruses: each attacking only its own host. It is remarkable that Dr Westerdijk's work led her to such modern conclusions at this very early stage of virus research.

Other plant diseases studied were fungus diseases of cucumber, pea (*Ascochyta pisi* on seed, Fusarium of the roots and Peronospora of the leaves), onion (*Sclerotium cepivorum*), barley and wheat (*Helminthosporium gramineum,* which probably could be controlled by a seed treatment with copper sulphate), potato (*Spongospora subterranea,* the cause of powdery scab; *Phytophthora erythroseptica, Rhizoctonia solani,* with which organisms field trials were carried out both with tuber and soil disinfection), apples (Penicillium on stored apples), flax (a root disease which we now know to be due to *Pythium megalacanthum*) and fruit trees (*Armillaria mellea* and *Fomes* spp., *Stereum purpureum,* the cause of silver leaf). A bacterial disease of Iris was also described. Some mites and insect pests (e.g. of pea) were not overlooked. Large weed-control field trials with iron sulphate were also carried out. The possibilities of the fungicides and insecticides known at the time were investigated.

In 1912 Dr Westerdijk was already aware that a potato disease called 'kringerigheid' (probably 'sprain' in English) had two different causes, one only being identical with the 'Eisenfleckigkeit' in Germany. She concluded that neither of the two when present on seed tubers were dangerous to the crop. Although 'kringerigheid' is probably a virus disease, it is still only a partly solved disease problem in the Netherlands. She frequently reported the presence of stem eelworms in diseased crop plants (e.g. potato, pea, narcissus, tulip, clover).

Every micro-organism found in connexion with plants interested her, especially in the first years of her directorship. In the Annual Report of 1909, for instance, she reported on trials carried out with 'nitragine', a concentrate of nodule bacteria of leguminous plants prepared by Hiltner. In field trials with lupins in the sandy dune area, seeds treated with a suspension of nitragine in water or milk produced taller plants than did untreated seeds. In other field experiments with lupins and beans the results were less striking. Laboratory investigations showed that some samples of nitragine not only contained root nodule bacteria but 11, 9 and 8 respectively other species of bacteria. In the same report she suggested that growers of orchids should order mycorrhizal fungi for trials with six different orchid species. 'Without these fungi it is impossible to grow e.g. Cattleyas from seed since the seedlings always die after a shorter or longer period.'

Later Dr Westerdijk was particularly interested in fungus diseases of trees. By 1916 she had already published a leaflet on damage to fruit trees caused by wood fungi; later publications with van Luijk give the results of studies on *Gloeosporium* spp. on oak and plane (1920, 1924), and *Nectria coccinea* and *Nectria galligena* on apple, poplar and beech (1923, 1924).

In 1928 Dr Westerdijk raised the question of whether the so-called Dutch elm disease is infectious. This disease always intrigued Dr Westerdijk; with one of her most gifted pupils, Dr Christina Buisman (who died at an early age), she wrote (1929) a report on this disease which destroyed so many beautiful elm trees along the canals of her beloved Amsterdam. After Miss Buisman had succeeded in carrying out the first artificial infections it was clear that *Ceratocystis ulmi* was the cause of the disease; but it remained a mystery for some time how this disease could spread so quickly from one tree to another. It was Dr J. J. Fransen, entomologist at Wageningen, who in 1931 described the role that Scolytus beetles played as vectors. Later Dr Westerdijk went on a collecting trip to Spain where the disease did not occur. Since then, much work has been done at Baarn on breeding resistant elm trees, first by Dr J. C. Went and now by H. Heybroek, who made a collecting trip to the Himalayas in 1960.

In 1937 Dr Westerdijk wrote an article on the desirability of testing deciduous and needle-leaved trees for disease resistance. This is laborious work and disappointments are frequent, as she found with the Christine Buisman elms. This elm was resistant to *Ceratocystis ulmi* but later appeared to be very susceptible to Nectria. Similarly, the apparently promising American breeding programme with chestnuts resistant to chestnut blight (*Endothia parasitica*) failed because these trees were later proved to be susceptible to the Chalaza disease of oaks. In 1937 Dr Westerdijk also published a paper on canker of poplars, probably caused by a combination of Nectria and a bacterium.

Since her nomination as professor in plant pathology in Amsterdam in 1930, the two professorships and the directorates of the Phytopathological Laboratory Willie Commelin Scholten and the Central Bureau of Fungus Cultures took so much of her energy that she had little opportunity to carry out research on her own. In later years her outstanding work was the stimulation and guidance of her students in their advanced post-graduate studies. When one reads the subjects of the 56 theses she supervised one finds many plant diseases on which she had already published short notes in the Annual Reports of the earlier years of the Laboratory, but which work had been left uncompleted due to lack of time. All this work, which cannot be cited in detail, indicates the wide range of Dr Westerdijk's interests.

Dr Westerdijk gave her last lecture in 1952, in a hall decorated with flowers where over 500 people were present to pay homage to their beloved professor and friend. On that occasion a Westerdijk Fund for the advancement of plant pathology and mycology was established. Her South African admirers offered her a trip to their country, which she gladly accepted. Prominent plant pathologists made contributions to the Westerdijk issue of the 'Tijdschrift over Plantenziekten' of which she had been co-editor for about 20 years.

Why was it that her lectures were so highly appreciated? In the first place because they were well prepared, read in a clear melodious voice, and finally because they had a stimulating effect on her audience. In her inaugural speech as professor

in Amsterdam in 1917 Dr Westerdijk said 'a phytopathological investigation, the knowledge of a disease and its control, must be based on the knowledge of the physiology of both the host plant and the parasite...'. 'What we need is a textbook of plant pathology in which emphasis is put on disease symptoms arranged in groups. Conceptions like anthracnose, canker, twig die-back, galls etc. must be better defined.' Later she published (1919) an extensive article on this subject jointly with Professor O. Appel. They considered that a system of plant diseases should serve special purposes; first and foremost it should be possible to recognize the natural connexion between the diseases. They tried to attain this by choosing the symptoms as a basis for the separate groups of diseases. Formerly plant diseases had always been grouped by the mycology of the parasites, or by the host plant. The latter still seems to be the best way for farmers and growers; but for scientific purposes the division according to well-defined symptoms has many advantages. In her lectures Dr Westerdijk therefore always kept to her own concept of grouping according to symptoms, much to the benefit of her students. In her inaugural lecture in 1917 she opposed Sorauer who put too much stress on the outer circumstances which weaken the host plant, but she realized quite well that these may be of great importance. The importance of breeding resistant varieties of plants was clearly brought out in this lecture. Dr Westerdijk put great stress on the importance of finding a scientific basis for resistance. Now, after almost half a century, we have not yet advanced much in this field, although many recent findings are promising.

In her inaugural speech of 1930 Dr Westerdijk remarked: 'The plant pathologist has apparently a much simpler task than the physician of men. A patient that one can cut into pieces without objection seems an easy subject as compared with a human victim who must be approached with care, not only physically but also spiritually. The diagnosis of plant disease is less urgent; by the lack of this urgency much has remained undeveloped in this direction. I don't believe there is one plant doctor who has sleepless nights for the sake of a bed of diseased green peas, although perhaps in his dreams he may be tormented by the unsolved problems of immunity in some varieties of peas....' 'How can one explain the still inharmonious growth of plant pathology? This discipline is not sufficiently rooted in other sciences, especially not in soil science and in biochemistry. The plant pathologist has to solve the disease problems of crops, the anatomy and nutritional requirements of which are still entirely unknown.' Does not this sound very modern even more than 30 years after these words were spoken?

Many of Dr Westerdijk's pupils found work in the fascinating field of plant pathology in Europe, and in other parts of the world. All her pupils consider it a great privilege to have had the opportunity to work under the guidance of this stimulating woman scientist. 'The time spent in this beneficial sphere of zest for work, of good cheer and joy of life remains of high value for the rest of one's life', wrote Miss L. C. Doyer, one of her pupils, in 1931. No wonder that many friends and many ex-pupils accompanied her on her last journey. Her grave was covered with flowers.

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A DISCUSSION ON ASPECTS OF CELL-SURFACE ADSORPTION PHENOMENA

HELD BY THE SOCIETY FOR GENERAL MICROBIOLOGY IN CONJUNCTION WITH THE NETHERLANDS SOCIETY FOR MICROBIOLOGY AT THE UNIVERSITY OF READING, 27 SEPTEMBER 1962

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Some Sorption Phenomena at Mammalian Cell Surfaces

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Although the nature of the cell surface is of obvious importance in any discussion of sorption phenomena it is not proposed to consider this in detail here (see Weiss, 1962*b*). In the present discussion 'cell surface' means simply the first part of the cell with which the adsorbend comes into contact. Concepts of the surface region are not clear, partly because of the difficulty in deciding where environment ends and cell surface begins. Functionally the cullular microenvironment should be considered as part of the cell surface, which implies that in the case of free cells, adsorption may initially be to an ionic 'cloud' or to water. For quantitative work on surfaces it is often necessary to have some idea of surface area. It is, however, quite impossible to estimate the true surface area of living mammalian cells partly because of the presence of irregularities such as microcrenations, microvilli and possibly the endoplasmic reticulum, which may increase cell surface area by many orders of magnitude without being reflected in measurements of cell diameters or volumes. It will be pointed out that surface shape may affect some adsorption phenomena; this presents analytical difficulties in that the free edges of living cells are continuously moving and changing shape.

Cells carry a net negative charge at their surfaces due to ionogenic surface groups and to ion-induced dipoles. The approach of two cells in a fluid medium, and the

adsorption of one by the other may therefore be considered initially in terms of energies of attraction and repulsion between two charged particles. A useful discussion of the physical chemistry of cell adhesion is given by Pethica (1961). Two cells approaching each other in a 'physiological' environment can be regarded as being attracted by a potential energy of attraction V_A, due to dispersion forces where

$$V_A = \frac{-Aa}{12x} \quad \text{(Verwey \& Overbeek, 1948)},$$

A is a constant which depends on the nature of the cell surface; a is the radius of the approaching surfaces and x is the distance separating them. Other attractive energies at this range of separation are thought to be of minor importance.

As the two cells get closer together (approximately 20Å) they are separated by a potential energy (repulsion) barrier, V_R, mainly due to ionic double-layers, where

$$V_R = \tfrac{1}{2}Da\psi^2 \log_e (1+e^{-Kx}),$$

D is the dielectric constant of the medium; a is the radius of curvature of the relevant parts of the cell surfaces which are separated by distance x; $1/K$ is the thickness of the ionic cloud and ψ is the surface potential. Without attempting to fit real values to this equation it can be seen that the repulsion barrier which tends to prevent adsorption is reduced when the radius of curvature (a) of the approaching cells becomes less, and similarly with the surface potentials (ψ) and the depth of the ionic (Debye-Hückel) cloud ($1/K$).

Bangham & Pethica (1960) have made the suggestion that the approach of cells via pseudopodial projections of low radius of curvature may represent a method whereby cells may penetrate the repulsion barrier to make contact. The properties of living and dead cells may differ in respect to this.

Reducing the surface potentials on erythrocytes results in agglutination, and has been described by Coulter (1920) and others.

The potential ψ in a liquid medium surrounding a surface with a potential ψ_0 decreases exponentially with the distance. $1/K$, the Debye-Hückel mean double-layer thickness, is that distance at which

$$\psi = 1/e \cdot \psi_0$$

$$\frac{1}{K} = \sqrt{\frac{DkT}{8\pi n e_1^2 V^2}} \quad \text{(Glasstone, 1948)}$$

where n = number of ions per ml.; e_1 = the electronic charge; V = valency of ions; D = dielectric constant of the medium and k = Boltzmann's constant. Therefore $1/K$, and hence the repulsion barrier to cell contact, can be reduced by increasing ionic strength and by introducing multivalent ions into the environment. The ionic double layer in relation to the erythrocyte has been discussed by Seaman & Heard (1960) and adsorption of viruses to a variety of surfaces has been affected in a manner predicted from this equation by altering these two variables (Valentine & Allison, 1959).

The systematic study of ionic effects is technically difficult with mammalian cells, since cells immersed in solutions of non-physiological osmolarity and chemical constitutions may not be considered normal in some respects. Wilkins, Ottewill & Bangham (1962) attempted to study flocculation in leucocytes in low concentrations

of sodium chloride, by maintaining the physiological tonicity with the non-electrolyte sorbitol. However, Pulvertaft & Weiss (to be published) showed that with some cells sorbitol may induce massive ingestion of fluid leading to gross vacuolation. Whether or not such cellular trauma inflicted during observations affects their validity is a difficult point to settle.

Another factor preventing cell contact in the 20 Å range may be solvation barriers around cells. There is no doubt that these zones can exist, but opinion is divided on their existence at the cell surface. If both ionic clouds and solvation barriers exist in this region, it might be expected that in environments of 'low' strength repulsion is primarily due to ionic clouds, and in environments of 'high' ionic strength (i.e. 'physiological' and higher) repulsion will be due mainly to solvation barriers.

In systems exhibiting contact phenomena, cells almost always seem capable of overcoming the various barriers before any evidence of biological specificity is seen. From this it is concluded that the initial contact between cells is non-specific, and that the physical bases for biological specificity in these circumstances are not to be found in considerations of forces operating over long and medium ranges.

After overcoming the obstacle of the repulsion barrier two cell surfaces can approach to less than 5 Å separation. At this range the most powerful attractive forces binding surfaces are primary chemical bonds having energies of the order of hundreds of kilocalories per mole. It should be noted that, *in vitro*, most mammalian cells will adhere strongly to the surfaces of pure gold, tantalum, platinum and polytetrafluorethylene (Weiss, 1962*a*) where primary chemical bonding appears unlikely. Other short range attractive phenomena include hydrogen-bonds and coulombic attractions.

Attempts have been made to 'explain' specific adsorption in terms of these various forces, relating them to chemical groups bearing particular spatial relationships one to another. When enzyme adsorption is considered, the work of Bangham & Dawson (1959) is of interest since it suggests that the positioning of certain enzymes at substrate surfaces, which is reflected in the magnitude of enzyme/substrate interaction, is dependent on the surface charge of the substrate among other factors. 'Fine' positioning of antibodies adsorbed on the cell surfaces may also be charge-dependent. Estimates of surface charge obtained from measurements of the electrophoretic mobility of various mammalian tissue cells show that this may vary appreciably with the growth rate of the cells (Heard, Seaman & Simon-Reuss, 1961; Eisenberg, Ben-Or & Doljanski, 1962). It seems conceivable that the final positioning of a closely approaching adsorbend to a cell surface could be brought about by internally regulated changes in surface charge. The specificity of much of the adsorption process in the case of tissue cells in a physiological environment is perhaps questionable, since *in vitro* tissue cells will adhere (adsorb) to a very wide range of non-toxic surfaces (Weiss, 1962*a*).

The separation of material from a cell surface may result from a reverse process to adsorption, in which case the term desorption may properly be used. On the other hand, surface material may be lost from cells, when the plane of separation is not the same as the plane of adhesion or adsorption between the adsorbend and cell surfaces. If cells growing on glass *in vitro* are detached from these surfaces, by shearing pressures transmitted through their fluid environment (Weiss, 1961*a*),

separation occurs by means of non-lethal surface rupture of the cells (Weiss, 1961*b*), and the cells leave species specific surface antigen behind on the glass (Weiss & Coombs, 1962). From these and other observations (Weiss, 1962*a*) it has been suggested that the active or passive separation of cells from cellular or non-cellular surfaces is accompanied by surface rupture.

In vivo, cells move actively through tissues by 'amoeboid' movement. Whatever propulsive mechanism is responsible for this type of movement, it is essential that some sort of adhesion or friction exists between the moving cell and the surfaces over which it moves. As cells are not infinitely extensible, amoeboid movement must be accompanied by making and breaking of cellular contacts with a surface. It has been suggested that breaking such a contact is not a 'desorption' of a cell (process) from a surface in the sense that it is a separation along the cell/surface interface, but that contacts are broken by rupture in the surface of the moving cell, or the surface over which it moves, or both. It can thus be visualized that active amoeboid movements of cells within tissues are accompanied by two-way exchange of surface materials, for which the term 'epipherocytosis' has been proposed (Weiss, 1961*a*, *b*). It has also been suggested that this may result in exchanges of surface antigens, antibodies and other 'information' during round-cell infiltration for example.

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The Mechanism of Influenza Virus Haemagglutination as a Model of Specific Receptor Adsorption

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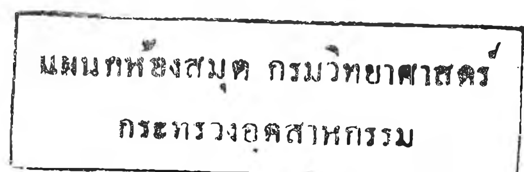
The classical work of Landsteiner established the concept of the chemically 'specific' receptor for antigen-antibody reactions. The comparable degree of specificity characterizing enzyme-substrate interactions made it highly probable that they were mediated by a basically similar structure. The biologist has usually been happy to picture such receptor structures as grotesque spikes, prongs, or even hooks and eyes projecting from the surfaces of antibody molecules or cells. Such simplicities have clearly never gone down very well with the chemists, who have worked unceasingly to create a more sophisticated and fundamental concept.

It is perhaps useful to summarize initially those features of specific receptor behaviour which can constitute common ground between biologists and chemists. The essential core of the receptor mechanism is the fact that molecular surfaces can become physically bound by the close mutual apposition of circumscribed elements of those surfaces. The element is understood to be defined by the spatial distribution of a particular chemical grouping, and to this extent it is perhaps wrong to talk about such receptor elements as being elements of a surface in a strictly two-dimensional sense, for it is clear that the structures involved are extended in three dimensions.

Current theory on specific receptor mechanisms seems to incline increasingly to the idea that mutual binding between homologous receptor sites takes place at specific binding 'points', and it is the spatial distribution of such 'points' within the structure of the chemical group that confers upon it its 'specific' properties. Thus the work of Friess and co-workers (Friess, Witkop, Durant, Reber & Thommesen, 1962) on the inhibition of the acetyl cholinesterase-acetyl choline system by muscarine isomers, led them to postulate 3-point binding of the inhibitor species to the catalytic surface of the enzyme. Earlier workers have postulated similar ideas from the results of the study of other enzymic systems. Clearly the more 'points' there are in the receptor element, the more 'specific' will the receptor configuration be, for the greater the number of points one defines positionally in an element of space, the smaller the number of linear patterns which will include all the points.

The forces acting at the binding points do not appear to be very clearly understood. The presence of ionogenic groupings will generate a potential field around the molecular surface on which the electrokinetic potential of the molecule as a whole must depend. This will be modified by solvation. It is clear, however, that if the distribution of its binding points in three dimensions determines the 'specificity' of the receptor, the points concerned must act independently as sites of attachment, and therefore the binding forces involved must be effective over a distance which is small relative to the over-all dimensions of the receptor. The effective range of these point binding forces must therefore be very much less than



the effective range of the electrostatic field. This leads to the idea that the forces involved are perhaps of the nature of van der Waals or London forces, obeying an inverse seventh power law. Thermodynamic data derived from antigen-antibody reactions indicate a large free energy change, the greater part of which, however, is derived from a correspondingly large increase in entropy. While the exact reason for this is obscure, it seems evident that the activation energy and binding energies involved are consonant with the formation of weak hydrogen bonds.

So much for the general features of the receptor concept. The suspicion that specific receptor mechanisms were concerned in the attachment of virus particles to the surface of the host cell was perhaps first crystallized by the demonstration of the remarkable, almost antigen-antibody-like, specificity governing the adsorption of bacteriophage to susceptible bacterial cells. The subsequent discovery of the agglutination of human and fowl red cells by influenza virus revealed a particularly attractive experimental system for the study of the receptor problem in relation to virus-cell attachment. The fact that the adsorbed influenza virus could elute itself spontaneously from the red-cell surface after initial adsorption, and that elution could subsequently leave the red cell surface bereft of the power to adsorb fresh virus, suggested enzymic action. Most virologists have been satisfied for some time that the influenza virus possesses a specific enzyme grouping on its surface. It is only comparatively recently, however, that the substrate, which constitutes the red-cell surface receptor, has been identified.

Many other macromolecular substances have been found which will bind on to the surface of the influenza virus, inhibiting thereby its capacity to agglutinate red cells; these are the so-called haemagglutination inhibitors. Certain of these inhibitors are attacked by the virus enzyme; the inhibitor molecule is released from the surface of the virus, and at the same time, free sialic acid appears in the system. Chemical study has shown that most of the known haemagglutination inhibitors obtained so far in any degree of purity contain sialic acid as a substituent group, linked by an α glycosidic bond, probably to a carbohydrate prosthetic group.

Sialic acid has subsequently been identified in the limiting membrane of the human and fowl red cell, and evidence has been produced to show that when the virus elutes from the surface of the red cell, free sialic acid is stripped from the red cell surface. Thus it was shown some years ago (Hanig, 1948; Stone & Ada, 1952) that the electrophoretic mobility of red cells was markedly reduced after the elution of previously adsorbed influenza virus. More recently, Cook, Heard & Seaman (1961) have shown that all the electrokinetic potential of the red cell surface can be ascribed to ionization of the free carboxyl groups of the sialic acid moieties distributed thereon. The removal of sialic acid groups alone, therefore, would account for all the change in electrophoretic mobility. It is clear, therefore, that the influenza virus receptor on the red cell must be sialic acid, and that the virus will bind to most molecular structures possessing this substituent group. Enzymic action at the site of adsorption leads to release of virus, free sialic acid and substrate residue.

It seems reasonable for a virologist to inquire: if the virus receptor (whatever it may be) binds on to sialic acid by union at specific points on the molecule, so that enzymic action can be initiated at the α glycosidic bond, why does the sialic acid become subsequently released? If in fact there is no intimate binding to the sialic acid molecule *per se*, but only to the α glycosidic bond, or its immediate vicinity,

why is binding so markedly affected by the presence of sialic acid? There is a good deal to suggest that other factors influence the interaction between enzyme and the sialic acid receptor. Thus the rate at which influenza viruses elute from the red cell surface can vary according to the species of the red cell. Some strains elute very much more rapidly from fowl red-cells than from human ones, though the link to be broken is presumably in each case an α glycosidic one (Smith & Cohen, 1956). It is possible, however, that more than one form of sialic acid is involved, and that the group is linked to different carbohydrate substituents. The local spatial distribution of these groupings at the cell surface is also likely to affect the ease with which the α glycosidic bond may be broken by the virus.

Specific antibody molecules will adsorb on to the surface of the virus and interfere thereby with the ability of the virus to haemagglutinate. There would seem to be a superficial analogy here with the haemagglutination inhibitors. That this is not so, is clearly evident from the fact that the specificity of influenza virus antibodies is not dependent on sialic acid substituent groupings. The effect of the antibody molecules on the simple adsorption receptor must depend upon subtle steric interferences.

We can see, from all this, how the behaviour of the virus-receptor system increasingly suggests that on a complex macromolecular surface, the behaviour of a simple chemical receptor group may perhaps be modified in many ways by the proximity on the same surface of other complex chemical configurations, and by the general spatial distribution of all these structures.

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The Bacterial Cell Wall. The Result of Adsorption, Structure or Selective Permeability?

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With advancing knowledge about the chemistry and physiology of cell structure, it is becoming profitable to speculate about the arrangement, biosynthesis and function of the various macromolecular components which make up the bacterial surface. Due obeisance is, of course, necessary to the well-known platitude that the surface is impossible to define accurately, and that all definitions in this respect are either arbitrary or operational. For present purposes the terms are operational, and we are interested in the make-up of the so-called bacterial cell wall. The thick, rigid layer which surrounds bacteria, governs their form and probably often the response of the environment to them; particularly when the environment is that of a living animal. All metabolites and extracellular products such as proteins and polysaccharides must pass either through or round this layer, as must bacteriophages on their way into the cell. Under the wall and closely applied to it is the delicate cytoplasmic membrane which itself has little or no ability to withstand the high osmotic pressure inside the cell when the wall is removed.

The picture now emerging is of a wall made of one or more main structural components, which confer rigidity and form to the wall, and a rather wide variety of other substances that can usually be removed without destroying the gross morphological structure (Archibald, Armstrong, Baddiley & Hay, 1961; Krause & McCarty, 1961; Weidel, Frank & Martin, 1961). The structural component in all bacteria so far examined contains two amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid, combined with the three amino acids, alanine, glutamic acid, and either lysine or α-ϵ-diaminopimelic acid; usually either glycine or aspartic acid is also present. Some of the alanine and all of the glutamic and aspartic acids have the D-configuration. These compounds are combined together to give one or more polymers called mucopeptides (Mandelstam & Rogers, 1959; Perkins & Rogers, 1959), and their chemistry has been reviewed several times recently (Work, 1961; Salton, 1961*a*; Rogers, 1962*a*). There is insufficient evidence to be certain of the precise structure and arrangement of these compounds, but it seems probable that the amino sugars are joined together to give polysaccharide chains which are linked together by peptides attached to the carboxyl group of the muramic acid. A possible macromolucular arrangement of the mucopeptides in the whole wall (Rogers, 1962*a*) is as sheets of polysaccharide fibres linked together by peptides attached to the carboxyl group of the muramic acid. Such an arrangement would have the advantage of great strength and rigidity combined with an open meshwork structure through which molecules could with relative ease diffuse or be carried by water flow. The minimum intervals between the peptide chains, assuming a cross-linkage of the type proposed by Ghuysen (1961) and Salton (1962) to occur on every muramic acid residue, would be the length of a disaccharide unit, i.e. 15–20 Å; the length

of the peptide chains would be about 20–30 Å. Mucopeptides are the principal mechanical supports in both Gram-positive and Gram-negative micro-organisms, but whereas they form a major portion of preparations of walls of the former organisms, of the latter they may constitute only 5–10 % of the weight (Mandelstam, 1961, 1962; Weidel *et al.* 1961). It seems that similar mucopeptides are also likely to be present in strains of blue-green algae such as *Phormidium uncinatum* (Frank, Lefort & Martin, 1962) and in rickettsia (Allison & Perkins, 1961).

The wall structure of Gram-positive organisms appears to be somewhat simpler than that of the Gram-negative forms, and there is a recent suggestion of a fundamental difference in arrangement of materials in the two groups (Clark & Lilly, 1962). In wall preparations from Gram-positive organisms the mucopeptides are major components, but surveys have shown (Cummins & Harris, 1956) that a variety of hexoses and pentoses are also present. How are these linked into the wall? Are they part of the basal structure, or are they parts of other polymers? If they are parts of separate polymers, are the polymers covalently linked to the mucopeptide or are they adsorbed, entangled or otherwise trapped by physico-chemical forces? If the mucopeptides and the other substances are linked together by covalent linkages to make a total structure called the wall, then presumably, apart from the enzymes necessary to polymerize precursors of the mucopeptides and of the other polymers, further enzymes may be necessary to link the different macromolecular components together. Biosynthetic pathways may be so inter-linked that all the polymers present in the wall may have to be synthesized simultaneously. There would also be genetic implications. Many of the immunological characters of the cells appear to be carried by the substances associated with the mucopeptides. If enzymes are necessary for linking the polymers on the wall together as well as for biosynthesizing them, the genetic change required to alter the specificity of the cell might be correspondingly increased. For example, if we have present besides the mucopeptide a polymer made of A, then by a single step mutation we could easily lose A. We could also obtain an organism containing an additional polymer B by, say, transduction or transformation. If, however, B must also be linked covalently to the mucopeptide, then such a change might be expected to be less easy since enzymes concerned with insertion of the substance into the wall might be involved in addition to those necessary for its synthesis.

Sufficient evidence has been obtained over the last few years to suggest that most if not all of the non-mucopeptide sugars present in wall preparations from Gram-positive organisms are present as part of separable polymers; ten years ago Holdsworth (1952) extracted an oligosaccharide containing arabinose, galactose and mannose from wall preparations of *Corynebacterium diphtheriae*. Many of these separable polymers also contain constituents in common with the mucopeptide. The polysaccharide from *Streptococcus haemolyticus* contains *N*-acetylglucosamine as well as rhamnose (Krause & McCarty, 1961). Teichoic acids from the walls of *Bacillus subtilis* (Armstrong, Baddiley & Buchanan, 1960, 1961), *Lactobacillus arabinosus* (Archibald, Baddiley & Buchanan, 1961) *Staphylococcus aureus* (Baddiley, Buchanan, Rajbhandary & Sanderson, 1962) contain D-alanine, and from the two latter organisms *N*-acetylglucosamine as well. Sometimes one of the components of these separable polymers is excessively labile to acid, and its presence has therefore not previously been recognized, e.g. glucuronic acid (Janczura, Perkins & Rogers, 1961), and amino

mannuronic acid (Perkins, 1963). The question then becomes: how are these polymers, which often bear net negative charges, held in the wall? Rather drastic means are usually necessary to separate them from the mucopeptide; wall preparations must be treated with hot dilute mineral acid, or cold, strong trichloroacetic acid, hot picric acid or hot formamide. None of these treatments can be said with certainty to leave labile covalent bonds intact. Examination of the state of the ribitol-teichoic acid in the wall of two strains of *S. aureus* (Rogers & Garrett, unpublished work) suggests strongly that it is mostly either entangled in the mucopeptide fibres or held there by hydrogen bonds. The role of ionic bonds is not likely to be important, since although the teichoic acid is strongly negatively charged, the mucopeptide appears likely to have few or no available basic groups (Salton, 1961*b*). Examination of the structure of this teichoic acid (Fig. 1) shows that after removal of the alanine, either by brief

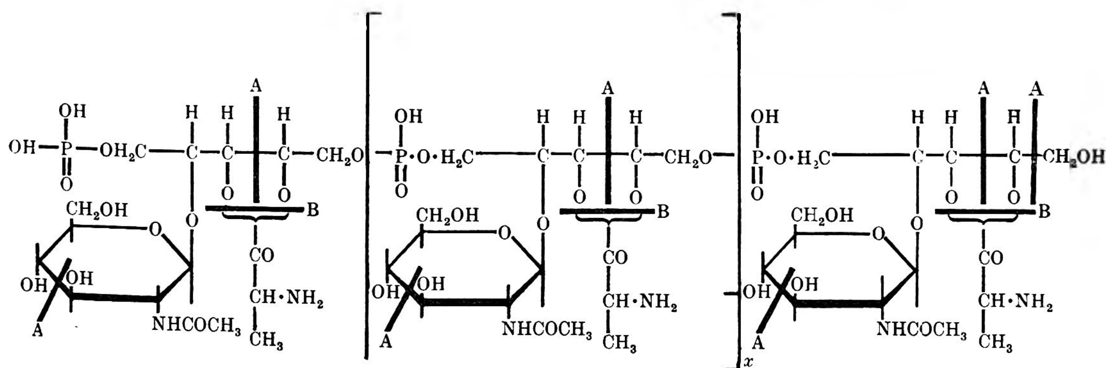
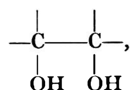


Fig. 1. The structure of the teichoic acid from the cell walls of *Staphylococcus aureus*. When alanine has been removed (see text), thus breaking the bond as by B, periodate can oxidize at the positions marked A.

treatment with 2N-ammonia at room temperature (Armstrong *et al.* 1961) or by hydroxylamine acting at pH 7·4 and room temperature (Kelemen & Baddiley, 1961), periodic acid, which oxidizes carbon to carbon bonds of the type



can attack at one place in the main polyribitol phosphate chain and one place in the *N*-acetylglucosamine molecule. If, then, the teichoic acid were linked to the mucopeptide in the wall, either via the sugar or via the ribitol, the amount of the periodate consumed would be reduced, unless the linkage was by the six position of the amino sugar. The phosphorus of the teichoic acid would never be removed from the wall by such treatment, whatever the linkage. An earlier suggestion (Mandelstam & Strominger, 1961), that the alanine of the teichoic acid is covalently linked to the mucopeptide, must be rejected, because the alanine can be removed without altering the extractability of the main polymer (Archibald *et al.* 1961 and present work). When dilute periodate was allowed to act upon a cell-wall suspension in 0·1 M-sodium acetate buffer at pH 5·8 and at 0–4°, a high proportion (about 80 %) of the phosphorus was removed within a few hours. By 24 hr. the consumption of periodate expected on the basis of the structure shown in Fig. 1 had occurred. Thus, it seems

likely that a high proportion of the polyribitol-*N*-acetylglucosamine units in the teichoic acid of *S. aureus* is not linked by frequent covalent bonds to the mucopeptide. The most likely hypothesis is that the molecules are mostly held in the mucopeptide meshwork by hydrogen bonding.

The importance of the mucopeptides in bacterial walls is made plain by the effect upon cells of the penicillins which inhibit their formation (cf. Rogers, 1962*b*, for review). It has also been shown that cytidine diphosphoribitol accumulates as well as presumed mucopeptide precursors when staphylococci are treated with penicillin (Clark, Glover & Mathias, 1959; Saukkonen, 1961). It is reasonable to suppose that this nucleotide is a precursor of the cell wall teichoic acid. Very large doses of penicillin partially stop the incorporation of ^{32}P into the cell walls of the organisms (Nathenson & Strominger, 1961). Recent examination (Rogers & Garrett, unpublished observations) has shown that a very low concentration of benzylpenicillin (0·05–0·1 μg./ml.) causes 30–40 % inhibition of teichoic acid synthesis by *Staphylococcus aureus* strain Oxford. The system used was similar to that of Mandelstam & Rogers (1959) and Rogers & Jeljaszewicz (1961). When, however, the penicillin concentration is increased even up to 10 μg./ml., little or no more inhibition occurs. This result is very different from that obtained for mucopeptide formation (Rogers, 1962*b*), which is already 80–90 % inhibited at a concentration of 0·1–0·2 μg./ml. of penicillin and 95 % inhibited by 0·2–0·3 μg./ml. Such a difference might be explicable by supposing that the formation of some of the teichoic acid is dependent upon mucopeptide biosynthesis. When mucopeptide synthesis is stopped, this part of the teichoic acid synthesis would be halted. Such a situation could arise if the polymerase for teichoic acid, although formed on the growing membrane in the region of cell division, were only active when adsorbed to formed fibrils of mucopeptide. In the region of cell division mucopeptide may well first be partly dissolved or disorganized by the 'endogenous' lytic enzyme to allow for the remodelling of the wall that fairly clearly occurs during division. Then, when the re-formation of mucopeptide fibrils is stopped by penicillin, formation of teichoic acid would also be halted for lack of sites to adsorb and activate the enzyme. Elsewhere in the wall the mature mucopeptide fibrils would not be disturbed, and the adsorbed polymerase would remain active. Such a hypothesis supposes that the cell wall itself is an active surface for the collection of enzymes necessary for formation of its own components. It might then also be supposed that some of the enzymes necessary for the final stages in the formation of mucopeptide fibrils are also contained in the preformed wall itself. If, for example, large but soluble mucopeptides were formed at the surface of the membrane in the region of cell division, and these were subsequently cross-linked together to form the final insoluble fibres by enzymes adsorbed in the wall, a simple model for cell growth can be imagined. The ends of the fibres which had been eaten into by the lytic enzyme would then, by virtue of final polymerase they have previously adsorbed, grow in length, pushing away from the point of cell division.

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Some Biologically Important Aspects of Adsorption of Antibody on to Tissue Cells

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The reaction between antibody against a constituent of the cell surface and the corresponding antigen will usually damage the cell, especially if complement is present. Although the mechanism of such damage is obscure, so far as this Discussion is concerned there is no problem as to how the interaction comes about. It is less easy to see, however, how antibody against a completely extraneous antigen should so be able to 'sensitize' tissues that subsequent interaction with the antigen results in damage to at least some cells, and so produces the familiar features of anaphylaxis.

Most studies on anaphylaxis have been carried out on guinea pigs, using either active sensitization (with antigen), or passive sensitization with rabbit antibody. For quantitative studies passive sensitization is essential, and rabbit antibody is a fortunate choice since almost all rabbit antibody sensitizes guinea-pig tissues (a property which is not shared by all antibodies from other species, as is discussed below). The nature of the cells that become sensitized in such a way as to manifest obvious damage on subsequent contact with antigen has not been exactly determined. Mast cells are certainly among them (Mota, 1959; Humphrey & Mota, 1959*a*); indeed, inasmuch as such cells are present in the connective tissue of most organs, and when damaged release powerful pharmacologically active substances, such as histamine and serotonin that act on smooth muscle and vascular endothelium, they may be the most important cells. However, mast cells constitute quite a small proportion of the cells in any tissue (less than 1 %), and there is at present no reason to suppose that antibodies sensitize mast cells exclusively. Anaphylaxis is accompanied by the release of other materials besides histamine and serotonin, such as 'slow reacting substance' (SRS-A) and bradykinin, and the origin of these last two is unknown (see review by Humphrey, 1962).

A characteristic feature of passive sensitization, either of the living guinea pig or of isolated tissues (e.g. lung, ileum, uterus) *in vitro*, is that the extent of sensitization, as judged by the severity of the manifestations on subsequent contact with antigen, increases with the duration of contact with the antibody. Although preformed antigen-antibody complexes *per se* are able to elicit at least some of the features of anaphylaxis, such as histamine release or smooth muscle contraction, they are very inefficient at doing so compared with antibody that has become 'fixed' on the tissues. Fixed antibody, on the other hand, is very efficient. Brocklehurst and I, using ^{131}I-labelled antibody administered intravenously to a guinea pig 48 hr. previously, found that the presence of as little as 20 mμg. of antibody per g. of wet tissue was sufficient to sensitize the ileum so that it responded to contact with antigen in an organ bath by giving a maximal contraction. Since such small amounts are involved, lying below the limits for detection by many tests for antibody, it is not

surprising that it was long thought that fixed antibody could exist in the absence of any antibody in the blood.

By studying the sensitization *in vitro*, by labelled antibody, of tissues whose response to antigen can be quantitated, it is possible to relate fixation of antibody to sensitization. Various tissues have been used in this way: lung fragments, from which the histamine released on contact with the antigen can be measured; mesentery fragments in which mast cell disruption can be estimated; and pieces of ileum, whose contraction in response to an excess of antigen is limited by the amount of antibody fixed and can be compared with contractions due to known amounts of histamine. These systems are used as models only, and should not be taken as reliable guides to the complex events of anaphylaxis in the whole animal.

Sensitization does not necessarily involve active uptake of antibody into cells. For example it occurs under circumstances in which active uptake is precluded, when tissues are left in contact with antibody in the cold or in the presence of reversible metabolic inhibitors. Furthermore, sensitized tissues can be de-sensitized (i.e. the antibody can be neutralized without damaging the cell) by contact with the antigen under similar circumstances. Both findings indicate that sensitization involves adsorption of antibody at the cell surface. Studies by Brocklehurst, Humphrey & Perry (1961) bore this out, inasmuch as uptake by lung fragments of purified ^{131}I-labelled rabbit gamma globulin (containing a high proportion of specific antibody) obeyed a typical adsorption isotherm. The tissue was maximally sensitized, however, when only a small fraction of the antibody ultimately adsorbed had been taken up. When such sensitized tissues were subjected to prolonged washing in Tyrode's solution, antibody was eluted, but never completely enough for the tissues to lose their sensitization. It might be expected that guinea-pig tissues would not be able to distinguish antibody from non-specific gamma globulin, and that antibody molecules would only be adsorbed in competition with similar non-antibody molecules. This proved to be the case, and the rate of uptake of antibody and extent of sensitization of the chopped lung were decreased by adding non-specific gamma globulin (but not other plasma fractions). Binaghi, Liacopoulos, Halpern & Liacopoulos-Briot (1962) have studied this aspect carefully, using a standardized technique for sensitizing pieces of ileum. They found that the gamma globulins of various species competed with rabbit antibody for sensitizing the ileum, but with decreasing effectiveness in the order rabbit, man, dog, guinea pig, rat, horse, cattle, pig, chick, goat. They also showed that the rate of sensitization varied little over the pH range 5·3–8·2 (Halpern *et al.* 1959), but was increased in isotonic media of low ionic strength (Binaghi *et al.* 1961), or in the presence of 0·8 M urea (Binaghi *et al.* 1959).

So far the story seems reasonably straightforward, except in so far as the important adsorption sites are not known and that there is quite strong circumstantial evidence that antibody which has been in contact with tissue for a longer time is not only more extensively but also more firmly adsorbed than that which has been in contact for a shorter time. This argues that adsorption sites are heterogeneous, and has been interpreted by Mongar & Schild (1962) as also implying a multi-point attachment. The story becomes complicated however when the capacity of different sera to sensitize guinea-pig tissues is compared. Differences are found both between and within species. For example, goat, chick, pig, cattle, horse and rat antibodies

nearly always fail to sensitize guinea-pig tissues for anaphylaxis (Humphrey & Mota, 1959*b*). Inasmuch as these are the kinds of gamma globulin which competed least well with rabbit antibody, it may be that they are not adsorbed in the right way or at the right sites. Nevertheless, the few studies which have been made on the gross uptake of such globulins on to guinea-pig tissues failed to reveal any obvious distinction between them and rabbit antibody.

Furthermore, even when some antibodies of a species (rabbit, man or even the guinea pig itself) sensitize well, others may not do so at all. For example, human macroglobulin (γ1M or β2M) antibodies, and human reaginic (skin-sensitizing) antibodies, which may belong to the class of β2A (γ1A) globulins, do not sensitize guinea-pig tissues. The capacity of human globulins of these kinds to become 'fixed' on to guinea-pig skin cells has been studied by Ovary, Fudenberg & Kunkel (1960) using the technique of direct and reversed passive cutaneous anaphylactic reactions. Such reactions depend upon sensitization of a localized area of the skin of the animal by intracutaneously injected antibody, or a suitably purified globulin fraction; and are elicited by subsequent intravenous injection of antigen, or of rabbit antibody against the globulin, in the direct or reversed reactions respectively. The animals also contain a suitable dye in their circulation, local extravasation of which reveals the presence and extent of any increased capillary permeability. Ovary & Karush (1961) showed that sensitization depended upon the presence in the rabbit antibody molecule of a part with certain structural peculiarities. Porter (1959) first showed that the splitting of a small number of peptide bonds and reduction of some of the S–S bonds in rabbit gamma globulin caused the molecule to fall apart into three approximately equal parts, two of which (fragments I and II) contained the antibody combining sites and were antigenically very similar, while the third (fragment III) was antigenically distinct and was unrelated to antibody activity. Human gamma globulin is similarly split into two 'slow' (S) and one 'fast' (F) fragments (see review by Fahey (1962)). The presence of rabbit fragment III or human fragment F are associated with the ability to fix complement; they are also essential for the antibody or non-specific globulin to become fixed on and to sensitize guinea-pig tissues. Inasmuch as human β2A (γ1A) and β2M (γ1M) globulins do not contain the same F fragment as the other gamma globulins their failure to sensitize guinea-pig tissues is at least partly explained. However much remains to be discovered. For example, guinea-pig antibodies formed early in immunization have been found to be unable to sensitize guinea-pig tissues, while those formed later do so very well (Ovary & Benacerraf 1962; White, Jenkins & Wilkinson 1963). These antibodies differ in electrophoretic mobility, but both have similar molecular weights and both fix complement.

In other species the situation is at least as complex and much less thoroughly explored. Thus it has long been known that most human antibodies do not sensitize human tissues in the same way as they would guinea-pig tissues. Those antibodies which do sensitize human tissues (reagins) are characteristically more labile to heat, and have a greater electrophoretic mobility than most other human antibodies, although their molecular weight and their general immuno-chemical nature is still a matter for dispute. In rats there is strong evidence that tissue-sensitizing antibodies are in some way different from others; they tend to be formed early in immunization, and especially when *Bordetella pertussis* has been used as an adjuvant

(Mota 1962; Humphrey, Austen & Rapp, 1963). Although the picture outlined above of a reversible adsorption of antibody onto cells as underlying sensitization may in principle be correct, there are certainly other, at present unknown, factors involved.

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Group N Streptococcal Phage Lysin

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Two stages of phage multiplications, the adsorption-penetration process and the lysis of the host, have recently been largely elucidated. For some time it has been postulated that an enzyme attached to, or contained in, the phage tail is involved in the adsorption process with at least some phage-host systems. Similarly, the earliest discovered effect of a lytic phage, namely the liberation of the mature phage particles (lysis of the host), is due to a lytic enzyme acting on the cell wall. Bronfenbrenner & Muckenfuss (1927) recognized that phage lysates contain a substance other than phage. This 'ferment-like' substance differed from phage because it only affected dead staphylococci. It was adsorbed to clay filters, inactivated on standing at room temperature, it was more heat labile than phage, and did not diffuse through collodion membranes. This description adequately fits the staphylococcal virolysin (or phage lysin) described by Ralston, Beer, Lieberman & Krueger (1955) which lysed only dead cells, although viable cells were lysed in the presence of the homologous phage.

Brown (1956) showed that phage-free lysates of coliphage T_6 could dissolve cells of *Escherichia coli* killed with chloroform, and Koch & Jordan (1957) demonstrated a free enzyme in T_2 phage lysates. Koch & Dreyer (1958) came to the conclusion that the phage lysin was a lysozyme. This was confirmed by Weidel & Katz (1961) and Katz & Weidel (1961) who purified both the bound and free coli T_2 phage enzymes; and Murphy (1960), working on similar lines, found that the bound and free 'megaterium' enzymes differed only in their pH optima.

Halo formation was observed by Naylor & Czulak (1956) with a lactic acid streptococcal phage, and by Murphy (1957; 1960) with *Bacillus megaterium* phage. They found that halos were due to an enzyme affecting the dead cells surrounding the phages without giving complete lysis. Murphy demonstrated that the enzyme was resistant to trypsin, pepsin, DNA-ase and RNA-ase but was inactivated by chymotrypsin. It is possible that the inhibition of a rhizobium phage by chymotrypsin reported by Kleczkowsky & Kleczkowsky (1954) may be due to the inhibition of a similar phage enzyme. Halo formation due to cell-wall lysis differs from the halos observed around plaques of mucoid strains of *Escherichia coli* by Sertic (1929), and since reported to occur with many other organisms. In these cases the halo is due to an enzyme hydrolysing capsular polysaccharides (Adams & Park, 1956).

Other aspects of the action of phage lysin are the 'nascent' phage phenomenon and 'lysis from without'. Nascent phage was first observed by Evans (1934) and shown by Maxted (1957) to be due to an enzyme liberated from the host at the same time as the phage. When some phages, e.g. coliphage T_2, are adsorbed to their host

at high multiplicity the cell wall can become so riddled with holes that the rigid structure collapses leading to 'lysis from without' (Weidel, 1951, 1958; Delbrück, 1940).

The contribution now presented is concerned with the enzyme found in phage lysates of group N streptococci and some aspects of phage and enzyme adsorption.

Purification and properties of phage lysin

In the first instance the enzyme was purified from high titre phage lysates of *Streptococcus lactis* ML 3 (10^{12} phage/ml.) by conventional methods—precipitation with acetone and salts and ion-exchange chromatography on Amberlite CG 50. Five hundred-fold purification was achieved with a yield of about 13% and no further purification could be obtained using a variety of fractionation procedures. A 10^{-4} dilution of this enzyme preparation lysed 4×10^8 viable cells/ml. in 0·1 M-phosphate buffer at pH 6·7 at 37° in 10 min. The enzyme was heat labile and appeared most active in the presence of 0·15 M-monovalent cations and at a pH of 6·6–6·9, conditions which are very similar to those for optimal phage adsorption.

This phage lysin resembled that of group C streptococci (Maxted, 1957), in lysing viable cells. It thus differed from all other known lysins since these are active only against cell walls or denatured cells. Lysis of viable cells was preceded by a lag period whose length varied inversely with the initial enzyme concentration; no delay was observed with cell-wall preparations.

The action of phage lysin on cell walls resulted in the almost complete solubilization of the walls, with the release of at least two components. One, not dialysable, contained all the sugars (rhamnose, glucose and galactose), about 80% of the amino sugars (muramic acid and glucosamine) and amino acids (glutamic acid, aspartic acid, lysine and alanine). The other was dialysable and, after acid hydrolysis, was found to contain the same amino sugars and amino acids. These would appear to be present in an amino sugar-peptide complex.

Dialysable acetyl amino sugar-peptide complexes were found after the action of either lysozyme or an enzyme from *Streptomyces albus* on walls of *Micrococcus lysodeikticus* by Ghuysen & Salton (1960) and *Bacillus megaterium* KM (Ghuysen, 1961). However, Maxted & Gooder (1958) failed to find low molecular weight compounds after the lysis of group A streptococcal cell walls with a group C streptococcal phage lysate. The release of dialysable substances from cell walls by this type of enzyme may depend on the number of secondary linkages between the muramate-peptide side chains of the amino sugar 'backbones'. These could be in the form of peptide bonds between neighbouring side chains, or links between the side chains and groups in adjacent polymers—of amino sugars or teichoic acids for example. Fewer diffusible products would be obtained by the action of enzymes on the more extensively cross-linked structures.

The effect of phage lysin on cell walls of group N streptococci appears to be very similar to that of lysozyme on *Micrococcus lysodeikticus* walls. The results are consistent with the breaking of glycosidic linkages between *N*-acetyl amino sugar-peptide compounds, some of which are not cross-linked.

Strain and group specificity

At an early stage it was evident that the ML 3 phage lysin was not strain specific although all strains of group N streptococci were lysed, the rate of lysis differed appreciably. Strains of group D were lysed at a lower rate and strains of groups A, B and C were not lysed. These findings correlate well with the results of Maxted (1957) who reported lysis of group A, B, C and E streptococci, and under certain circumstances of group H, by a phage lysate of a strain of group C. It appears therefore that two or more types of streptococcal phage lysin occur.

Further evidence of the lack of strain specificity was obtained when a second phage lysin, prepared from a phage unrelated strain *Streptococcus lactis* c10 was compared with the first. Although they had the same pH optima for activity and resistance to inactivation by heat, and had almost identical activation energies of 28 kcal. mol.$^{-1}$, their activity spectra were different.

Ralston, Beer, Lieberman & Krueger (1961) demonstrated that staphylococcal phage lysin is produced during maturation of the phage particles. Using a lactic acid streptococcus (ML 3), susceptible to two phages (ML3: large plaques; 712′: minute plaques), we found that the two separately produced lysins had distinct patterns of activity against a number of test strains. This suggests that the specificity of phage lysin is determined by the phage.

Inhibition of phage lysin

Crude phage enzyme preparations readily lysed viable cells of phage unrelated strains, but not the host itself (in buffer) until the residual phage was removed by repeated centrifugation. Addition to purified enzyme preparations of the homologous phage at a multiplicity of 30–50 resulted in complete inhibition of activity. This behaviour contrasts with that of staphylococcal and megaterium lysins, in that these lyse viable cell suspensions only in the presence of their homologous phages (Ralston *et al.* 1955; Murphy, 1957).

Inhibition of phage lysin by phage indicates that phage and enzyme compete for adsorption sites that are either identical or very closely linked. Heterologous phages failed to inhibit the phage lysin, although so far every phage has been adsorbed by every strain. Heterologous phages must therefore be adsorbed on different sites. At 0° both homologous and heterologous phages are eluted by nutrient medium whose electrolyte concentration is above that permitting adsorption. At 30° no elution occurs. Phage-resistant mutants of these streptococci adsorb their phages; in this they resemble the behaviour of T_1 phage-resistant mutants of *Escherichia coli* strain B.

Reiter & Oram (1962) showed that suramin inhibits phage adsorption and now, as one would predict, we have found that suramin similarly inhibits phage lysin. As non-specific adsorption is also inhibited it appears that suramin masks the whole cell surface.

Escherichia coli B has been the subject of most of the research on phage receptor sites and their isolation (Weidel, 1958). It may well be that the relatively simple cell walls of the Gram-positive cocci will provide valuable additional material.

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Electrical Counting Characteristics of Several Equivolume Micro-organisms

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SUMMARY

Several micro-organisms of similar initial size ($0{\cdot}25\mu^3$) were used to study the intra-species and inter-genus variations in the electronic counting characteristics and population distribution as functions of culture age. A Coulter Counter Model A was used to count populations of *Staphylococcus aureus* SM, *Escherichia coli* (Sias), an *E. coli* variant, *E. freundii* (8454), and an *E. freundii* variant. Initial inocula into brain heart infusion broth were from agar slopes. Cultures were incubated at 37° for various growth periods from 4 to 24 hr.; centrifuged at 1500 *g* for 25 min, and the deposit resuspended in 0·9 % (w/v) NaCl twice, then resuspended in 10 ml. saline, diluted $1/10^4$, shaken, and counted in the Coulter counter at maximum gain on aperture current settings (a.c.s.) 4, 5 and 6 with threshold settings from 5 to 100. Population distributions were made with a phase-contrast microscope in a Petroff-Hauser counting chamber. The numbers of aggregates containing 1, 2, 3, 4 and > 4 organisms were recorded as percentage of total populations. Counts were established for all bacterial populations tested as well as for the $0{\cdot}81\,\mu$ diameter latex sphere counting standard. With a technique based on the addition of random variables, true bacterial population counts could be formulated. Some of the actively dividing populations displayed characteristic sensitivities which were dependent in part on the aperture current setting. It is concluded that intra-species as well as inter-genus variations exist. These variations are the result of factors such as: the given organism, its age, distribution and growth rate at the time of count, dilution and counting media, aperture diameter and magnitude of the current field in the electronic counting system.

INTRODUCTION

The counting of bacteria in suspension by a method which gives immediate values for population size is desirable. The study of subtle and rapid changes in the growth of given bacterial populations has been limited. Preliminary reports of progress in the field of electronic counting of bacteria have appeared recently (Toennies, Iszard, Rogers & Shockman, 1961; Truant, Brett & Merckel, 1962). Our previous work on this problem has shown the necessity of working with populations of one organism by using basic microbiological techniques, and by referring frequently to established standards (Swanton, Curby & Lind, 1962). As with many complex functions, certain general relationships exist which make possible the solution of the function under special conditions. The present paper is concerned with the rationale involved in attaining a practicable means of counting bacterial

suspensions by using the Coulter Counter and to show the results obtained when applying this method to counting different micro-organisms of similar initial volume ($0{\cdot}25\mu^3$).

METHODS

Staphylococcus aureus SM, *Escherichia coli* Sias, an *E. coli* variant, *E. freundii* (8454), and an *E. freundii* variant were grown in brain heart infusion broth (supplied by Difco Laboratories Inc., Detroit, Michigan, U.S.A.) and followed for periods from 6 min. to 48 hr. Pour plates were made for each culture at the time of inoculation and at 1 hr. intervals thereafter. Simultaneously, counts were taken on a Model A Coulter Counter, adjusted for maximum gain, from 0·5 ml. samples of the

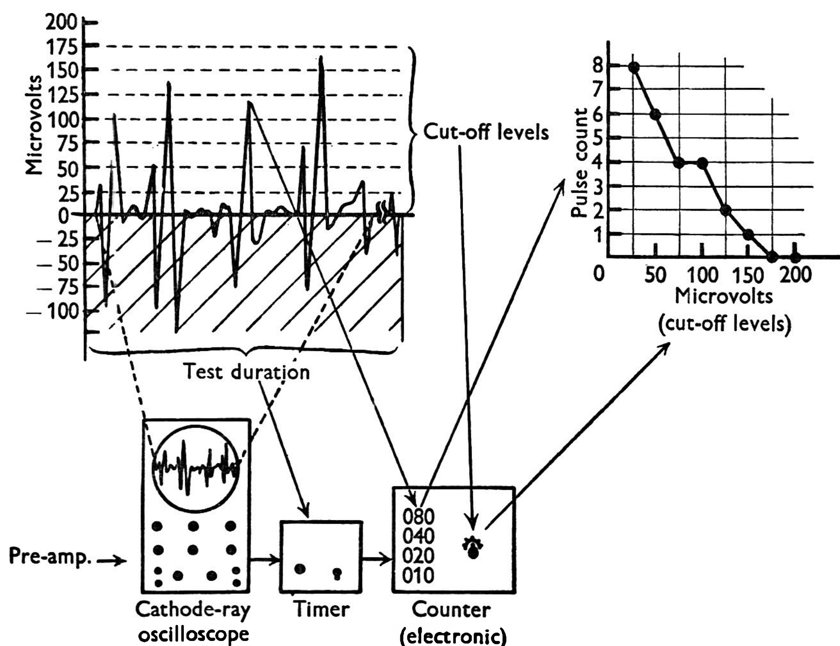

Fig. 1. Pictorial diagram of typical pulse counting equipment together with a graph insert (upper right) showing how the data are displayed in subsequent figures. For simplicity a linear rather than a semi-log or log-log relationship is depicted. The counter used in our experiments has a fixed volume rather than a fixed time interval control, but for ease of graphic explanation a fixed time unit is shown.

growing culture diluted in 0·9% (w/v) sodium chloride solution. All counts were made through a 30μ diameter aperture of the apparatus at threshold settings from 5 to 100 for aperture current settings (a.c.s.) 4, 5 and 6. Population distributions and visual counts were made on samples introduced into a phase-illuminated Petroff–Hausser counting chamber. The electronic counting method used for this study utilized two volumes of a fluid several hundred times more conductive than the particles which were to be counted. The two volumes of fluid were continuous through a precision hole ($30\ \mu$ diameter) drilled in a thin, non-conductive wall. In each of the volumes a non-reactive platinum electrode was submerged, and a fixed current electric circuit was completed between them through the fluid suspension.

A known volume (0·05 ml.) of fluid with its suspended particles was caused to flow through the aperture. As each particle passed through the orifice, there was a transient change in resistance. A voltage increase (current × resistance) occurred each time a particle passed through the aperture, the amplitude of the voltage pulse generated being proportional to the aperture-filling properties of the particle (mainly size and shape). These voltage pulses were amplified and passed through a circuit which controlled the minimum voltage that was registered on a connected pulse-counter. Figure 1 shows the counting circuitry; at the upper right a method of graphically indicating the results obtained as the threshold cut-off value is increased is shown. The voltage pulses generated as the particles were pulled through the aperture were amplified and displayed on the cathode-ray tube. Pulses of similar polarity were passed into a counting circuit through a unit which controlled the test interval as a function of time or, as in our experiments, of volume. The effect of varying the threshold cut-off value may be followed (Fig. 1) in the expanded view of the image on the cathode-ray tube superimposed on the pulse height grid (upper left part of figure). It will be noted that 8 pulses in the test duration were 25 μV. in amplitude or greater. This was displayed as a pulse count of 8 at a threshold cut-off value of 25 on the graph in the upper right part of the figure. There were 4 pulses having amplitudes of 75 μV. or greater; in fact, the amplitude of these pulses was greater than 100 μV. Thus for both cut-off values the pulse count was the same. This flat portion of the graph will be referred to as a plateau, and the portion to the right of it as the tail-off of the curve. The curve at the 75 μV. threshold value made a positive flexure, while at the 100 μV. value it flexed negatively. Families of pulse count *versus* threshold curves were generated for each of our micro-organisms; one curve was noted for each 1 hr. interval in the growth period.

An analysis of the particle distribution producing a curve of the form described above is shown in Fig. 2. A complete treatment of the analysis of the addition of two randomly presented functions upon which this analytic method is based will be the subject of a subsequent publication. The dark line on the graph in Fig. 2*a* is the resultant of two random (stochastic) variable curves indicated in dotted lines (Dixon & Massey, 1957). The straight line with the negative slope would be generated if the particles in the fluid volume counted were randomly distributed as to rate and order of presentation, size, shape, and some factors of lesser importance. When all particles are uniform, but again presented randomly with respect to rate and order, the curve with the wide plateau is generated. The horizontal position of the plateau on the resultant curve is determined by the effective size of the particles. Using a 30 μ diameter aperture under the aforementioned test conditions the tail-off of the particle count *versus* threshold setting curve for 0·81 μ diam. latex spheres occurred at a threshold setting of 6 for an a.c.s. of 5, and at a threshold setting of 9 for an a.c.s. of 6. Under the same conditions, the plateaux of the curves generated by 2·85 μ diam. latex spheres extended through threshold settings of 100 for a.c.s.'s of both 5 and 6 with a tail-off which began at 95 for a.c.s. 4. The maximum threshold setting seen under test conditions at which the tail-off began for bacteria studied was 90 for a.c.s. 6. It was therefore possible to follow the growth of the organisms studied through their entire size increase in the growth cycle, and to estimate that the 30 μ diameter aperture under test

conditions described here will allow for the analysis of particles having space-filling properties equivalent to spherical volumes ranging from 0·25 to 12·00μ^3. The width and the vertical position was also altered by factors which were affected by the frequency of the pulses as well as the pulse amplitude. It should be pointed out that these factors limited the use of the analytical technique to be discussed to pulse input rates of about 6×10^3 pulses/sec. or less. Pulses generated at frequencies greater than this tended to superimpose in time and increase the apparent amplitude of the total pulse pattern. This theoretical limit was in accord with the findings of Brecher *et al.* (1962), who reported an increase in pulse counts at 8×10^3 erythrocytes/sec. especially at higher threshold values. The particles ordinarily found as

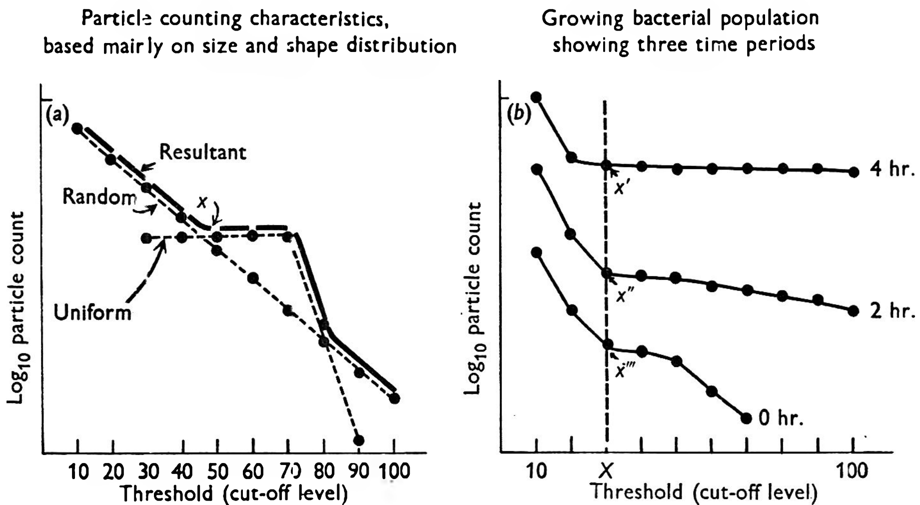

Fig. 2. (*a*). The resultant of the superimposition of populations of randomly and uniformly sized particle populations. Point x shows the threshold level at which the count in a growing micro-organism population should be most reliable. (*b*). The typical growth curves generated from a growing bacterial population. In practice the cut-off level (X) which best approximates point x in graph a will be used. Thus, particle counts for 0, 2 and 4 hr. will be taken as points x', x'' and x''' respectively.

impurities in suspension media used for counting were randomly distributed with respect to size and to some extent shape. The presence of these particles did not affect the total count of particles of uniform size analysed by the method described in this paper, provided their contribution did not cause the total pulse count/sec. to exceed the 6×10^3 limit.

The bacterial populations grown in pure cultures showed counting characteristics which followed those indicated in Fig. 2*b*. A reliable particle count could be made when enough uniform particles were present to cause a positive flexure in the curves. The mean percentage standard deviation for all counts within a pulse population range of $1{\cdot}5 \times 10^2$ to $3{\cdot}0 \times 10^3$ pulses/sec., and based on random sampling of the variance over a 6-month period under conditions recommended in this paper was found to be 1·945 within a minimum of 0·314 and a maximum of 6·565. An electrical count having a variance smaller than that found for its associated standard plate count was possible if one took as a point of count the pulse count for the threshold

value at the base of the first positive flexure on any of the curves; point x in Fig. 2*a*, and points x', x'', x''' in the curves in Fig. 2*b*. In mixed population analysis any subsequent positive flexures must also be considered. For all curves in a family generated from any micro-organism population, this threshold (X in Fig. 2*b*) can be taken as constant. To demonstrate the capabilities of this analytical procedure, no correction factors were used on any of the data presented.

RESULTS

Figures 3–7 show comparisons of growth characteristics, some population distributions for two cultures of *Escherichia coli*, two of *E. freundii* and one of *Staphylococcus aureus*; and the effect of the magnitude of current flow through the counting orifice (aperture current) on the reproducibility of the electrical count. Changing the aperture current setting (a.c.s.) control on the counter was observed to have an effect on the recorded particle count at times in the growth cycle of some of the organisms studied (Swanton *et al.* 1962). It can be demonstrated that this effect

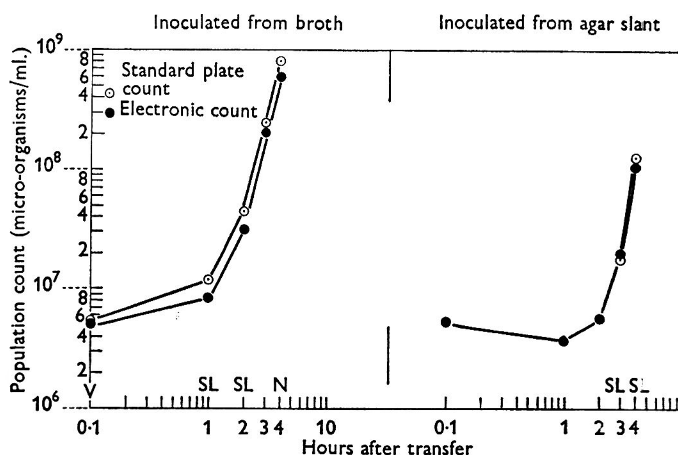

Fig. 3. Comparison of plate counts and electronic counts obtained from populations of *Escherichia coli* Sias. The notations V, SL and N above the abscissa indicate times at which the organisms were found to be, very, slightly, or not detectably sensitive to the current field in the counting media.

is associated with a general sensitivity of the micro-organisms to a variation in the magnitude of some electrical field property in the counting chamber. Until more definite information concerning this phenomenon can be obtained, we will refer to it as an electromotive force (E.M.F.) sensitivity. Figure 3 shows an *E. coli* and compares populations initiated from two inoculation sources. The degree of E.M.F. sensitivity, as measured by the failure of plateaux of curves in the graphs of particle count versus threshold setting to superimpose for aperture current settings of 4, 5, and 6 will be seen just above the abscissa: V for very sensitive; SL for slightly sensitive; N for no detectable E.M.F. sensitivity. The filled circles represent the population counts made within 1 min. after the electronic count began, while the open circles indicate the average values for plate counts read 24 hr. later. The maximum growth rate for this organism was 2·05 divisions/hr. when inoculated from broth and 2·02 divisions/hr. when inoculated from an agar slope. The *E. coli*

variant results shown in Fig. 4 were from extended periods of study. Petroff-Hausser counts were made at the points in time indicated by the triangles. Population distributions given in percentage of singlets (S), doublets (D), triplets (T), quadruplets (Q), and greater than quadruplets (> Q), appear in legends beside

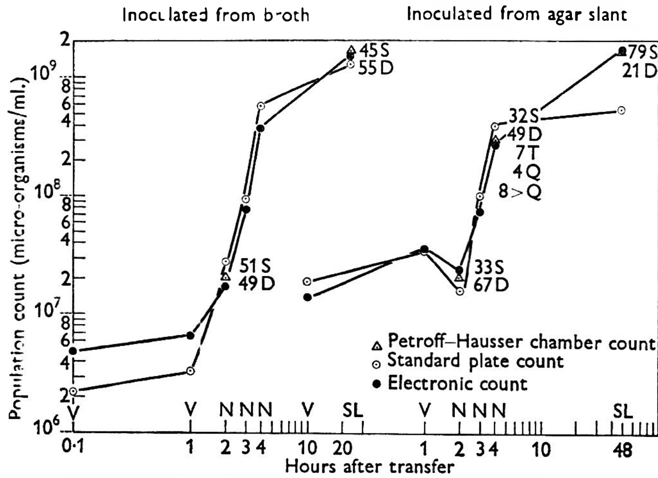

Fig. 4. Comparison of plate counts, electronic counts and Petroff–Hausser chamber counts obtained from populations of an *Escherichia coli* variant. The notations V, SL, and N above the abscissa indicate times at which the organisms were found to be: very, slightly and not detectably sensitive to the current field in the counting medium. The numbers beside the triangles indicate the percentage of singlets (S), doublets (D), triplets (T), and the quadruplets (Q), and greater than quadruplets (> Q) making up the population at the time of count.

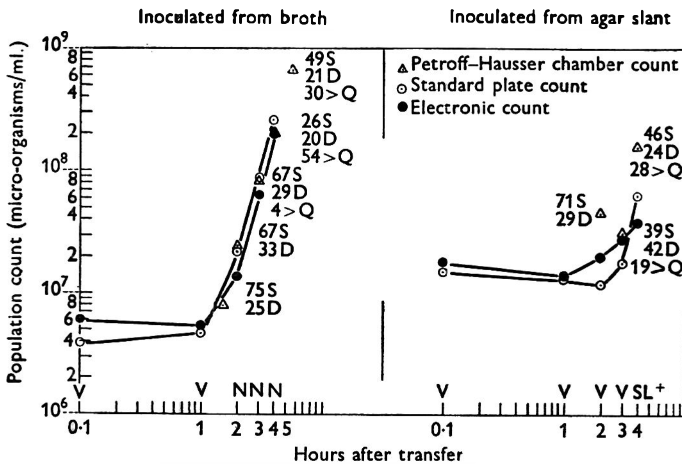

Fig. 5. Comparison of plate counts, electronic counts, and Petroff–Hausser counts obtained from populations of *Escherichia freundii* 8454. The notations V, SL, and N above the abscissa indicate the times during population growth at which the organisms were found to be: very, slightly, and not detectably sensitive to the current field in the counting medium. The numbers beside the triangles indicate the percentage of singlets (S), doublets (D), triplets (T), quadruplets (Q), and greater than quadruplets (> Q) making up the population at the time of count.

each triangle. At the 48 hr interval for the culture grown from agar, the plate count was lower than the electrical count and the Petroff–Hausser count. This was probably because some dead organisms had accumulated which would not have been differentiated by methods based on physical counting techniques. This

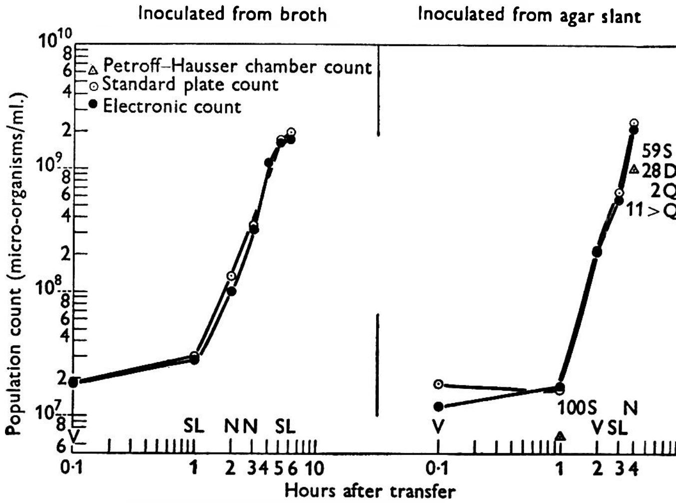

Fig. 6. Comparison of plate counts, electronic counts, and Petroff–Hausser counts obtained from populations of an *Escherichia freundii* variant. The notations V, SL, and N above the abscissa indicate the times during population growth at which the organisms were found to be: very, slightly, and not detectably sensitive to the current field in the counting medium. The numbers beside the triangles indicate the percentage of singlets (S), doublets (D), triplets (T), quadruplets (Q), and greater than quadruplets (> Q) making up the population at the time of count.

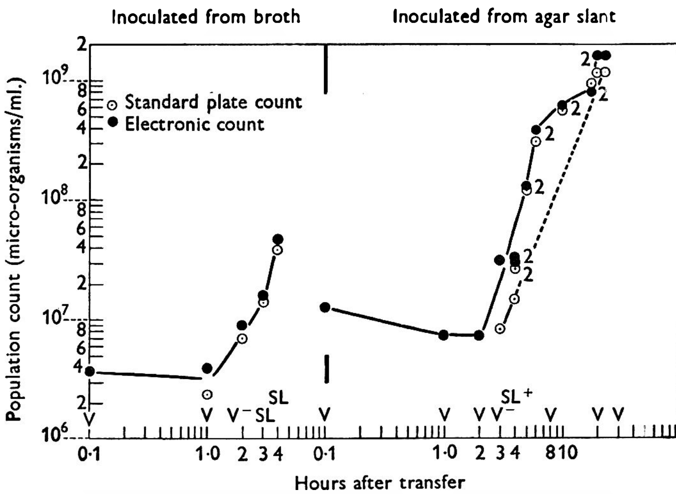

Fig. 7. Comparison of plate counts and electronic counts obtained from populations of *Staphylococcus aureus* SM. The points having the notation 2 in the graph of the micro-organism grown from agar are plotted from a culture of the same stock two months after the other agar slope data were obtained. The notations V and SL indicate the times at which the cells were found to be very and slightly sensitive to the current field in the counting medium.

difference can be seen to be slight in the 24 hr. sample from the culture grown from broth; the maximum growth rate was 2·00 divisions/hr. for the population started from broth and 2·17 divisions/hr. for the population initiated from an agar slope. *E. freundii* 8454 (Fig. 5) resembled *E. coli*, although it was also like *S. aureus* in its E.M.F. sensitivity. The plate counts may be related to the E.M.F. sensitivity. If the culture was highly sensitive, the plate count was consistently lower than the simultaneous electrical count. When no E.M.F. sensitivity was demonstrated the reverse occurred. The action of the population initiated from the agar slope was not clear. Subsequent studies may clarify the reasons for this behaviour. The maximum growth rate for the broth source culture for this bacterium was 1·80 divisions/hr. The maximum growth rate for the agar source culture was 1·14 divisions/hr. The population distributions indicated considerable clumping at times during growth. Because of the shape of these organisms and properties of the clumps, this did not affect the counting reliability. The *E. freundii* variant in Fig. 6 exhibited a sharp drop in E.M.F. sensitivity for the culture inoculated from broth. The sensitivity began to return at 6 hr. as the growth rate diminished from a maximum of 1·88 divisions/hr. The Petroff–Hausser counts taken at the 1 and 4 hr. test periods for the population started from an agar slope show the same growth rate (2·46 divisions/hr.) as that calculated from the other counting methods; however, the counts are lower. Further study will be necessary to explain or dismiss this as a recurrent phenomenon for this organism. *S. aureus* SM seen in Fig. 7 displayed continuously high E.M.F. sensitivity and again the electrical counts were higher than their plate count mates. The growth rate for these populations attained a maximum of 1·22 divisions/hr. for the population started from broth and 1·46 divisions/hr. for the populations inoculated from agar slopes. Two cultures inoculated from agar slopes were plotted together in this figure. The 0 and 4 hr. interval and the 24 hr. point were plotted from one culture; the 4 and 20 hr. interval from a culture started from the same stock 2 months later. The results indicated constant growth characteristics for this organism.

DISCUSSION

Several facts were apparent from the results. *Staphylococcus aureus* and one *Escherichia freundii* showed E.M.F. sensitivity throughout their growth period. All the micro-organisms studied exhibited the greatest E.M.F. sensitivity before the rapid growth phase. E.M.F. sensitivity had some effect on the electronic *versus* plate count. This sensitivity held regardless of polarity of the electrodes, thus showing that this phenomenon was not related to a charge on the organisms, but, rather, was determined by an active process within the organisms just before cell division. All of the Escherichia populations studied displayed similar growth characteristics. Population distribution did not seem to have an effect on the electrical count; however, the tendency to clog the aperture increased as the percentage of greater than quadruple units became larger. All Escherichia species examined showed normal frequency distribution for singlets and doublets except at the time of maximum growth. *S. aureus* did not follow the normal distribution. As would be expected in theory, by using the analytical method here discussed, the electronic count correlation with the standard plate count remained high. Because of the shift in population distribution during portions of the growth cycles

of some of the bacteria studied, any technique based on a Poisson distribution cannot be used throughout the entire growth period. That correction for population distribution is not necessary is encouraging, since it extends the range of types of micro-organisms which may be counted electronically.

CONCLUSIONS

It is concluded that it is possible to count growing bacterial populations electronically even though intra-species and inter-genus variations exist. These variations are the result of factors such as: the given organism, age, distribution and growth rate at the time of count, dilution and counting media, aperture diameter and magnitude of the current field in the electronic counting system.

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Growth (without Multiplication) of *Mycobacterium lepraemurium* in Cell-free Medium

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SUMMARY

Leprosy bacilli have not been cultivated with certainty in any cell-free medium, but the medium now described consistently supported considerable elongation of the organisms of murine leprosy (*Mycobacterium lepraemurium*), though without evidence of multiplication. The mean length of the bacilli doubled in about the generation time obtaining in host cells (7–14 days) and quadrupled before the bacilli became degenerate after about 2 months. The obligate acidity and other factors concerned in this growth were investigated, and are discussed in relation to the problem of the extracellular cultivation of leprosy bacilli.

INTRODUCTION

In the present context, 'growth' of a bacterium is defined as a gradual 'increase in bulk by the process of organic life', without this necessarily being accompanied by multiplication. (The distinction is illustrated by Nicolay (1585): 'Great bushes, and wilde brambles, which in process of time...were so growen and multiplyed'.) A cell-free medium is defined as one without intact tissue cells.

Continuous intracellular multiplication of *Mycobacterium lepraemurium*, the rat leprosy bacillus (used often as a laboratory model in leprosy studies), has recently been obtained in cultures of rat fibroblasts (Rees & Garbutt, 1962), but this species has never been shown unequivocally to multiply at all in any cell-free nutrient medium (Eddy, 1937; Gray, 1952). We have similarly failed with many such media, and have moreover found with the electron microscope that most of the bacilli are degenerate (dead) within 2–3 weeks at 37°. However, by adding sucrose to a concentration of 10% to a liquid medium (modified from that of Dubos & Davis, 1946) that is used in these laboratories for subsurface culture of *Mycobacterium tuberculosis*, Hart & Valentine (1960) obtained an increase in bacillary length in *M. lepraemurium* and a delay in its degeneration. We now report the further development of this work, which provides evidence of a living process, even though no multiplication has been detected.

METHODS

Source of the bacilli

The bacilli were provided by Dr R. J. W. Rees and his co-workers:

(*a*) *From infected mice.* The liver of a mouse of the albino P strain which had been inoculated intravenously 4–6 months previously with *Mycobacterium lepraemurium* (Douglas strain), and which had usually received suramin to enhance the infection,

was homogenized (see Hart, Rees & Valentine, 1962*a*), and the bacilli partly freed from the tissue components by the method of Garbutt, Rees & Barr (1962) and then suspended in physiological saline solution containing 1 % albumin (bovine plasma fraction V, Armour), to give about 2×10^9 acid-fast bacilli/ml., as counted by the method of Hart & Rees (1960). The organisms were well dispersed and predominantly single.

(*b*) *From infected tissue cultures.* A suspension, in Hanks balanced salt solution, of rat fibroblasts containing bacilli of *M. lepraemurium* (Garbutt *et al.* 1962) was centrifuged at 1200 rev./min. (250 *g*) for 5 min. to deposit the tissue cells, which were concentrated by removing an appropriate amount of the supernatant fluid. In some experiments the bacilli were released from the cells by exposure to ultrasonic vibration (400 kc./sec. supplied by a 500 W. generator) for 1 min. In the present work tissue culture-grown bacilli were used much less frequently than bacilli from infected mouse livers. Unless otherwise stated, the infected mouse liver was the source for inoculation into cell-free medium.

Table 1. *Final concentrations of ingredients of the present medium for studying elongation of* Mycobacterium lepraemurium *bacilli*

Ingredient	% (w/v)
Casamino acids (Difco)	1·6
Asparagine	0·75
Na_2HPO_4	0·14
KH_2PO_4	0·06
Sodium citrate	0·08
$MgSO_4,7H_2O$	0·03
Glycerol	2·9
Albumin	0·18*
Sucrose	7·4

* 0·15 % when the inoculum was from tissue culture.

Present medium

The basal medium was composed of: Difco (Bacto) 'Casamino acids' (certified), 29 g.; *L*-asparagine monohydrate, 13·5 g.; anhydrous Na_2HPO_4, 2·5 g.; KH_2PO_4, 1·0 g.; trisodium citrate, 1·5 g.; $MgSO_4,7H_2O$, 0·6 g.; glycerol, 53 ml.; de-ionized water to 1000 ml.; adjusted with HCl or NaOH to pH 6·2 or as required; autoclaved at 115° for 20 min. This autoclaved solution was distributed in 4·5 ml. amounts into 1 oz. (28 ml.) screw-capped bottles, to which were added 0·25 ml. of 5 % aqueous solution of albumin (fraction V) sterilized by filtration; 1·2 ml. of autoclaved 50 % (w/v) sucrose solution; solutions of any test substances; water to 7·9 ml.; 0·2 ml. of the bacillary suspension from mouse liver homogenate or tissue culture, giving a final total volume of 8·1 ml., containing about 5×10^7, or 10^6–10^7, bacilli/ml., respectively. The final concentrations of the ingredients in the complete medium are shown in Table 1; in addition a small amount of liver or fibroblast tissue from the inoculum was inevitably present.

Assessment of growth of bacilli in medium

A bacillary suspension in the medium was immediately fixed by adding formaldehyde to a concentration of 1 %; this served as a sample of the inoculum (for a base

line) and was stored at 4° (no significant change in the length of the bacilli occurred during storage). Other suspensions in the same medium were incubated, at 37° unless otherwise stated, for periods up to 2 months and were then similarly fixed. The inoculum sample and the incubated suspensions were diluted 1/8 in water (to decrease the sucrose concentration) and then centrifuged at 2000 *g* for 30 min. in 8 ml. Pyrex tubes. The deposited bacilli were dispersed in about 0·1 ml. of supernatant fluid by exposure to ultrasonic vibration (400 kc./sec.) for 1 min.

Samples of the deposits were examined in the electron microscope, adjusted to a magnification of 10,000 by means of standard spheres (diam. 0·26 μ) of polystyrene latex. A series of concentric circles, the circumferences of which were spaced by 0·5 cm., drawn on the fluorescent screen on which the final image was seen, allowed the lengths of the bacilli to be estimated to the nearest 0·1 μ. The lengths of 100 or more bacilli from each sample were measured in this way, and the frequency distributions of lengths and their means calculated. At the same time the proportion of bacilli which appeared degenerate (McFadzean & Valentine, 1959) was estimated. In addition, the bacillary deposits were stained by the Ziehl–Neelsen method and examined with the light microscope; a grading of length was made without formal measurement, and, using the modified stain described by Rees & Valentine (1962), the presence of degenerate bacilli was assessed. (Unless otherwise stated, bacillary lengths and proportions degenerate refer to the results obtained with the electron microscope.)

RESULTS

Optimal conditions for elongation of the bacilli of Mycobacterium lepraemurium

The nutrient medium used by Hart & Valentine (1960) required a high concentration of sucrose (or glucose) in order to obtain substantial lengthening of the bacilli. However, the results were irregular. They were somewhat better when Fe^{3+} was added to 15 μg./ml. and Mg^{2+} was increased to 14 mM (Hart, Rees & Valentine, 1962*b*); but these additions were not necessary with the present medium. In the latter, the Casamino acids were increased 6 and the asparagine 25 times; the sucrose was 7·4 %; and the solution had a specified acidity (see below). Elongation was then more rapid, more uniformly distributed in the bacterial population, and reached a higher final figure; and the results were regularly reproducible.

The acidity of the medium was critical for elongation to occur. Figure 1 shows the mean length and the degree of degeneration of the bacilli, obtained from infected mouse liver, after 17 days of incubation at 37° in the medium at different initial pH values (the pH value remained stable during this period). The greatest elongation (mean length nearly 4 μ) occurred around pH 6·0–6·4, with a rather sharp peak; and there was virtually none (mean length about 2 μ compared with 1·8 μ in the original inoculum) at pH 7·2 and more, or at pH 5·0 and less. The curve for the proportion of bacilli which were degenerate (assessed by electron microscopy) was nearly a mirror-image of that for lengthening; the majority were degenerate after incubation above pH 7·2 and below pH 5·5, whereas over 80 % (compared with over 90 % in the original inoculum) were still not degenerate at pH 6·4. It has not been possible to separate the association between acidity and lengthening and that between acidity and decreased degeneration; the optimum pH value was about the same for both and, moreover, all alterations so far made to the medium which

resulted in abolition of the elongation at pH 6·2 were accompanied also by degeneration similar to that seen at neutrality.

On the basis of these findings, a value of about pH 6·2 was specified for the medium when elongation was desired, and of about pH 7·2 when it was not. The progress, in a typical experiment, of elongation and degeneration of the bacilli (from infected

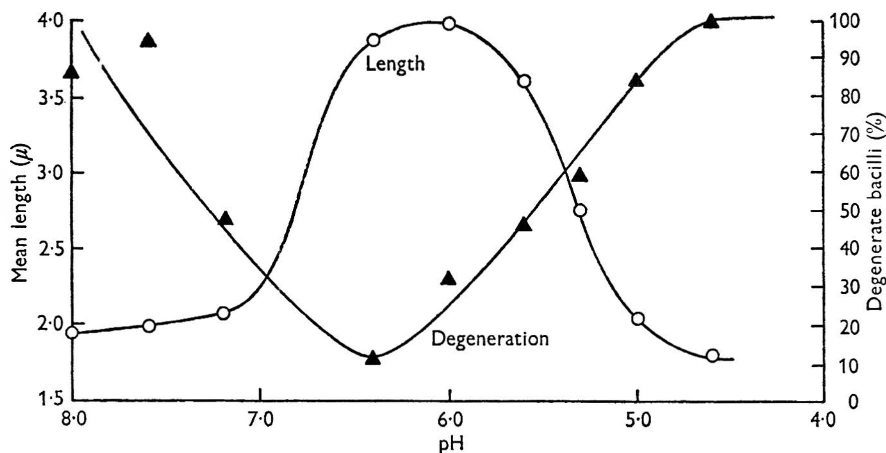

Fig. 1. The lengths (○) and proportions degenerate (▲) of *Mycobacterium lepraemurium* organisms after incubation at 37° for 17 days in the medium adjusted with HCl or NaOH to different pH values. The mean length of the bacilli in the inoculum was 1·8 μ and less than 5 % were degenerate.

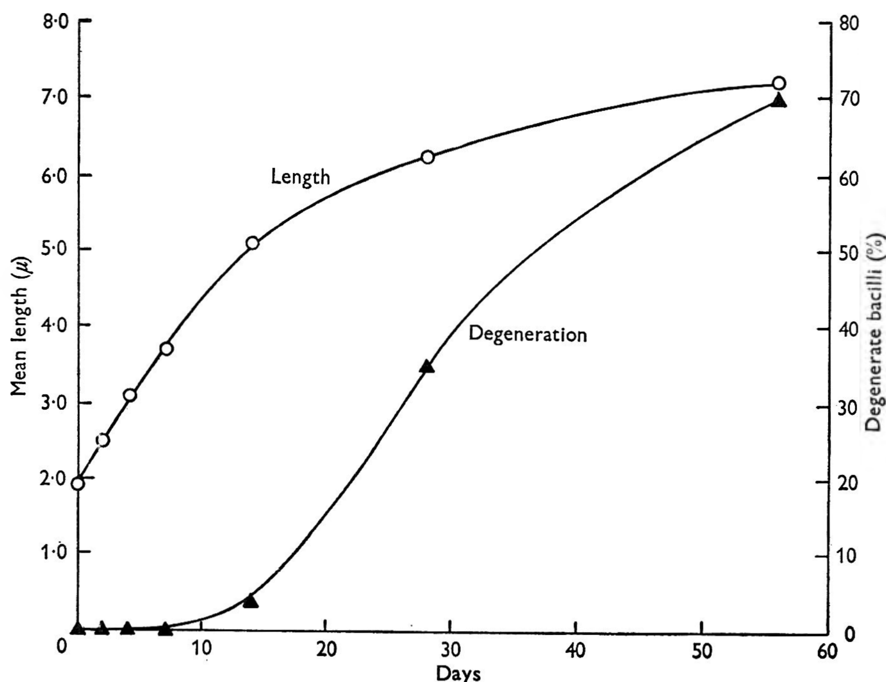

Fig. 2. The lengths (○) and proportions degenerate (▲) of *Mycobacterium lepraemurium* organisms after different periods of incubation at 37° in the medium at pH 6·0.

mouse liver) during 2 months of incubation at 37° in the medium at pH 6·0 is shown in Fig. 2. Some lengthening was evident by 2 days. The mean length of 1·9 μ in the original inoculum doubled by 7 days; after between 7 and 14 days the rate of increase gradually decreased, and the mean length at 60 days was 7·1 μ. The proportion of organisms which were degenerate (initially less than 5 %) was still less than 10 % at 7–14 days; it then increased gradually, reaching 35 % by 30 days and 70 % by 60 days, by which time elongation had virtually ceased. Hence degeneration, already marked at 2 weeks at pH values unfavourable to elongation (Fig. 1), was postponed but not avoided under these optimum conditions.

The frequency distribution of lengths of bacilli in the original inoculum is compared (Table 2) with that after incubation for 17 days in the medium. Initially, only 4 % of bacilli measured more than 3 μ and 32 % more than 2 μ; after the incubation the corresponding percentages were 62 % and 87 %. The increase in mean length is clearly due to a general shift and not merely to a few very long bacilli.

Table 2. *Length distribution of* Mycobacterium lepraemurium *organisms before and after incubation for* 17 *days at* 37° *in the medium at* pH 6·0

Length (μ)	% bacilli	
	Initially	After 17 days
0–1	0	0
1–2	68	13
2–3	28	25
3–4	4	25
4–5	0	17
5–6	0	8
6–7	0	4
7–8	0	4
8–9	0	2
9–10	0	2

The necessary constituents of the medium were investigated further, first, by adding albumin and sucrose in the same concentrations to phosphate buffer pH 7·3 and 6·2, the phosphate being finally M/50 and M/125 (that in the medium was M/70). After incubation for 17 days in these four buffer mixtures, there was no appreciable increase in average length of the bacilli at either pH value, whereas the complete medium showed none also at the higher pH value, but more than trebling of initial mean length at the lower pH value. The proportion of organisms which were degenerate was 5 % in the inoculum, 60 % or more in all the four buffer mixtures after incubation for 17 days, as compared with only 10 % at pH 6·2 in the complete medium. Next, to a similar buffer mixture at pH 6·2 (phosphate M/70; albumin and sucrose as before) glycerol was added to 2·9 % (w/v), as in the complete medium. Some elongation was now evident after incubation (though less than in the complete medium), whereas there was again none in the buffer mixture without glycerol.

The microscopic appearances of *Mycobacterium lepraemurium* in the conditions described are illustrated. Plate 1 shows, by electron microscopy: a normal bacillus from infected mouse liver (Pl. 1, fig. 1); a degenerate bacillus such as is produced by incubation for 2 or more weeks in phosphate buffer, or in the complete medium at pH 7·2 (Pl. 1, fig. 2); a long apparently healthy bacillus as produced in the

medium at pH 6·2 (Pl. 1, fig. 3); a long bacillus which has eventually degenerated (Pl. 1, fig. 4). Plate 2 shows, by light microscopy: bacilli from mouse liver immediately after inoculation into the medium (Pl. 2, fig. 5), and also the bacilli after incubation for 17 days at pH 6·2 (Pl. 2, fig. 6) and at pH 7·4 (Pl. 2, fig. 7); bacilli in tissue culture cells as inoculated into the medium (Pl. 2, fig. 8), and their appearances after incubation for 14 days at pH 6·1 (Pl. 2, figs. 9, 10). In the light microscope, long bacilli in the early stages showed a characteristic beaded appearance of the stained material which made assessment of degree of degeneration more difficult than in the electron microscope; degeneration in short bacilli, as well as in long bacilli at a later stage, was easier to assess from the gross irregularity of the stained material.

Increase in weight of the bacilli

The dry weights of the bacilli could be estimated from the electron micrographs by using electron-scattering theory (Valentine, 1962). In a typical experiment, the mean dry weights so obtained before and after 2 weeks of incubation in the medium at acid reaction were: bacillus inoculated into the medium $= 5{\cdot}9 \times 10^{-11}$ mg.; bacillus after incubation for 14 days in the medium $= 12{\cdot}0 \times 10^{-11}$ mg. During this 14-day period the mean length of the bacilli increased from 2·1 to 4·6 μ, i.e. almost in the same proportion as the weight. In the same time there was usually a slight (*c.* 20 %) increase in the width of the bacilli.

The effect of unfavourable environment on subsequent ability to elongate

The bacilli were incubated in the medium for various periods at a pH value either too large or too small for elongation to occur; the pH value was then changed to the optimum pH range by adding acid or alkali. Thus, after 1 day at pH 7·7, followed by 2 weeks at pH 6·0, the bacilli lengthened moderately well, whereas when this change in pH value was made (from pH 7·7 or 7·2 to pH 6·0) after 1 week, they lengthened little or not at all. Similar results were obtained when 1 week at pH 4·4 or 4·8 was followed by 2 weeks at pH 6·3. On the other hand, organisms incubated in the medium at pH 6·2 for 1 week, and then transferred to fresh medium at pH 6·2 and 7·0 for a further 2 weeks, continued to lengthen at the lower pH value but not at the higher. Finally, bacilli which were incubated in phosphate buffer mixtures (see above) for 2 weeks, whether at pH 6·2 or 7·3, and then (when more than half were seen to be degenerate by microscopy) transferred to the complete medium at pH 6·2, showed no subsequent elongation. It may be concluded that, when the pH value or the composition of the medium was unfavourable to elongation, not only did degeneration (as seen by microscopy) rapidly ensue (see above), but the ability to lengthen when subsequently the pH value or the composition of the medium was made favourable was soon diminished and abolished.

The natural resistance of *Mycobacterium lepraemurium* to killing by alkali was tested by exposing a bacillary suspension (from infected mouse liver) in 0·5 or 0·05 % albumin saline to 0·1 N or 0·02 N-NaOH for 30 min. at 35°, and washing the organisms, before inoculating them into the medium at pH 6·2. Resistance to mineral acid was tested by exposing a suspension in 0·025 % albumin saline to 0·1 N-HCl for 2 hr. at 37°, neutralizing, and then inoculating into the medium at pH 6·2. In both cases the capacity for lengthening was still evident, though

diminished. In this resistance, more particularly to acid, *M. lepraemurium* resembles *M. tuberculosis*, in which species this property is well known.

The effect of temperature

Bacilli were kept in medium at 42°, 37°, 34°, 24° and 4° for a month. The elongation at 34° was slightly less than at 37°, and there was none at the other temperatures. In another experiment samples of a bacillary suspension from infected mouse liver in the usual 1 % albumin saline were kept at 37° and 4° for 14 days, samples being withdrawn periodically from each lot of suspension and inoculated into the medium at pH 6·0, which was then incubated for 14 days at 37° and the bacilli examined for lengthening. Bacilli sampled from the 37° suspension on days 0 and 2 showed lengthening to a similar and considerable degree in the medium, but those sampled at day 5 lengthened hardly at all. In contrast, bacilli from the 4° suspension showed considerable lengthening when sampled up to day 8, and a little when sampled on day 14.

Tissue contamination

Mycobacterium lepraemurium is intracellular *in vivo*, but the method of preparation of the bacillary suspensions from mouse liver breaks up the tissue cells. Thus electron and light microscopy have not revealed any intact liver cells in samples of bacilli examined either before or after incubation in the medium for 14 days. It was, nevertheless, conceivable that some few liver cells were necessary for elongation to occur. This was excluded by the finding that lengthening could occur after treatment with NaOH (p. 48), which decreased the total cold trichloracetic acid-precipitatable material of the liver in the final bacillary suspension by 80–90 %. Such digestion not only would have destroyed any whole cells but also would have removed most of the remaining cell fragments.

Towards further exclusion of an essential role of tissue cells, a suspension of *Mycobacterium lepraemurium* was specially freed from liver remnants by additional differential centrifugation, and then tested for bacillary elongation in the usual manner; this was found to be similar to that of a normal preparation of bacilli. With a similar object, bacilli from a tissue culture were released from the fibroblasts by ultrasonic vibration, and the suspension washed twice and resuspended in 1 % albumin saline; again lengthening occurred.

Although it seems unlikely, therefore, that contaminating tissue played an essential part in the bacillary growth in the complete medium, it is possible that the residual liver in the preparations from mice contributed some of the nutritional requirements for growth.

Stale medium

Bacilli were incubated for 2 weeks in the complete medium at pH 6·2; the suspension was then centrifuged and fresh bacilli inoculated into the supernatant fluid, and incubation carried on for a further 2 weeks. In another experiment a fresh suspension was inoculated into the supernatant fluid from some of our earlier medium which had been in contact at 37° with bacilli for 2 months. In both experiments the fresh bacilli lengthened in the old supernatant fluid. 'Staling' of the medium appeared therefore not to be the cause of failure to multiply, nor of the eventual degeneration of lengthening bacilli.

Alterations to the medium

Many additions were made to the present medium in the hope of obtaining division and multiplication of the bacilli, or a further delay in their degeneration, but these additions, among which was mycobactin (the growth factor for *Mycobacterium johnei*), were ineffective both at neutrality and in the acid pH range optimal for lengthening. Substitution of other carbon sources for sucrose, in high or low concentrations (e.g. glucose (Hart & Valentine, 1960), sorbose or erythritol) gave no advantage. Omission of the sucrose altogether usually decreased the degree of elongation, though not much, but the results were then less regular and uniform. Omission of glycerol led to a much more pronounced diminution in elongation. On the other hand, some elongation (at pH 6·2) still occurred (both with well-washed tissue-culture bacilli and with liver-contaminated bacilli from mice) when the asparagine and Casamino acids were omitted, leaving salts, glycerol, albumin, sucrose, i.e. a mixture rather like the buffer mixture with glycerol (p. 47) and giving similar results.

Results in other media

A few conventional media were tested for their capacity to promote the lengthening of *Mycobacterium lepraemurium*. These included Hedley Wright broth, glycerinated broth, and Hanks's basal salt solution with human cord serum. At about pH 7 and 6, and both with and without sucrose, the bacilli degenerated within a few weeks without obvious lengthening.

The effect of isoniazid

As is well known, isoniazid suppresses the *in vitro* multiplication of *Mycobacterium tuberculosis*; this substance at 1 μg./ml. also completely inhibited the elongation of *M. lepraemurium* at pH 6·0–6·8. On the other hand, a substrain obtained from an infected mouse and which showed isoniazid resistance *in vivo* required 25 μg. isoniazid/ml. for complete inhibition (Hart *et al.* 1962*b*).

Total count of acid-fast bacilli

The possibility of significant changes in the total population of acid-fast bacilli was examined by Dr R. J. W. Rees (method of Hart & Rees, 1960). The counts were made immediately after inoculation into the medium and also after incubation for 17 days at pH 7·0 and at pH 6·0. About 80 bacilli were counted in each case, sufficient to detect almost certainly (P = 0·001) a change of 50 % or more in the population. In fact, the counts diminished slightly from $1{\cdot}7 \times 10^7$/ml. initially to $1{\cdot}5 \times 10^7$/ml. (pH 7·0) and $1{\cdot}4 \times 10^7$/ml. (pH 6·0) after 17 days, but the differences were not significant ($P > 0{\cdot}15$). Almost certainly, therefore, any increase or decrease in the population during the period of maximum bacillary elongation was less than 50 % and consequently unimportant or non-existent.

DISCUSSION

Gross elongation of the bacilli of many bacterial species *in vitro* has been frequently described, the common factor being a change in the physical or chemical environment unfavourable enough to prevent multiplication (division) while still permitting

growth. Mycobacteria are no exception, e.g. *Mycobacterium johnei* in culture medium containing minimal concentrations of its growth factor mycobactin (Hart, 1958) and *M. tuberculosis* in medium made toxic by urea (Hart, unpublished) or when incubated in certain buffer mixtures (see below). There would be little point in describing elongation in *M. lepraemurium* in a cell-free medium were it not that multiplication has not been achieved for this species in any such medium. Consequently, growth in the mass and length of *M. lepraemurium* bacilli may be an advance towards the possibility of obtaining complete *in vitro* cultivation; and, unlike the case with other bacteria, the physical and chemical conditions required for maximum elongation can be a guide to those most favourable for this organism.

That the elongation described represents an active vital process and not a passive stretching, or a mere accumulation of material from the medium, is supported by the present evidence—in particular, the gradual progress to a doubling of length in about the time required for one generation cycle in living mice or rats or in fibroblast cultures, namely 7–14 days (Hilson & Elek, 1957; Rees & Garbutt, 1962), the suppression of the process by low concentrations of isoniazid, and the failure of isoniazid to do so with a substrain which was isoniazid-resistant *in vivo* (Hart *et al.* 1962*b*).

Although the bacillary preparations from mice contained some liver material, the ability of the bacteria to lengthen after treatment with sodium hydroxide (which would have digested any whole liver cells still present and removed most of the cell fragments) appears to exclude any analogy with the cell-micro-organism association of tissue culture. On the other hand, the small amounts of residual tissue may have made a nutritional contribution towards the growth.

Elongation of *Mycobacterium lepraemurium* can be observed as a normal process within the host cells in the initial phase of slow multiplication in progressive murine leprosy infection in mice (Hilson & Elek, 1957; Chang, 1960; Hart *et al.* 1962*a*) and in monocytes maintained *in vitro* (Chang, 1961). We have noted abnormal elongation in a minority of the population of the slow-growing *M. johnei* in cultures in or near foci of multiplying short bacilli. However, the extracellular lengthening which we can now obtain with *M. lepraemurium* is far more striking, in that almost all the bacilli are affected, most of them finally reaching three to five times their initial lengths. This abnormal elongation, terminated eventually by degeneration, is presumably a consequence of inability to divide. We have observed a possibly analogous, though less marked, elongation without obvious multiplication in *M. tuberculosis* incubated for several weeks at 37° in phosphate buffer (pH 6·0) containing albumin and sucrose (a mixture which had given some lengthening of *M. lepraemurium* if glycerol was added, see p. 47); the *M. tuberculosis* bacilli remained short in buffer alone.

The present medium was developed from the Dubos-type formula (without Tween 80), but has a much higher content of amino acids, a high concentration of sucrose, and an acid pH value (about pH 6·2). None of the limited number of other media so far tested has given similar results either at neutral or at acid pH values, with or without sucrose. On the other hand the sucrose, while beneficial in the present medium (optimum about 10%; Hart & Valentine, 1960), is not essential for the bacillary elongation; consequently the mode of its action in such high concentration is perhaps not of particular interest. Of the other constituents of the

present medium, glycerol appears to be important for obtaining elongation, but the role of the asparagine and Casamino acids is less certain, since their omission still allowed some lengthening to occur with organisms from infected mouse liver and also with well-washed tissue-culture bacilli. However, the relatively small amount of nitrogen required for growth could have been provided under these conditions by remnants of liver cells or tissue-culture fibroblasts in the preparations, by the albumin, by the autolysis of bacterial cells, or even from the air as has been suggested to explain a multiplication of *Mycobacterium tuberculosis* in a 'nitrogen-free' medium (Hedgecock & Costello, 1962).

The narrow range of pH values for optimum elongation (pH 6·0–6·4) was less surprising than the virtual absence of elongation at about pH 7·2. For, although the optimum range for multiplication of *Mycobacterium tuberculosis* has been reported to be in the range pH 6·0–6·5 (Bekierkunst, 1957; Hedgecock & Costello, 1962), both this species and the more exacting *M. johnei* give profuse yields when cultivated at neutrality; moreover, the elongation of *M. tuberculosis*, mentioned above, was observed at pH 7·0, also.

The only method of testing chemotherapeutic agents in a murine leprosy infection is *in vivo*, or, latterly, in tissue culture. Complete inhibition of elongation of bacilli of *Mycobacterium lepraemurium* by isoniazid suggests that the doubling or more of mean length which occurs in the first 1–2 weeks of incubation in the present medium without the drug, and which is easily appreciated in the light microscope, could be used as a means for a preliminary assessment *in vitro* of the activity of potentially antileprosy agents for use in man; this possibility is being investigated. However, it does not necessarily follow that this bacillary elongation, since it is not accompanied by division, can provide information relevant to activity against a progressive infection in the animal body.

We are much indebted to Dr R. J. W. Rees for providing the bacillary suspensions and for his advice and encouragement. We also thank Dr J. Mandelstam for advice on certain biochemical points. A criticism by Professor D. G. Evans suggested an additional experiment and we are grateful to him.

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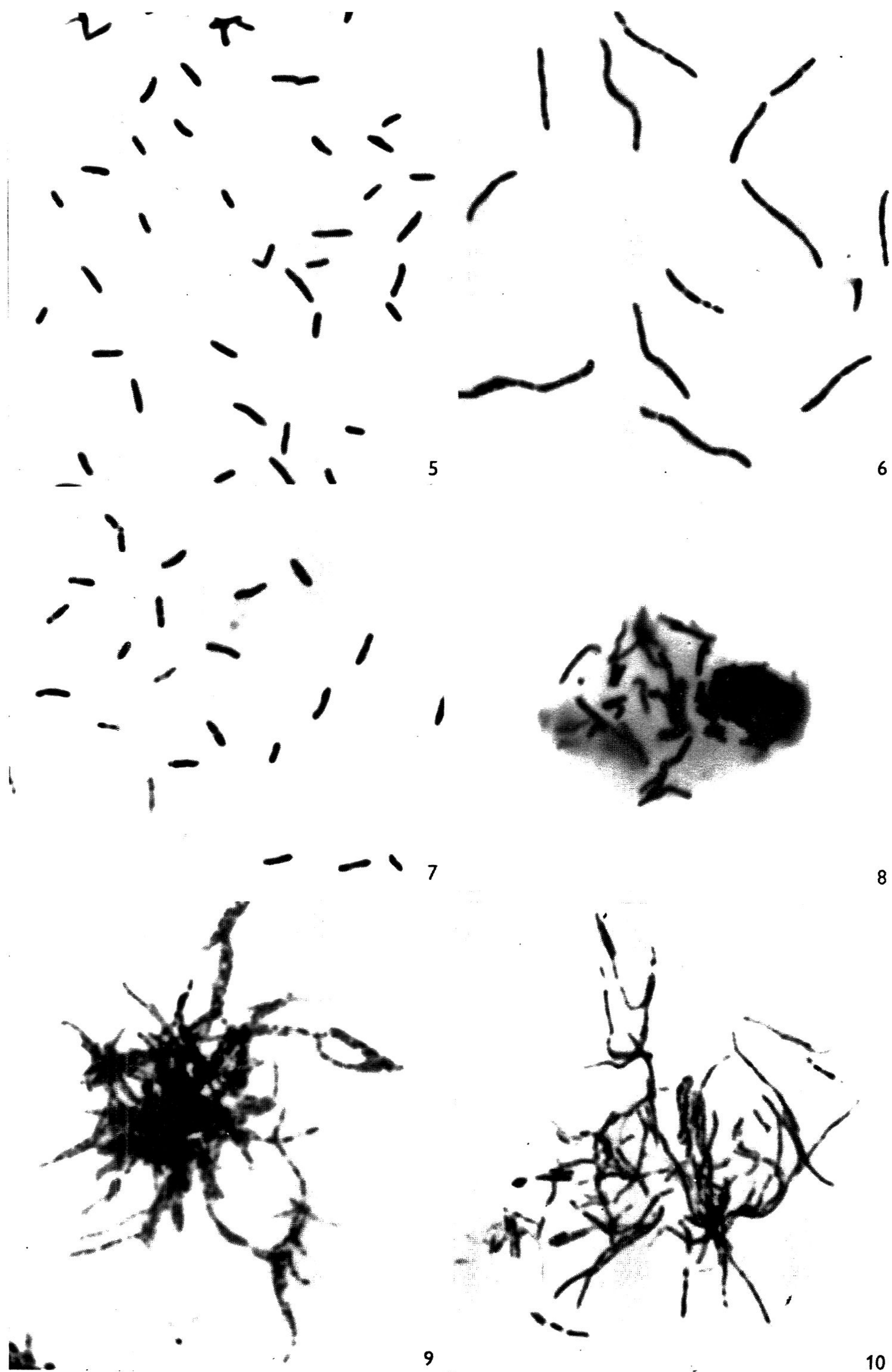

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EXPLANATION OF PLATES

PLATE 1

Electron microscope pictures of *Mycobacterium lepraemurium* before and after incubation (× 27,000).

Fig. 1. A normal bacillus from infected mouse liver.

Fig. 2. A bacillus after incubation for 28 days in phosphate buffer. A typical degenerate appearance.

Fig. 3. A bacillus after incubation for 14 days in the medium at pH 6·2. It has lengthened without yet showing a degenerative appearance.

Fig. 4. A bacillus after lengthening, followed eventually by an appearance typical of degeneration.

PLATE 2

Light microscope pictures (Ziehl–Neelsen stain) of *Mycobacterium lepraemurium* before and after incubation in the medium (× 1900).

Fig. 5. Normal bacilli obtained from infected mouse liver. The organisms are well dispersed and predominantly single. They are homogeneously stained.

Fig. 6. Similar bacilli after incubation for 17 days at pH 6·2. They are long and many have a beaded appearance.

Fig. 7. Similar bacilli after incubation for 17 days at pH 7·4. They have not lengthened and many are unevenly stained.

Fig. 8. Normal bacilli in tissue culture fibroblasts.

Figs. 9, 10. Similar bacilli after incubation for 14 days at pH 6·1. The fibroblasts have largely autolysed, the organisms are long and some appear beaded.

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Reverting and Non-Reverting Rough Variants of *Bacillus anthracis*

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SUMMARY

Non-capsulated variants of *Bacillus anthracis* have been said not to revert to the capsulated state. However, in the present work, capsulated revertants were isolated from about half the non-capsulated strains tested either by exposure to phage Wα, which attacks only non-capsulated organisms, or by passage in mice.

INTRODUCTION

Virulent strains of *Bacillus anthracis* form mucoid colonies of capsulated organisms when grown under appropriate conditions (Nungester, 1929; Schaefer, 1936; Sterne, 1937*a*, *b*; Thorne, Gomez & Housewright, 1952). After several days' incubation, these colonies may show rough outgrowths which contain non-capsulated avirulent variants presumably arisen by mutation (Stamatin, 1934; Schaefer, 1936; Sterne, 1937*a*, *b*; Chu, 1952). Examination of rough variants isolated from many different strains of *B. anthracis* has led to the belief that they never revert to the virulent capsulated type, even on animal passage (Bail, 1915; Sterne, 1937*a*, *b*; Chu, 1952); and since they retain the toxigenic (see Smith & Keppie, 1955), and thus the immunizing, activity of the parental type (Bail, 1915; Stamatin & Stamatin, 1936; Stamatin, 1937), they have been widely and successfully used during the last 25 years for the immunization of domestic animals (Sterne, 1939*a*).

Non-reverting mutants are especially convenient for genetic experiment, since parental types can only appear following recombination. Consequently, various known methods of gene transfer were tested for their ability to transmit the C+ genes which determine capsule production to a rough (C−) recipient strain. Any C+ recombinants were to be selected by phage Wα, which attacks non-capsulated, but not capsulated, organisms (McCloy, 1951). This experiment led to the unexpected finding of mucoid colonies on control plates which had received only bacteria of the non-capsulated (C−) strain, and phage Wα. These colonies could not have arisen by transduction (Zinder & Lederberg, 1952), since the phage stock had been grown on a genetically identical strain. Thus it seemed that, contrary to what has been generally accepted, at least some C− variants were able to revert to the C+ form. Further tests on a number of rough variants arising independently from several anthrax strains have shown that many, though not all, revert to the capsulated virulent form. It should be added that each of the C− variants was purified by three successive single spore isolations before being tested for reversion, so as to

exclude contamination by C+ organisms of the parental culture. Thorne (1960) briefly mentioned the appearance of C+ colonies in rough cultures exposed to phage, and Wright, Puziss & Neely (1962) observed the emergence of C+ clones while studying the formation of protective antigen by C− strains in a chemically defined medium.

METHODS

Media. Nutrient broth was made of Lab. Lemco 0·1 % (w/v), Tryptone (Oxoid) 0·2 % (w/v), Peptone (Oxoid) 1 % (w/v) and NaCl 0·7 % (w/v). Nutrient agar contained nutrient broth solidified with 1·5 % (w/v) Davis N.Z. agar. Cultures incubated in air, particularly those used for the production of spore suspensions, were often grown on plates containing 2 parts nutrient agar and 1 part 1·5 % (w/v) solution of liver extract (Oxoid). For production of capsules, the bacteria were grown on nutrient agar containing either 20 % (v/v) horse serum or 0·025 M-$NaHCO_3$ and incubated in air + CO_2 (i.e. about 30 % of the air replaced by CO_2).

Spore suspensions. A confluent growth of bacteria on liver agar was incubated for several days at 37° and then suspended in distilled water and incubated for some further days (Hardwick & Foster, 1952). Suspensions were sometimes lightly centrifuged to remove clumps; only those in which the spores were well dispersed and in which no vegetative bacteria could be seen were finally used.

Phage stocks. Stocks of phage Wα grown on non-capsulated variants of the atypical strain Davis (McCloy, 1958) were used in most experiments, but occasionally phage grown on two other strains was used, as described in the next section. Lysates were freed from bacteria by filtration through Seitz filters, and had titres of 5×10^9 to 2×10^{10} plaque-forming particles/ml.

Strains of Bacillus anthracis *tested. Isolation and purification of C− clones.* Eleven strains tested were received in the C− state and produced only rough non-mucoid colonies on serum or bicarbonate agar incubated in air + CO_2. These comprised: (1) 2 strains used as vaccines, CN 1874 and CN 3518 (respectively 34F2 and V8R1 from Ondestepoort, South Africa), received from Mr H. Proom, The Wellcome Research Laboratories, Beckenham, Kent; (2) 7 strains received from Dr G. Ivánovics, Universitatis Medicae Szegediensis, Szeged, Hungary, 5 of which had been reported as non-reverting to the capsulated state (Ivánovics & Földes, 1958); (3) 1 strain received from Dr J. Davis, Public Health Laboratory, County Hall, Westminster Bridge, London; (4) 1 strain from the author's stocks. Nine strains when received produced a mixture of mucoid and rough colonies, or only mucoid colonies; C− variants were then isolated by picking a rough colony or a rough outgrowth from a mucoid colony. The C− strains were rigorously purified in view of Sterne's (1937*a*, *b*) opinion that any apparent reversions to capsulation observed either by himself or other workers (Schaefer, 1936) were in reality the result of incomplete purification of the rough isolate, which had not been entirely freed from capsulated bacteria carried over from the parent strain. Three successive single-colony isolations were therefore made by allowing the C− clone to sporulate, plating the resultant spore suspension for single colonies, and repeating the procedure twice more before accepting a C− clone as pure. This ensured that, in each cycle, each colony arose from a single spore and not from entangled chains of vegetative forms.

Isolation of C+ bacteria from C− strains. Serum or bicarbonate agar plates were spread with 0·2 ml. of undiluted phage Wα preparation and 10^8–10^9 spores of the C− strain, and then, as quickly as possible, transferred to an anaerobic jar in which 20–30% (v/v) of the air was then replaced with CO_2. Spores, which do not adsorb the phage, were used as inocula, since it was thought that in this way any C+ organisms which might be present would remain resistant to the phage until they reached the atmosphere of CO_2 in which they could develop the protective capsule. Mucoid colonies which appeared were subcultured several times to free them from contaminating phage, and before being tested for virulence were shown to be phage-sensitive (on ordinary agar in air) and non-lysogenic (Ivânovics, 1962).

Virulence tests. The C+ strains were tested at doses of about 10^4, 10^3, 10^2 and 10 spores, and the C− at doses of about 10^7, 10^6, 10^5 and 10^4 spores, three mice being used per dose which was given intraperitoneally in 0·2 ml. sterile distilled water. At the same time, viable counts were made on ordinary agar and on serum or bicarbonate agar incubated in air + CO_2; the C+ strains produced an occasional C− colony, but with the C− strains, only rough non-mucoid colonies were seen. Mice which died were examined *post mortem*, and a sample of heart blood cultured on serum or bicarbonate agar in air + CO_2.

RESULTS

Selection with phage grown on the same strain. Non-capsulated (C−) variants were isolated from the C+ strains 2160s and Davis (McCloy, 1958). These strains are asporogenous and greatly attenuated from causes other than the absence of a capsule. Strain 2160s is a smooth colony variant (Nungester, 1929; Sterne, 1938) isolated from a virulent strain, 2160, by growth in broth containing calcium chloride (Renaux, 1952). Since these strains did not form spores, the C− variants were simply put through three successive single-colony isolations with plating on serum agar in air + CO_2 in order to detect any possible mucoid sectors in the colonies. The third strain tested, 1444, produced spores. Stocks of phage were grown on each of these three C− strains, and each strain yielded a small proportion (about 10^{-7}) of C+ colonies when plated with phage which had been grown on itself. Since the strain tested and the last host of the phage were identical, the C+ colonies could only have arisen by selection, and this conclusion was confirmed for strain 1444 by isolating C+ bacteria from the blood of mice fatally infected with large doses of the C− strain. Since strains 2160s and Davis were virtually avirulent even in the C+ state, no attempts were made to isolate C+ from C− bacteria of these strains by animal passage.

Tests with phage grown on C− strain Davis. Table 1 shows the results of tests on 30 C− variants isolated from 21 strains of *Bacillus anthracis*. C+ bacteria were found in 17 of the 30 C− cultures at frequencies of 7×10^{-9} to 10^{-6}. Eleven of the C− variants were also inoculated into mice, and all but one produced some fatal infections in high doses of 10^6 or more spores. With 5 of the 11 strains, C+ bacteria were found in the heart blood *post mortem*; fatal infections with the other 5 yielded only C− bacteria.

Differences between independent C− isolates. The most obvious difference was that C+ revertants were obtained from some, but not all, C− variants, even when these were isolated from the same C+ parental strain. When several independent C−

variants were isolated from five C+ strains, the fractions giving C+ revertants were $\frac{1}{3}$, $\frac{1}{3}$, $\frac{2}{3}$, $\frac{3}{3}$, and $\frac{1}{2}$, respectively. The change C+ to C− thus seems to resemble variation in other characters which may or may not revert, where the non-reverting mutation is often due to deletion or inversion in the genome (see Demerec & Hartman, 1959).

Table 1. *Tests with phage Wα grown on C−* Bacillus anthracis *strain Davis*

Strains received as	No. of strains	No. of independent C− isolates tested	No. of C− variants yielding C+: (i) with phage	(ii) in mice	(iii) total
C+	10	19	12/19*	3/4	12/19
C−	11	11	5/11	2/7	5/11

* Number of strains tested.

C− variants appear more readily in some C+ strains than in others (Sterne, 1937*b*) which is probably at least in part because C− bacteria can grow out from the mucoid parent colony more readily when they form long chains. Typical rough outgrowths were never seen with one of the C+ strains examined, but only indentations or narrow borders of non-mucoid growth around the mucoid colonies. The reason for this was most probably that, under conditions unsuitable for capsule production, the colonies of this strain were rather smooth instead of having the rough form associated with long chains of bacteria. These non-mucoid areas contained organisms which killed about 50% of mice given 10^5 spores, although there was no evidence from microscopic examination that traces of capsule were still present, as found by Schaefer (1936) in some of his variants. Highly mucoid C+ revertants were found in some fatally infected mice, and could also be isolated by culture with phage Wα.

DISCUSSION

Conditions favouring the production of mucoid colonies and short bacillary chains by capsulated (C+) forms of *Bacillus anthracis* are considered to confer a selective advantage on non-capsulated (C−) variants which eventually project outwards from the colony towards new sources of nutrient (Chu, 1952). C+ revertants are not seen when C− bacteria are simply cultured in air+CO_2, which may partly explain why C− variants have so long been stated to be non-reverting. Another observation which supported this belief was the absence of fatal infections in cattle and other animals vaccinated with C− cultures (Sterne, 1939*a*, *b*). The present results show that at least half the C− variants isolated from C+ strains revert to C−, and also that the kind of C− variant produced (reverting or non-reverting) is not determined by the C+ parental strain. C+ revertants are recovered only when C− cultures are exposed to a selective agent like phage Wα, which attacks non-capsulated, but not capsulated, bacteria (McCloy, 1951), or to the anti-bacterial mechanisms present in mice which preferentially kill C− organisms. Mice may select C+ bacteria more efficiently than other animals, for their immunity to anthrax may depend on anticapsular antibody (Tomcsik & Ivánovics, 1938; Ivánovics, 1938; Sterne 1939*b*; Gladstone, 1946), unlike other animals such as the guinea-pig, rabbit or sheep (Stamatin, 1937; Tomcsik & Ivánovics, 1938;

Ivánovics, 1938; Sterne, 1939*a*, *b*; Gladstone, 1946; Staub & Grabar, 1943; Smith, Zwartouw & Harris-Smith, 1956). Moreover, in the guinea-pig at least, immunity develops so rapidly after a dose of C− spores (Sterne, 1939*b*) that C+ revertants might not be able to cause a fatal infection if they did not arise soon after inoculation.

Schaefer (1936), reported the isolation of unstable, as well as of stable, non-capsulated variants, but no revertants were recovered from several rough variants examined by Stamatin (1934, 1937; Stamatin & Stamatin, 1936), from more than nine variants isolated by Sterne (1937*b*, 1939*a*) and from the many more tested by Chu (1952). This is surprising in view of the present results, but it must be mentioned that the two vaccine strains, CN 1874 and CN 3518, originally from Dr Sterne's collection, did not revert in the present tests. These and other rough variants used for active immunization are not likely to have been derived from C+ strains that gave only non-reverting C− variants. Dr Sterne tells me that his C+ strains were chosen at random, and that the explanation may be that he tested as possible vaccines only those C− cultures which remained free from capsulated bacteria, and dismissed any C+ bacteria as contaminants carried over from the original C+ parental strain.

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The Characterization of a Series of Lactobacillus Bacteriophages

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SUMMARY

The properties of four *Lactobacillus casei* phages and eleven *L. fermenti* phages are presented. Most of these phages are species specific; some have a limited intraspecies action. The two groups of phages are serologically distinct, but the members of each group are related serologically. All the phages have slow adsorption rates. The *L. casei* phages are citrate insensitive, while some of the *L. fermenti* phages are citrate sensitive. The *L. fermenti* phages have shorter latent periods than the *L. casei* phages and this correlates directly with the generation times of their hosts. The phages are large and have a tadpole appearance.

INTRODUCTION

The first phage active on lactobacilli was reported by Kopeloff (1934); this was isolated from sewage. No further details were provided apart from the statement that it attacked certain strains of *Lactobacillus acidophilus* and *L. bulgaricus* and did not attack R variants of these strains. Kiuru & Tybeck (1955) isolated two phages from starters used in the manufacture of Swiss cheese. These phages had very restricted host ranges. The one was active on a strain of *L. lactis* and the other on two strains of *L. helveticus*. Meyers, Walter & Green (1958) isolated from saliva a phage active on a strain of *L. casei*; this phage was characterized by Walter (1958). Coetzee, de Klerk & Sacks (1960) and Coetzee & de Klerk (1962) reported the isolation of 14 Lactobacillus phages from sewage; subsequently one more phage (no. 780) active on strains of *L. casei* was isolated, also from sewage. This paper reports the properties of these 15 phages.

METHODS

Media. The media used have been described (Coetzee *et al.* 1960; de Klerk & Coetzee, 1962).

Phages. The methods used for the assay of phage, the preparation and assay of antiphage sera, the determination of adsorption rates, latent periods and burst sizes and the isolation of phage-resistant mutants were those of Adams (1959). The phages are identified according to the number of the original host organism. These

are 41, 69, 206, 222, 222a, 276, 315, 514, 517, 535, 547 isolated on *Lactobacillus fermenti* hosts and 300, 316, 356, 780 isolated on *L. casei* hosts. Phage lysates were prepared by inoculating 40 ml. of a young broth culture from a plaque. After overnight incubation the cultures were clear and had plaque forming titres of about 1×10^9/ml. Phage suspensions were stored above 0·1 vol. of chloroform at 4°. In some adsorption experiments the broth was supplemented with vitamin-free pancreatic casein digest (Nutritional Biochemicals Co., Cleveland, Ohio) 10 g./l. and/or 50% (v/v) of Hanks balanced salt solution (Weller, Enders, Robbins & Stoddard, 1952). In other experiments Tween 80 was omitted from all media. The influence of sodium chloride on the stability of the phages was tested by the method of Kiuru & Tybeck (1955).

Citrate sensitivity. The citrate sensitivity of the phages was expressed as their relative efficiency of plating (e.o.p.) on media supplemented with 2% (w/v) sodium citrate.

Heat inactivation. Heat inactivation constants were determined by maintaining the phages at 55° in broth and assaying samples at intervals. The samples were brought to 20° before plating.

Electron microscopy. Phage lysates were purified and concentrated by differential centrifugation (alternate cycles at 12,350*g* for 90 min. and 3090*g* for 10 min.) in a Servall centrifuge (model SS-4). Purified suspensions (plaque forming titre 1×10^{12}/ml.) were sprayed on to collodion-covered grids, air dried, shadowed with a gold+palladium alloy and examined with a Philips (Model EM 100) electron microscope.

Organisms. In addition to the host strains of lactobacilli used, 37 strains of *Lactobacillus acidophilus*, 14 of *L. casei*, 11 of *L. casei* var. *rhamnosus*, 12 of *L. salivarius*, 29 of *L. plantarum*, 27 of *L. brevis*, 35 of *L. fermenti* and one of *L. helveticus* strains were used in host range experiments; they were identified by the methods previously employed (Coetzee *et al.* 1960).

Generation times. The generation times of the lactobacilli were determined by total counts (de Klerk & Coetzee, 1962).

RESULTS

All the phages were stable in the presence of chloroform, thus differing from a series of *Clostridium perfringens* phages (Smith, 1959) and Brucella phages (McDuff, Jones & Wilson, 1962).

Electron microscopy. Electron micrographs were taken of the four *Lactobacillus casei* phages and four of the *L. fermenti* phages. These revealed tadpole morphologies. The *L. casei* phages had larger heads (average dimensions 105×87 mμ) than the *L. fermenti* phages (average dimensions 83×64 mμ). The tails of the former were slightly shorter and thicker than the *L. fermenti* phages (average dimensions 194×21 mμ and 210×18 mμ respectively). The average over-all length of the phages was nearly the same (299 and 293 mμ respectively.) All the phages showed terminal knobs like *Listeria monocytogenes* phages (Sword & Pickett, 1961).

Adsorption rates. The adsorption of all the Lactobacillus phages followed first-order reactions. The *L. fermenti* phages all adsorbed slowly (Table 1) and their adsorption rates could not be correlated with their other properties. The *L. casei*

phages (Table 2) also had slow adsorption rates, with the exception of phage 356, which differed in many respects from the other *L. casei* phages. The adsorption of all the phages was unaffected by the omission of Tween (White & Knight, 1958) or the addition of co-factors to the media. The optimal concentration of organisms was $1 \cdot 0 \times 10^9$ colony forming units/ml.; smaller values than this slowed the adsorption rate.

Table 1. *Some properties of* Lactobacillus fermenti *phages*

Phage no.	E.o.p. on citrate media	Growth constants at 37°: Latent period (min.)	Growth constants at 37°: Burst size	Adsorption constants (ml./min.) at 37°	Heat inactivation constants at 55°
41	1	83	82	$2 \cdot 3 \times 10^{-10}$	0·044
315	1	77	60	$1 \cdot 5 \times 10^{-11}$	0·156
514	1	85	88	$1 \cdot 5 \times 10^{-11}$	0·620
222	1	85	63	$4 \cdot 0 \times 10^{-10}$	0·643
222a	1	97	30	$2 \cdot 8 \times 10^{-11}$	0·098
69	0·0001	99	35	$5 \cdot 7 \times 10^{-12}$	0·088
206	0·0002	72	100	$5 \cdot 7 \times 10^{-11}$	0·066
517	0·0001	72	90	$2 \cdot 6 \times 10^{-11}$	0·087
535	0·0001	85	73	$1 \cdot 6 \times 10^{-11}$	0·093
547	0·0003	87	60	$1 \cdot 6 \times 10^{-11}$	0·112
276	0·0001	72	70	$1 \cdot 2 \times 10^{-11}$	0·095

Table 2. *Some properties of* Lactobacillus casei *phages*

Phage no.	E.o.p. on citrate media	Growth constants at 37°: Latent period (min.)	Growth constants at 37°: Burst size	Adsorption constants (ml./min.) at 37°	Heat inactivation constants at 55°
300	1	220	20	$5 \cdot 0 \times 10^{-12}$	0·190
780	1	220	20	$2 \cdot 1 \times 10^{-11}$	0·204
316	1	220	6	$3 \cdot 3 \times 10^{-11}$	0·442
356	1	180	33	$3 \cdot 2 \times 10^{-9}$	0·104

Plaque morphology. The *Lactobacillus casei* phages formed minute clear plaques of average diameter 0·3 mm. and a shelving edge. The *L. fermenti* phages formed similar but larger plaques. Phages 41, 69, 276, 535 and 547 formed plaques of average diameter 0·5 mm. and phages 206, 222, 222a, 315, 514 and 517 formed plaques of diameter 1 mm. The plaques of individual phages varied somewhat in size and this variation may be explained (McDuff *et al.* 1962) as due to vagaries of adsorption. The phages are large and the smallness of the plaques was ascribed to this factor, but the slow adsorption rates may be a contributory factor (McDuff *et al.* 1962).

Serology. The inactivation of the phages by antisera followed first order kinetics; Table 3 shows the neutralization constants of the phage antisera. These constants are low. A second series of rabbits was immunized with the phages but their sera yielded lower K values. Table 3 shows that the *Lactobacillus casei* phages and *L. fermenti* phages are serologically distinct and are classified into groups I and II respectively.

Host range. The results of host range experiments have already been reported (Coetzee *et al.* 1960; Coetzee & de Klerk, 1962). These indicated that the phages were not only genus specific but practically species specific. The present results confirmed these findings. Individual *L. fermenti* phages productively lysed 25–40 % of the 35 *L. fermenti* organisms and caused abortive infection with host killing in 14·3–66 % of these organisms. The *L. casei* phages productively lysed 12–40 % of the 25 *L. casei* spp. tested, and caused abortive infection with host death in 15 % of these organisms. A few of the *L. fermenti* phages caused abortive infections with host killing of 3 *L. acidophilus*, 3 *L. casei* and 3 *L. brevis* organisms, and productive

Table 3. *Neutralization constants of Lactobacillus phage antisera at* 37°

Dilutions of homologous and heterologous phage antisera were mixed with the phages at 37°. Phage was assayed at intervals. The proportion of phage surviving was plotted against time. The survival was a first order reaction and from this neutralization constants were calculated.

Phage antiserum no.	Phage no.														
	41	69	206	222	222a	276	315	514	517	535	547	300	780	316	356
	Minutes^{-1}														
41	4	3	2	4	4	4	4	4	4	4	4	0	0	0	0
69	8	11	3	10	10	6	9	8	10	10	10	0	0	0	0
206	8	5	9	6	6	7	2	3	4	3	2	0	0	0	0
222	12	12	5	11	11	7	12	11	13	11	12	0	0	0	0
222a	15	14	9	12	21	17	13	16	16	18	7	0	0	0	0
276	3	5	8	4	4	8	4	3	5	4	3	0	0	0	0
315	3	3	1	3	3	3	9	4	5	4	2	0	0	0	0
514	3	1	1	2	4	1	4	2	3	4	3	0	0	0	0
517	4	5	2	5	6	3	7	4	4	3	5	0	0	0	0
535	10	8	4	8	8	8	10	9	14	12	6	0	0	0	0
547	3	3	6	5	3	2	3	3	3	4	6	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	76	60	3·6	1·2
780	0	0	0	0	0	0	0	0	0	0	0	29	30	3·0	1·0
316	0	0	0	0	0	0	0	0	0	0	0	17	12	72	29
356	0	0	0	0	0	0	0	0	0	0	0	0·80	2·0	10	16
	L. fermenti phages. Serological group II											*L. casei* phages. Serological group I			

infections in 2 *L. casei* and 3 *L. brevis* strains. The *L. fermenti* host no. 296 on which *L. casei* phage 300 caused lytic infection (Coetzee *et al.* 1960) has been lost and extra-species activity of the *L. casei* phages is limited to the action of 3 of these on one or more of 3 *L. plantarum* organisms. The host ranges of the eleven *L. fermenti* phages differ from one another, while those of two of the *L. casei* phages (nos. 300, 780) are identical. With the exception of the few inter-species abortive and productive infections just mentioned, host range experiments support the serological grouping of these phages into two distinct groups.

Citrate sensitivity. The *Lactobacillus casei* phages did not require Ca^{2+} for adsorption (Table 2). *L. fermenti phages* 41, 222, 222a, 315 and 514 had an e.o.p. value of 1 in the presence of sodium citrate, while phages 69, 206, 276, 517, 535 and 547 had a plating efficiency of 10^{-4} (Table 1).

Heat inactivation. Heat inactivation of the phages at 55° followed first order

kinetics; the constants varied from 0·044 to 0·643 (Tables 1, 2). These values did not correlate with other properties of the phages.

Latent period. The latent periods of the *Lactobacillus casei* phages at 37° ranged from 180 min. (phage 356) to 220 min. (phages 316, 300 and 780); the burst sizes were below 50 (Table 2). The latent periods of all the *L. fermenti* phages were less than 100 min. (Table 1) and the burst sizes were between 50 and 100 (Table 1). The latent periods correlated directly with the generation times (McDuff *et al.* 1962). The generation time of *L. casei* organisms at 37° was 62 min. as compared with 50 min. for the *L. fermenti* organisms. By premature lysis of the host organisms with chloroform (Séchaud & Kellenberger, 1956) *L. casei* phage could be detected at 85 min. and *L. fermenti* phages were demonstrated in this manner after 60 min.

Effect of sodium chloride. Like the phages of Kiuru & Tybeck (1955) the *Lactobacillus casei* phages showed no significant decrease in plaque forming units when suspended in 25% (w/v) NaCl solution for 2 days, while the *L. fermenti* phages were unstable. The titre of all the phages of this latter group decreased by more than 90% under these conditions.

Table 4. *Cross-resistance grouping of citrate-insensitive* Lactobacillus fermenti *phages*

Phages were added to organisms and after adsorption for 15 min. unadsorbed phage was assayed. The contents of the adsorption tube were then diluted 1/100 in fresh pre-warmed broth and maintained at 37°. Samples were assayed at intervals.

Bacteria no.	Phage no. 41	315	514
41/ 41	−	−	+
315/315	+	−	−
514/514	+	+	−

+ = Productive infection; − = adsorption, but no phage production.

Table 5. *Cross-resistance grouping of citrate-sensitive* Lactobacillus fermenti *phages*

Phages were added to organisms and after adsorption for 15 min. unadsorbed phage was assayed. The contents of the adsorption tube were then diluted 1/100 in fresh pre-warmed broth and maintained at 37°. Samples were assayed at intervals.

Bacteria no.	Phage no. 69	206	517	535	547
69/ 69	−	+	+	+	+
517/517	−	+	−	+	−
547/547	+	+	+	+	−
206/206	−	−	+	−	−
535/535	−	−	−	−	−

+ = Productive infection; − = adsorption, but no phage production.

Cross-resistance tests. Cross-resistance tests afforded another method of distinguishing phages with similar properties. Phage-resistant mutants of *Lactobacillus casei* strains 300 and 780 and of *L. fermenti* strains 41, 69, 206, 514, 517, 535

and 547 were obtained on first attempt and are presumably one-step mutants. The phages adsorbed at the same rate to the resistant mutants, but no progeny phage was liberated and the bacteria were unaffected (Garen & Puck, 1951). All the mutants were of this type. No phage non-adsorbing mutants of these strains were obtained. Resistant mutants of *L. casei* strain 316 and *L. fermenti* strains 222, 222a, 276 were not obtained despite repeated attempts at selection. *L. casei* phages 300 and 780 could not be differentiated by cross-resistance tests and, as the other properties examined are similar, they are presumably identical. Table 4 shows that *L. fermenti* 41/41 is also resistant to phage 315. *L. fermenti* 315/315 is also resistant to phage 514 but is lysed by phage 41. *L. fermenti* 514/514 is lysed by phages 41 and 315. Table 5 presents the results of cross-resistance tests with a group of citrate-sensitive *L. fermenti* phages. Some of the mutants again show multiple resistance (Demerec & Fano, 1945) but the lytic pattern enables phages 69, 517 and 514 to be differentiated from one another. Phages 206 and 535 have the same host range on these mutants but can be differentiated by plaque size.

DISCUSSION

In recent years interest has been shown in phages other than those which attack organisms of the family Enterobacteriaceae. Smith (1959) described phages which attacked strains of *Clostridium perfringens*; Sword & Pickett (1961) described *Listeria monocytogenes* phages. Brucella phages were described by McDuff *et al.* (1962) and Bacillus phages have been characterized by Meynell (1962). A revival of interest has also been shown in the mycophages, Streptococcus phages and Staphylococcus phages (Groman, 1961). All these phages have properties of a similar nature to the T phages. The Lactobacillus phages are no exception. The characteristics of the four phages isolated on homofermentative hosts correspond to descriptions of the *Lactobacillus casei* phage characterized by Walter (1958). Thus Walter's phage has the same over-all length as our *L. casei* phages, although the tail is decidedly shorter and lacks a terminal knob. His phage also forms small plaques and has a host range limited to one strain of *L. casei*. The latent period (140 min.) is shorter than the latent periods of our *L. casei* phages but the burst size is similar. The long latent periods of the *L. casei* phages approach those of certain mycophages (Bowman & Redmond, 1959). Walter's phage had the same heat sensitivity as our *L. casei* phages. Apart from *L. casei* phage 356, the other phages investigated had slow adsorption rates which could not be increased. Walter (1958) encountered the same phenomenon with his phage. Our media contained Tween 80 and it was hoped to increase the rate by omitting it (White & Knight, 1958). This omission had no effect. Kiuru & Tybeck (1955) investigated their phages along different lines, but their two phages were relatively stable at 55° and like our *L. casei* phages were also not inactivated by 25% (w/v) NaCl solution.

The phages isolated on heterofermentative hosts differ in some respects from the *Lactobacillus casei* phages. The most striking properties of the former phages are the larger plaques, the very much shorter latent periods and their inactivation by 25% (w/v) NaCl solution. These eleven phages are serologically related but are not homogeneous as regards citrate sensitivity.

The Lactobacillus phages usually have only a limited intra-species host range.

No phage like phage α (Meynell, 1962), which lyses all strains of *Bacillus anthracis*, was isolated. The limited host-range of the phages may be helpful in devising a phage-typing scheme for lactobacilli; but, despite repeated attempts, no phages which productively lyse *L. acidophilus* or *L. salivarius* strains have been isolated from sewage, vaginal secretions or saliva. We have also not succeeded in primarily isolating phages on strains of *L. plantarum* and *L. brevis*.

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The Decomposition of 4-(2,4-Dichlorophenoxy)butyric Acid by *Flavobacterium* sp.

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SUMMARY

A *Flavobacterium* sp. isolated from soil and grown in media containing 4-(2,4-dichlorophenoxy)butyric acid (4-(2,4-DB)) metabolized 2,4-dichlorophenol and 4-chlorocatechol without a preliminary period of induction. The initial oxidation of 4-(2,4-DB) was rapid, but the rate declined to a value equivalent to that observed for butyric and crotonic acid oxidation. The bacterium produced 2,4-dichlorophenol, 4-chlorocatechol and butyric and crotonic acids when grown in the presence of 4-(2,4-DB). It is proposed that the initial step in 4-(2,4-DB) oxidation involves a cleavage of the ether linkage rather than β-oxidation of the aliphatic moiety.

INTRODUCTION

Several of the phenoxyalkyl carboxylic acids which are used as herbicides are decomposed in soil by microbial action. By means of a plant bioassay technique, Burger, MacRae & Alexander (1962) established a relationship between molecular structure and the persistence in treated soil of phytotoxic phenoxyalkyl carboxylic acids, but such studies do not indicate whether the inhibitory effects result solely from residual pesticide or whether new phytotoxic substances appear as a result of microbial degradation of the added compound. To establish the pathway of decomposition, investigations have been made with cultures of some of the bacteria responsible for the detoxication in natural environments. For example, Steenson & Walker (1957) proposed that 2,4-dichlorophenol and 4-chlorocatechol were intermediates in the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) by strains of Achromobacter and *Flavobacterium peregrinum*, and Evans & Smith (1954) reported the production of 2-hydroxy-4-chlorophenoxyacetate and 4-chlorocatechol in cultures of a soil bacterium which metabolized 4-chlorophenoxyacetic acid. Webley, Duff & Farmer (1957) demonstrated the conversion of 4-(4-chlorophenoxy)-butyric acid to β-hydroxy-4-(4-chlorophenoxy)butyric acid by *Nocardia opaca*; these results, together with the more recent observations of Taylor & Wain (1962), suggest that the aliphatic moiety of phenoxyalkyl carboxylic acids is metabolized by β-oxidation. The present paper is concerned with work on the decomposition of 4-(2,4-dichlorophenoxy)butyric acid (4-(2,4-DB)) by a Flavobacterium isolated from soil. The data suggest that the molecule is initially degraded by cleavage of the ether linkage rather than by β-oxidation of the fatty acid.

METHODS

The bacterium, which was isolated and described by Burger *et al.* (1962), was grown in a medium containing: 4-(2,4-DB), 0·5 g.; $(NH_4)_2HPO_4$, 0·5 g.; yeast extract (Difco), 0·5 g.; KCl, 0·2 g.; $MgSO_4.7H_2O$, 0·2 g.; $FeSO_4.7H_2O$, 0·001 g.; and distilled water, 1·0 l. For the isolation of intermediates, the same medium was used, but 4-(2,4-DB) was provided as sole source of carbon and energy. For manometric investigations, 5-day cultures grown with forced aeration were harvested by centrifugation, washed twice in 0·02 M phosphate buffer (pH 7·0) and suspended in the same buffer. Oxygen uptake was measured at 30° with air as gas phase (Umbreit, Burris & Stauffer, 1957). Each respirometer flask contained 1·0–2·5 μmole substrate, 50 μmole phosphate buffer (pH 7·0) and the equivalent of about 15 mg. dry wt. bacteria in a total volume of 3·0 ml. To the centre well was added 0·2 ml. of 20 % (w/v) KOH. The indicated rates of oxygen uptake have been corrected for endogenous gas exchange. Following completion of the manometric measurements, the contents of each flask were centrifuged to remove the bacteria, and the supernatant fluid was analysed for chloride and the decrease in ultraviolet (u.v.) absorbency at the wavelength at which the compound showed maximum light absorption.

Chloride was determined by the colorimetric method of Bergmann & Sanik (1957). Ultraviolet absorption analyses were made with a Beckman spectrophotometer, Model DU. Phenolic substances were detected by the chromatographic method of Lederer (1949), the spots being located either by photographing the chromatograms under u.v. radiation in the range 275–300 mμ, or by spraying the chromatograms with diazotized sulphanilic acid followed by sodium carbonate (Lederer, 1949). The technique of Reid & Lederer (1951) was used for showing the presence of fatty acids.

Samples to be characterized by gas chromatography were dissolved in diethyl ether and introduced into a Barber-Coleman gas chromatograph (Model 10) fitted with an argon ionization detection cell containing 56 μc. ^{226}Ra. A U-shaped column of heavy walled borosilicate glass tubing, 5 mm. i.d. and 6 ft. long, was packed with the partitioning medium, which employed Silicone 710 on Chromasorb W, 80–100 mesh, in a ratio of 1:5 (w/w). The operating parameters were: column temperature, 162°; cell temperature, 235°; flash heater, 206°; and argon flow rate, 46 ml./min.

RESULTS

Sequential induction

In preliminary trials it was observed that 4-(2,4-DB)-grown Flavobacterium oxidized the herbicide most readily in the vicinity of pH 7·0, and this pH value was therefore used in subsequent studies. The oxidation was rapid at pH 8·0 but was nil at pH 6·0. The rate of O_2 uptake was linear with time around pH 7.

Previous work (Burger *et al.* 1962) indicated that the enzymes concerned with 4-(2,4-DB) degradation by this organism were inducible. Using the technique of sequential induction (Stanier, 1947), several compounds were examined as possible intermediates in the metabolism of 4-(2,4-DB) by Flavobacterium cells grown in the liquid medium containing 4-(2,4-DB) as carbon source. The substrates tested

included 2,4-dichlorophenol, 4-chlorocatechol, 2,4-D, hydroxyhydroquinone and chlorohydroquinone. Oxygen uptake, carbon dioxide evolution, chloride release and disappearance of the u.v. absorption specific for the substrate were used as criteria for the oxidation of the test compound.

Non-proliferating bacteria which had been grown in a 4-(2,4-DB) medium consumed O_2 at a rapid linear rate with 4-(2,4-DB) and 2,4-dichlorophenol as substrate, and no induction period was required (Fig. 1). This suggests that 2,4-dichlorophenol may be an intermediate in the degradation of the herbicide. The u.v. absorption characteristic of these two compounds was lost, and 102 and 113 % of the bound chlorine in 4-(2,4-DB) and 2,4-dichlorophenol, respectively, was recovered as free

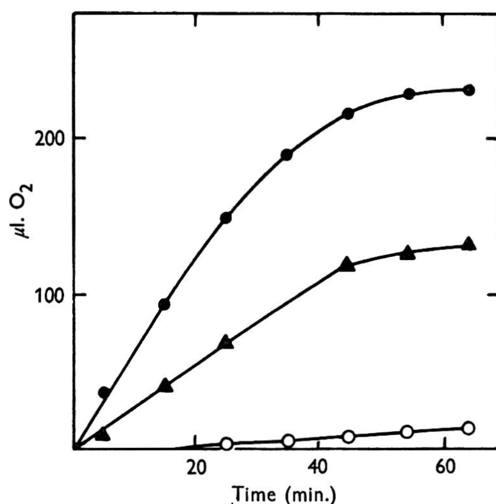

Fig. 1

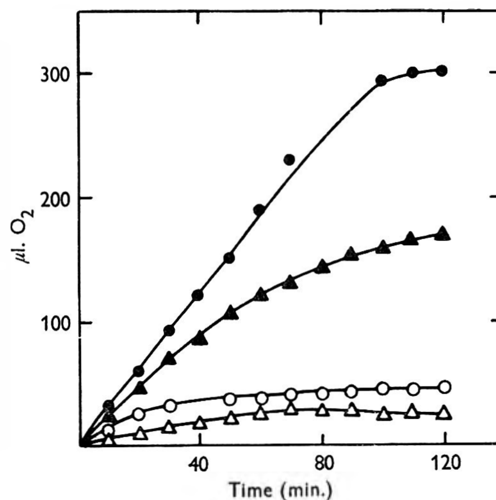

Fig. 2

Fig. 1. Oxidation by 4-(2,4-DB)-grown Flavobacterium of 4-(2,4-DB) (●—●), 2,4-dichlorophenol (▲—▲) and 2,4-D (○—○). The three substrates were supplied in quantities of 2·0, 1·05 and 2·0 µmole, respectively.

Fig. 2. Oxidation by 4-(2,4-DB)-grown Flavobacterium of 4-(2,4-DB) (●—●), 4-chlorocatechol (▲—▲), hydroxyhydroquinone (○—○) and chlorohydroquinone (△—△). The substrates were supplied in quantities of 2·18, 1·14, 1·70, and 2·43 µmole, respectively.

chloride. The latter substrate was completely metabolized, the theoretical value of 6 µmole O_2 being consumed per µmole of 2,4-dichlorophenol. The oxidation of 4-(2,4-DB) was incomplete, by contrast, and only about 6 µmole of O_2 were utilized per µmole of this substrate; the theoretical value is 10·5.

If the initial steps of degradation of 4-(2,4-DB) involve β-oxidation of the aliphatic moiety, it would be expected that the bacterium should have been induced to metabolize 2,4-D. The results in Fig. 1 show no significant O_2 consumption in the presence of 2,4-D. Moreover, there was neither loss of u.v. absorption nor liberation of chloride when the 4-(2,4-DB)-induced organisms were exposed to 2,4-D. The lack of activity on exogenously supplied 2,4-D may have resulted from a permeability barrier.

In the presence of 4-chlorocatechol, the rate of O_2 consumption was rapid, and no period of induction was detected (Fig. 2). For each µmole 4-chlorocatechol

supplied, 6 μmole of O_2 disappeared; this is the theoretical value for complete oxidation. The gas consumed before the marked decrease in the rate of 4-(2,4-DB) oxidation was equal to about 6 μmole O_2/μmole substrate. At the end of the experimental period, 96 and 105 % of the bound chlorine of 4-(2,4-DB) and 4-chlorocatechol was liberated, and the u.v. absorption of the aromatic nuclei of the two molecules had disappeared. There was no change of optical density in the u.v. region when the bacteria were incubated with chlorohydroquinone or hydroxyhydroquinone, and no chloride was liberated by the organism from the former compound. The O_2 consumption in the presence of the quinones was only slightly above the endogenous gaseous exchange, which was 63 μl. O_2 in 2 hr., and may have resulted from a stimulation of endogenous respiration.

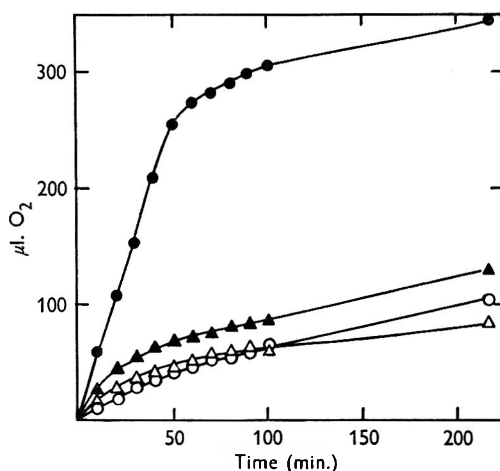

Fig. 3. Oxidation by 4-(2,4-DB)-grown Flavobacterium of 4-(2,4-DB) (●—●), butyrate and crotonate (▲—▲), β-hydroxybutyrate (○—○) and acetate (△—△). The substrates were supplied in quantities of 2·00, 1·95, 1·95, 2·44 and 6·75 μmole, respectively.

Oxidation of aliphatic acids

Cells of 4-(2,4-DB)-grown Flavobacterium were tested for ability to oxidize aliphatic acids. None of the compounds examined was metabolized readily (Fig. 3). There was no difference in activity between butyrate and crotonate. It is interesting that following the disappearance of sufficient O_2 to account for the complete metabolism of the aromatic portion of the herbicide molecule, the rate of O_2 uptake upon 4-(2,4-DB) declined to a value equivalent to that obtained for butyrate, crotonate and β-hydroxybutyrate. The initial rapid oxidation may thus have reflected the degradation of the aromatic ring, while the subsequent slow rate may have represented the decomposition of the aliphatic moiety.

Isolation and identification of intermediates

To demonstrate the presence of metabolic intermediates, the Flavobacterium was inoculated into a medium containing 4-(2,4-DB) as the sole carbon source. Samples of the cultures were taken at intervals and tested for phenolic substances by using diazotized sulphanilic acid and sodium carbonate. When a strong yellow

colour was obtained with these reagents, the cultures were adjusted to pH 2·0 with hydrochloric acid and steam distilled. The distillate was brought up to pH 8·0 with sodium hydroxide and evaporated to dryness in vacuum, the residue taken up in diethyl ether and applied to paper chromatograms, which were developed in *n*-butanol saturated with $5\,N$-NH_4OH. Parallel chromatograms were run using authentic 2,4-dichlorophenol and 4-chlorocatechol. When the development of the chromatograms was complete, the spots were located either by photographing under u.v. radiation or by spraying with diazotized sulphanilic acid followed by sodium carbonate. Two spots were revealed, one of R_f value 0·74, equivalent to that of 2,4-dichlorophenol, and a second of R_f 0·82. The R_f value of an authentic sample of 4-chlorocatechol was 0·81.

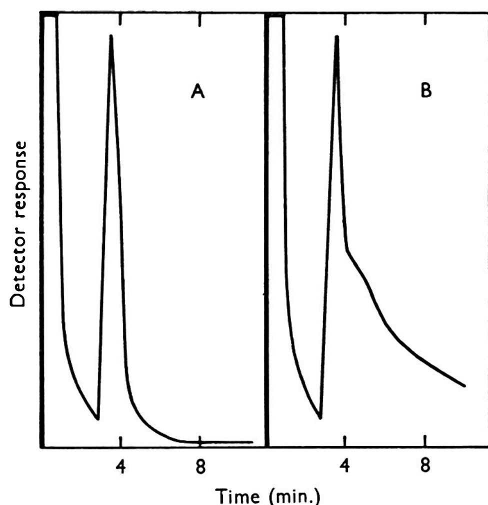

Fig. 4

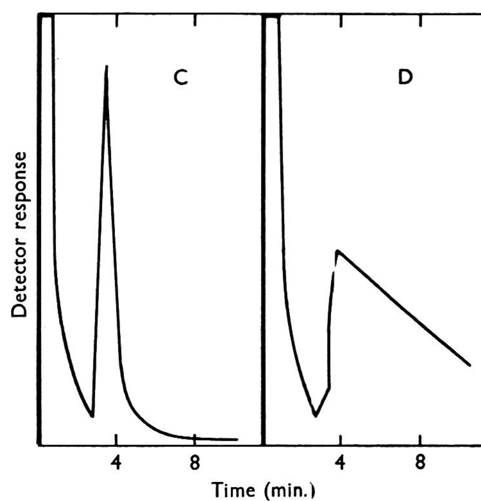

Fig. 5

Fig. 4. Gas chromatograms of authentic 2,4-dichlorophenol (A) and extracts of culture filtrate (B).

Fig. 5. Gas chromatograms of the phenolic fraction (C) and the acid fraction (D) obtained from Flavobacterium culture filtrates.

Additional steam distillates of the culture were prepared in the same way, and the dried material was taken up in 0·1 N-hydrochloric acid. The u.v. absorption spectrum of the sample was compared with that of authentic 2,4-dichlorophenol; the spectra were identical with symmetrical peaks at about 285 mμ.

Gas chromatograms were prepared from an ethereal solution of the dried steam distillate obtained in an identical manner. Samples (10 μl.) of the standard 2,4-dichlorophenol in diethyl ether were injected into the gas chromatographic column. The authentic compound yielded a normal peak with a retention time of 3·5 min., or a retention volume of 161 ml., and no tailing. In contrast, the material from the culture revealed a peak at 3·5 min., a shoulder at 4·25 min. and a considerable degree of tailing (Fig. 4).

The dried material was taken up in 5 % sodium hydroxide, the solution saturated with carbon dioxide and then extracted with ether to separate the phenolic com-

ponent. The remaining solution was adjusted to pH 2·0 with hydrochloric acid and again extracted with ether to obtain a second fraction. The water in the ether fractions was removed by drying over anhydrous sodium sulphate; 10 μl. samples were used for gas chromatography. The chromatograms obtained for these two fractions are shown in Fig. 5. The phenolic fraction showed a peak identical with that of the authentic sample of 2,4-dichlorophenol. The second fraction showed a peak at 4·25 min. with a small shoulder at 3·5 min., suggesting incomplete removal of the phenolic constituent. Although the identity of the peak at 4·25 min. had not been established by gas chromatographic analysis, the crystalline material did exhibit an odour similar to crotonic acid. A sample of the original 4-(2,4-DB) substrate showed no peaks in a 30 min. development, establishing that the herbicide sample was free of 2,4-dichlorophenol.

Metabolism of the butyrate moiety

Washed suspensions of Flavobacterium which had been grown in the 4-(2,4-DB) medium were incubated in 50 ml. of solution containing 100 μmole unlabelled 4-(2,4-DB), 0·09 μg. 4-(2,4-DB) carrying a ^{14}C label on the carboxyl carbon (50·6 mc./mg.) and 1·0 mmole phosphate buffer (pH 7·0). Carbon dioxide-free air was passed through this suspension and samples were withdrawn periodically to determine the quantity of ^{14}C remaining in the reaction vessel. At the time at which only half of the ^{14}C remained in the bacterial suspension, sufficient concentrated sodium hydroxide was added to give a final concentration of 5% (w/v) NaOH. This solution was saturated with carbon dioxide, extracted with ether, the aqueous residue adjusted to pH 2·0 and then re-extracted with ether to give a fraction containing organic acids. The acids were converted to ammonium salts, and these were applied to paper chromatograms together with authentic 4-(2,4-DB), butyrate, crotonate, acetate, propionate and β-hydroxybutyrate and the chromatograms developed with n-butanol that had been equilibrated previously with an equal volume of aqueous 1·5 N-NH_4OH. Only two spots appeared on chromatograms of the acid fraction after spraying with 0·04% (w/v) brom cresol purple (Reid & Lederer, 1951). These two spots had R_f values of 0·87 and 0·66, the known 4-(2,4-DB), butyrate and crotonate giving R_f values of 0·87, 0·65 and 0·66, respectively. By passing chromatogram strips through a chromatograph strip counter (Atomic Accessories, Bellerose, N.Y., Model RSC-5B), it was found that both spots carried the ^{14}C-label.

The paper was sprayed with a dilute solution of potassium permanganate, and the spot with R_f value 0·66 was resolved into two portions. The first of these, which decolorized the permanganate, had an R_f value of 0·66 and corresponded with the standard for crotonate. The second portion, which did not decolorize the permanganate, had an R_f value of 0·65, identical with that of butyrate.

DISCUSSION

The manometric data indicate that 4-(2,4-DB)-induced Flavobacterium metabolized 2,4-dichlorophenol and 4-chlorocatechol without a preliminary induction phase, but not 2,4-D, chlorohydroquinone or hydroxyhydroquinone. The oxidation of 2,4-dichlorophenol and 4-chlorocatechol was complete, whereas the initially

rapid rate of O_2 disappearance in the presence of 4-(2,4-DB) decreased when the quantity of O_2 consumed was about equal to that required for the oxidation of only the aromatic portion of the molecule. The new rate was the same as that obtained whether the substrate supplied to the bacterium was butyrate or crotonate. Consistent with these findings was the evidence for the formation of 2,4-dichlorophenol, 4-chlorocatechol, butyrate and crotonate by suspensions of the organism incubated with the herbicide.

On the basis of these observations the most plausible hypothesis for the mechanism of 4-(2,4-DB) decomposition by the organism used is that the initial attack on the molecule involves a cleavage of the ether linkage, with the formation of 2,4-dichlorophenol and butyric acid. The 2,4-dichlorophenol is then dehalogenated at the *o*-position, and the resultant 4-chlorocatechol is readily and completely degraded, possibly with the formation of *β*-chloro-cis, cis-muconic acid. The butyric acid is probably metabolized at a slow rate by *β*-oxidation.

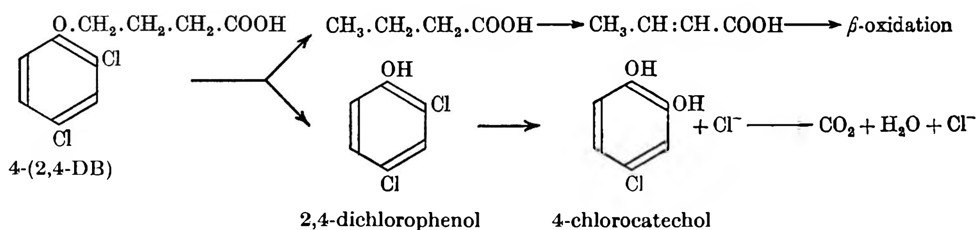

Fig. 6

The present findings differ from those of Webley *et al.* (1957) and Taylor & Wain (1962), whose isolates of Nocardia, Pseudomonas and Micrococcus metabolized the aliphatic moiety of phenoxyalkyl carboxylic acids while the fatty acid was still attached to the aromatic ring. It would thus appear that such molecules can be metabolized by at least two pathways, one being initiated by a *β*-oxidation sequence, as in the strains of Nocardia examined, the second by a cleavage of the ether linkage. The existence of two pathways may have an ecological significance, since *β*-oxidation of herbicides of this type would lead to the formation of new phytotoxic compounds. The alternate pathway would result in a detoxication of the applied herbicide by microbial action.

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The Role of Penicillinase in Determining Natural and Acquired Resistance of Gram-Negative Bacteria to Penicillins

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SUMMARY

The development of resistance to ampicillin (aminophenylacetamido-penicillanic acid) and penicillin G was investigated by selecting resistant variants of strains of *Escherichia coli*. Resistance occurred in stepwise manner. The parent strains contained minute amounts of penicillinase and production of the enzyme by resistant variants increased in relation to increase in resistance. Evidence was obtained that production of penicillinase was responsible for this increase in resistance. By using resistant variants and naturally occurring ampicillin-resistant organisms, it was found that production of only small amounts of penicillinase was sufficient to confer a high degree of resistance to the Gram-negative bacteria examined. In contrast, Gram-positive bacteria required much greater quantities of penicillinase for high grade resistance. It is suggested that an important reason for this difference is the complex lipid-containing cell wall of Gram-negative bacteria, which slows penetration by penicillin and enables small amounts of penicillinase to protect the cell. The penicillinase of all the Gram-negative organisms investigated was found to be a β-lactamase and to be more active against penicillin G than against ampicillin. This provides one explanation for the greater activity of ampicillin against Gram-negative bacteria.

INTRODUCTION

Since the isolation of 6-aminopenicillanic acid (Batchelor, Doyle, Nayler & Rolinson, 1959) many new penicillins have been synthesized. One of these, amino-phenylacetamidopenicillanic acid (ampicillin) is of theoretical and therapeutic importance because it is much more active against several Gram-negative bacteria than is penicillin G (Rolinson & Stevens, 1961). In preliminary studies a wide range of Gram-negative bacteria were examined for sensitivity to ampicillin and penicillin G. It was found that those organisms sensitive to ampicillin were also invariably sensitive to penicillin G, although the minimum inhibitory concentration for penicillin G was always 5 to 10 times greater than that for ampicillin. Similarly, Gram-negative bacteria highly resistant to ampicillin were always highly resistant to penicillin G. It has recently been demonstrated that, like penicillin G, ampicillin inhibits the incorporation of cytoplasmic cell wall precursors (Rogers & Mandlestam, 1962) and we have observed that ampicillin causes cytological changes in Gram-

negative bacteria similar to those produced by the action of penicillin G (Duguid, 1946; Hughes, Kramer & Fleming, 1946; Lederberg, 1957). It seemed reasonable to assume, therefore, that the mode of action of ampicillin is similar to that suggested by Park & Strominger (1957) for penicillin G, namely, to interfere with cell wall synthesis by preventing the formation and incorporation of muramic acid-peptide. It has long been known that Gram-negative bacteria may produce penicillinase (Abraham & Chain, 1940) and in the present work we have studied the part played by this enzyme in determining natural and acquired resistance to penicillins. Our findings have enabled us to suggest reasons for the relatively greater activity of ampicillin than other penicillins against Gram-negative bacteria.

METHODS

Media. Nutrient broth was made of Lab-Lemco meat extract 0·1 % (w/v), Tryptone (Oxoid) 0·2 % (w/v), peptone (Oxoid) 1 % (w/v), NaCl 0·7 % (w/v). Nutrient agar contained nutrient broth solidified with 1·5 % (w/v) Davis New Zealand agar.

Organisms. Standard laboratory strains of *Escherichia coli* (designated C3, CV, K28) were used in many of the experiments. The characteristics of these organisms were described in detail previously (Brumfitt & Heptinstall, 1960). A number of other Gram-negative bacteria isolated from patients with acute urinary infections, and penicillinase-producing staphylococci from infected lesions, were also used. Cultures of bacteria were preserved on agar slopes at 4°.

Penicillins. Penicillin G was obtained from Glaxo Laboratories Ltd. (Greenford, Middlesex) and methicillin, ampicillin, 6-aminopenicillanic acid (6-APA) and 3*o*-chlorophenyl-5-methyl-4-isoxazolyl penicillin (BRL 1621) from Beecham Research Laboratories (Brockham Park, Surrey). BRL 1621 is one of the isoxazolyl series of penicillins (Doyle, Long, Nayler & Stove, 1961) which combines the properties of penicillinase resistance with acid stability (Knudsen, Brown & Rolinson, 1962).

Standard penicillinases. *Bacillus cereus* penicillinase (Distillers Company Ltd., London) was used as a source of β-lactamase. A thick toluene-killed suspension of an amidase-producing coliform organism was kindly supplied by Dr F. R. Batchelor (Beecham Research Laboratories). This enzyme was stable when stored at 4° and was used as a standard amidase preparation.

Sensitivity testing. Screening tests for selection of naturally occurring ampicillin-resistant organisms were carried out by placing dried impregnated paper discs on well-dried nutrient agar plates previously flooded with a log-phase bacterial culture. In order to standardize the inoculum the optical density of the bacterial culture was measured in the Hilger absorptiometer and the culture then diluted so as to give a bacterial count of about 10^6 organisms per ml. Ampicillin (25 μg./disc) was used and an organism was considered to be resistant when there was no zone of inhibition after incubation for 18 hr. at 37°.

The minimum inhibitory concentration (MIC) for a particular organism was determined by adding a standard inoculum (about 10^4 bacteria) of mid log-phase culture to tubes containing serial dilutions of the antibiotic in broth. The lowest concentration of antibiotic which completely inhibited growth as judged by the absence of turbidity after incubation for 18 hr. at 37° was taken to be the MIC.

Selection of resistant variants. Resistant variants of ampicillin-sensitive strains were selected by serial subculture on nutrient agar containing increasing concentrations of ampicillin. At the same time the mutational frequency for selection of variants at a particular concentration of ampicillin was calculated by comparing the number of colonies on the ampicillin-containing medium with a viable count carried out by preparing tenfold dilutions of the bacterial suspension and inoculating antibiotic-free medium. For comparison with the ampicillin-resistant variants, penicillin G-resistant variants of the parent strains were selected by the same technique.

Detection and estimation of penicillinase production

Screening tests for penicillinase production were made on broth cultures which were incubated for 6–18 hr. at 37° when the test organism was killed by the addition of toluene to a final concentration of 10 % (v/v). These killed cultures were incubated with penicillin G for periods of 1–4 hr. at 37° and the residual penicillin was then assayed by the microbiological cup-plate method with *Sarcina lutea* (ATCC 9341) as test organism. This method was also used to compare the susceptibilities of penicillin G, ampicillin, methicillin, BRL 1621 and 6-APA to the penicillinases produced by various bacterial strains. Of the penicillins tested penicillin G was found to be the most susceptible to Gram-negative penicillinases and therefore was used as substrate for their estimation.

To compare the penicillinase activities of different preparations more accurately than was possible by the method described above, several procedures were tried, including that of Perret (1954), the spectrophotometric assay as described by Saz, Lowery & Jackson (1961), the method of Haight & Finland (1952) and a micro-assay method based upon that described by Pollock (1957). The micro-assay method was the only one which allowed small amounts of penicillinase to be measured sufficiently accurately for our purposes and will be described.

A sample (2 ml.) of penicillinase solution was added to 8 ml. of nutrient broth containing penicillin G 20 μg./ml., and the mixture incubated at 37°. At intervals of 15 or 30 min., samples (1 ml.) were withdrawn, diluted with 9 ml. of ice-cold 0·01 M-potassium phosphate buffer (pH 7·0) and the residual penicillin assayed in triplicate by the cup-plate assay with *Sarcina lutea* as test organism, and comparing zones of inhibition with those obtained using standard penicillin G solutions over the range 0·25–2·0 μg./ml. Assay plates were kept at 4° for 2–4 hr. before overnight incubation at 37°. Penicillin was estimated without the need for a specific penicillinase inactivator, because of the decrease of enzyme activity on sampling due to lowering of the temperature to 0° and the 1 in 10 dilution of substrate (Pollock, 1957). Penicillinase activity was estimated in terms of the rate of inactivation of penicillin G under standard conditions. This measurement was made from a graph in which residual penicillin was plotted against time.

Relationship between penicillin resistance and penicillinase production

The relationship between penicillinase production and resistance to penicillins was first demonstrated by comparing the MIC value obtained for ampicillin and penicillin G with large inocula (about 10^6 bacteria) with the values obtained with small

inocula (about 100 bacteria). By using the micro-assay method described, we subsequently carried out more accurate estimations of penicillinase activity with Seitz (SB) filtrates of 6–18 hr. broth cultures of *Escherichia coli* cv and its resistant variants. Preliminary experiments showed that there was no appreciable adsorption of this Gram-negative penicillinase by Seitz (SB) pads.

Determination of cell-bound and extracellular penicillinase

The organism to be tested was incubated for 18 hr. at 37° in 500 ml. nutrient broth. The bacteria were then deposited by centrifugation at 3000 rev./min. for 20 min. and the supernatant fluid passed through a Seitz (SB) filter. The penicillinase activity of the filtrate was then estimated; this was taken to represent extracellular penicillinase.

In order to remove any remaining traces of extracellular penicillinase from the bacterial deposit it was then washed three times by centrifugation in 0·01 M-phosphate buffer (pH 7·0) at 4° and resuspended in distilled water. Similar viable counts were obtained before and after this treatment, showing that no appreciable lysis of bacteria had occurred. The washed suspension was then divided into two equal portions. One was treated with toluene to prevent further bacterial growth. The other portion was treated in the Mullard ultrasonic 2 kW. disintegrator for 30 min., the suspension divided and half of it centrifuged at 27,000 rev./min. (65,900 ***g***) for 20 min. and the supernatant removed. The penicillinase activity of the toluene treated bacteria, the suspension of disintegrated bacteria and the supernatant fluid from the suspension of disintegrated bacteria was then estimated. To see whether the ultrasonic treatment used to disintegrate the bacteria did not also inactivate the penicillinase liberated, a Seitz filtrate of a broth culture containing penicillinase was subjected to treatment in the ultrasonic disintegrator. Treatment identical with that needed to break the bacteria caused no detectable loss of penicillinase activity. It was also found that the addition of toluene to a Seitz filtrate containing Gram-negative penicillinase had no effect on the activity of the enzyme.

Induction of penicillinase. Tests for inducibility of penicillinase were carried out in broth cultures of *Escherichia coli* cv and resistant variants of it which had been incubated for 6–10 hr. at 37°. In different experiments either ampicillin, penicillin G, methicillin or BRL 1621 was added to make a final concentration of 0·5–2·0 μg./ml. and incubation at 37° then continued for a further 1–8 hr. Penicillinase activity was estimated in the whole broth culture after the addition of toluene and in the filtrate after Seitz filtration. As a control for basal penicillinase activity estimations were performed in parallel on broth cultures to which no penicillin had been added.

Identification of penicillinase. Penicillinases are either β-lactamases or amidases (Rolinson *et al.* 1960). The distinction between these two enzymes was based upon the finding that the 6-aminopenicillanic acid (6-APA) formed following the action of amidase on penicillin can readily be converted into penicillin G by simple chemical treatment (Batchelor *et al.* 1959), whereas the penicilloic acid formed by the action of β-lactamase cannot.

An overnight broth culture of the organism to be tested was incubated for 1–4 hr. with a known amount of penicillin G and a sample withdrawn for assay of residual penicillin by the cup-plate method. To convert any 6-APA to penicillin G,

anhydrous sodium bicarbonate and 10% (v/v) phenylacetyl chloride (British Drug Houses Ltd.) in dry acetone were added to the rest of the culture to give final concentrations of 3% (w/v) and 1% (v/v), respectively. This mixture was allowed to stand at room temperature for 15 min. and the penicillin G content then assayed as before. Failure to demonstrate increase in penicillin after reconstitution was taken to indicate that the penicillinase was a β-lactamase. The validity of this assumption was confirmed by testing several penicillinase-producing strains for ability to act upon 6-APA, which is inactivated by β-lactamase but not by amidase. Broth cultures of several penicillinase-producing strains were incubated with known amounts of 6-APA and the extent of inactivation estimated by reconstitution of residual 6-APA to penicillin G.

Antisera. O antisera to the standard Escherichia strains were prepared by the method described by Edwards & Ewing (1957).

Pathogenicity tests. A comparison was made between *Escherichia coli* CV parent strain and a resistant variant with an MIC of 2500 μg./ml. ampicillin. Four separate experiments were carried out in which groups of five mice (18–20 g.) received an intraperitoneal injection of 0·1 ml. bacterial suspension containing 10^8, 10^6, 10^4 and 10^2 log-phase bacteria. Each batch of five mice was housed in a separate cage and the mortality was determined 4 days after inoculation. All results were used in the final analysis. LD 50 values were calculated by the method of Reed & Muench (1938). Bacterial suspensions were made in saline and in every case viable counts were carried out.

RESULTS

Selection of resistant variants of Escherichia coli *in the laboratory*

Escherichia coli variants resistant to penicillin G and to ampicillin were selected without difficulty with the three Escherichia strains CV, C3 and K28. Resistance developed in a stepwise fashion; variants were selected most readily from strain CV. The parent strain (CVP) of this organism had an MIC of 7·5 μg. ampicillin/ml., but after only three subcultures in the presence of increasing concentrations of ampicillin a resistant variant with an MIC of 400 μg. ampicillin/ml. (strain CVR 400) was obtained and after six subcultures a resistant organism with an MIC of 2500 μg. ampicillin/ml. (strain CVR 2500) was selected. The greatest single step increase in resistance to both ampicillin and penicillin G by *E. coli* strain CV was tenfold, and the mutational frequency for selection of resistant mutants with ten times the resistance of the parent strain was about $1/10^8$ bacteria. With strains C3, K28 and others, increase in resistance occurred in smaller steps, but the mutational frequency for selection of resistant mutants was similar. Investigation of a number of parent strains and their resistant variants showed no differences in colonial appearance, biochemical reactions, agglutination by specific O antisera, growth curves or mouse pathogenicity. Resistant mutants were stable and no significant change in resistance was detected after twenty subcultures on antibiotic-free medium.

Cross-resistance

Table 1 compares the MIC values obtained with three penicillins by using *Escherichia coli* CV parent strain (CVP) and two resistant variants (CVR 400, CVR 2500)

as test organisms. These variants had been selected by subculture in the presence of ampicillin, but it can be seen (Table 1) that there was complete cross-resistance with penicillin G. Similarly a variant of strain CV selected by growth in presence of penicillin G showed cross-resistance to ampicillin. When the MIC values of *E. coli* CV to ampicillin and penicillin G are expressed as a ratio this is very similar for the parent organism and for its resistant variants (Table 1). This ratio was approximately the same whether ampicillin or penicillin G was used for the selection of the variants. The numerous organisms tested and found to be naturally resistant to ampicillin were also always resistant to penicillin G. This shows that the difference between resistance of these bacteria to ampicillin and penicillin G is quantitative rather than qualitative. In contrast, the factors responsible for resistance of variants to ampicillin and penicillin G did not cause increased resistance to antibiotic BRL 1621, although in this case the parent strain was itself highly resistant (Table 1).

Table 1. *Antibacterial activity of different penicillins against resistant variants of* Escherichia coli *strain* CV

Organism	Minimum inhibitory concentration (MIC µg./ml.)			Ratio MIC ampicillin to penicillin G
	Penicillin G	Ampicillin	BRL 1621	
CV parent strain (CVP)	30	7·5	450	1:4
Ampicillin-resistant variants of CV				
CVR 400	1400	400	450	1:3·5
CVR 2500	9000	2500	450	1:3·6
Penicillin G-resistant variant of CV	750	200	450	1:3·7

Production of penicillinase by resistant variants

During the initial stages of selection of ampicillin-resistant variants of *Escherichia coli* CV it was found that when the concentration of ampicillin added to the nutrient agar just exceeded the MIC value for the parent strain, some of the resistant variants selected were surrounded by small satellite colonies. The MIC value of ampicillin for the colonies which were surrounded by satellites was much higher than that for the parent strain, whereas the MIC value for the satellite colonies was the same as for the parent strain. This suggested that the resistant variants of strain CV were producing penicillinase. This was confirmed by the screening test for penicillinase production: penicillinase activity shown in broth cultures of resistant variants of all three laboratory strains of *E. coli* (CV, C3, K28), but not in cultures of the parent strains.

Variants selected by subculture in the presence of ampicillin or penicillin G produced penicillinase, but the enzyme was much more active against penicillin G. For this reason penicillin G was always used as substrate when accurate estimations of penicillinase activity were required. This was necessary because even highly resistant variants produced only small amounts of penicillinase. For example, 1 ml. of an 18 hr. broth culture of the variant strain CVR 400, which had an MIC of 400 µg. ampicillin/ml. and 1400 µg. penicillin G/ml., inactivated only 2 µg. ampicillin and 50 µg. penicillin G after 1 hr. at 37°. However, production of small amounts of penicillinase could regularly be correlated with a high degree of resistance.

The ampicillin-resistant variant (strain CVR 400) was grown in nutrient broth at

37°. Samples were removed at hourly intervals and the bacteria removed by Seitz (SB) filtration. Estimation of the penicillinase activity of the filtrate by the micro-assay method showed a progressive increase between 4 and 10 hr. A comparison was also made between the amount of penicillinase present in whole cultures and the amount present in a Seitz filtrate of the same culture. Estimations were made at hourly intervals and it was found that the filtrate had a penicillinase activity only about 10% less than the whole culture. This was true even at 4 hr. when the penicillinase concentration began to increase. Experiments with the ampicillin-resistant variant CVR 2500 gave similar results.

Relationship between penicillin resistance and penicillinase production

Estimation of the penicillinase activities of variants of *Escherichia coli* strain CV with increasing degrees of resistance to ampicillin, showed that the degree of resistance was proportional to the amount of penicillinase produced (Table 2). The

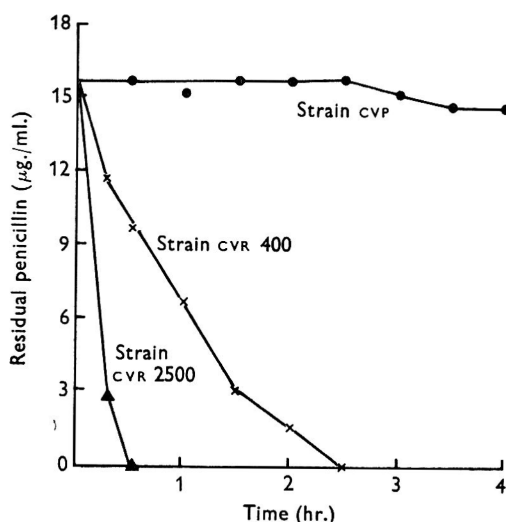

Fig. 1. Penicillinase activity in *Escherichia coli* strain CV and resistant variants (CVR 400, CVR 2500) with M.I.C. values of 400 and 2500μg. ampicillin/ml., respectively.

relationship between penicillinase production and resistance is also shown in Fig. 1, in which CV parent strain and the two resistant variants CVR 400 and CVR 2500 were used. These three organisms grown under identical conditions in nutrient broth were subsequently shown to contain similar numbers of bacteria. The actual experiment was carried out on Seitz filtrates of these cultures.

It was possible to show penicillinase activity in the same resistant variants (CVR 400, CVR 2500) by using the spectrophotometric method of Saz *et al.* (1961) and by the method of Haight & Finland (1952). Indications of differences in the penicillinase activities in variants with increasing degrees of resistance were observed. However, such differences could not be measured as precisely as by the micro-assay method. No penicillinase activity was detected in the parent strain or in variants with lower degrees of resistance. By using the micro-assay procedure

and a heavy suspension of bacteria (equiv. 13·8 mg. dry wt./ml.) which had been subjected to ultrasonic disintegration, we consistently found traces of penicillinase activity in the parent strain CVP.

Table 2. *Antibacterial activity of ampicillin and penicillinase activity in bacteria with natural and acquired resistance*

		MIC ampicillin (μg./ml.)	Penicillinase activity (μg. penicillin G inactivated in 1 hr. at 37°)	Ratio of minimum inhibitory concentration to penicillinase activity
Escherichia coli				
CV parent strain (CVP)		7·5	< 1·0	> 7·5:1
Ampicillin-resistant variants of strain CV				
CVR 400		400	50	8:1
CVR 2500		2500	150	16·6:1
Naturally resistant organisms				
Aerobacter aerogenes	(i)	900	75	12·0:1
	(ii)	50	5·5	9·1:1
Escherichia coli	(i)	550	40	13·8:1
	(ii)	50	4·5	11·1:1
Proteus spp.	(i)	750	50	15·0:1
	(ii)	300	12·5	24·0:1
Pseudomonas aeruginosa	(i)	500	25	20·0:1
	(ii)	50	3·5	14·3:1
Staphylococcus aureus		250	100	2·5:1

Table 3. *Antibacterial activity of different penicillins when using large or small inocula*

	No. of bacteria in inoculum	Minimum inhibitory concentration (μg./ml.)	
		Penicillin G	Ampicillin
Escherichia coli			
CV parent strain (CVP)	10^6	65	10
	10^2	25	5
Ampicillin-resistant variants of CV			
CVR 400	10^6	1,500	600
	10^2	900	350
CVR 2500	10^6	12,000	2,750
	10^2	7,000	2,250
Staphylococcus aureus	10^6	250	200
	10^2	7·5	10

Influence of inoculum size

It can be seen in Table 3 that the size of inoculum produced only a small effect on the MIC values for the CV parent strain and two of its resistant variants (CVR 400, CVR 2500) when tested against penicillin G and ampicillin. Although in every case the MIC value was higher with an inoculum of 10^6 organisms than with 10^2 organisms, this effect was small as compared with that found with a penicillinase-producing strain of *Staphylococcus aureus* (Table 3). The difference in inoculum effect for *S. aureus* and *Escherichia coli* strain CVR 400 was shown in another way. Samples (0·02 ml.) of serial tenfold dilutions of washed cultures of these organisms were

dropped on to nutrient agar containing 200 μg. ampicillin/ml. With the staphylococcus growth was only possible with a large inoculum, whereas with *E. coli* CVR 400 growth occurred from a small inoculum, giving rise to single colonies.

Proportion of cell-bound to extracellular penicillinase

With *Escherichia coli* strains CVR 400 and CVR 2500, penicillinase activity in Seitz-filtrates was found to be 90 % of that of the whole bacterial cultures in broth, the remaining 10 % being detected in the washed bacterial suspensions.

The penicillinase activity of the washed bacterial suspensions was found to be 2·5 times greater after ultrasonic disintegration than before. This increase in activity following disintegration was taken to represent bound intracellular penicillinase, because the increase was unaffected by removal of the bacterial cell walls by centrifugation at 27,000 rev./min. Therefore, even allowing for bound intracellular penicillinase, in whole broth cultures of the two strains tested, about 75 % of the total penicillinase was extracellular.

Inducibility of penicillinase

With *Escherichia coli* parent strain CV and the two resistant variants CVR 400 and CVR 2500, no increase in penicillinase activity was detected after attempted induction with the four penicillins used. In fact, when induction was attempted with the penicillinase-resistant penicillins, methicillin and BRL 1621, a progressive fall in penicillinase activity was observed, although the concentration of penicillin was too low to inhibit growth. There was no significant fall in penicillinase activity when ampicillin or penicillin G was used.

Table 4. *Ampicillin resistance and penicillinase production in Gram-negative bacteria isolated from patients with urinary tract infections*

Organism	No. of isolates tested	No. isolates resistant to ampicillin (25 μg. disc)	No. resistant isolates tested	No. isolates showing penicillinase activity
Escherichia coli	427	80 (19 %)	15	6 (40 %)
Atypical coli	24	9 (38 %)	6	2 (33 %)
Proteus spp.	154	62 (38 %)	25	9 (36 %)
Aerobacter aerogenes	16	16 (100 %)	8	8 (100 %)
Pseudomonas aeruginosa	27	27 (100 %)	6	3 (50 %)

Penicillinase activity in naturally resistant Gram-negative organisms

Gram-negative bacteria isolated from 640 patients with urinary tract infections were examined for resistance by means of discs containing 25 μg. of antibiotic. A random selection of the organisms found to be resistant were also tested for penicillinase production by the screening method. The results are summarized in Table 4, where it is seen that all strains of *Aerobacter aerogenes* and a significant proportion of the other organisms showed a definite penicillinase activity. Comparisons made under standard conditions showed that there was considerable variation in the amount of penicillinase produced by different strains. When MIC values for these penicillinase-producing strains were determined by using a standard inoculum of 10^4 bacteria, it was found that the degree of resistance was proportional

to the amount of penicillinase produced. Table 2 shows this relationship to hold both with naturally resistant organisms and with those selected in the laboratory; also that production of only small amounts of penicillinase was associated with a high degree of resistance in Gram-negative bacteria. It is also seen in Table 2 that *Escherichia coli* strain CVR 2500, which produced amounts of penicillinase of the same order as a strain of *Staphylococcus aureus*, required very much larger concentrations of ampicillin to inhibit its growth.

Susceptibility of different penicillins to different penicillinases

Table 5 shows the susceptibilities of different penicillins to the actions of penicillinases produced by a variety of bacteria when tested under standard conditions at pH 7·4. The penicillinases of the resistant variant of *Escherichia coli* selected in the laboratory, and of ampicillin-resistant Gram-negative organisms isolated from patients, inactivated penicillin G much more readily than ampicillin. Methicillin and BRL 1621 were highly resistant to the action of these penicillinases. The penicillinases produced by *Bacillus cereus* and *Staphylococcus aureus* inactivated ampicillin at least as readily as penicillin G. The standard amidase preparation showed properties similar to that of penicillinase from Gram-negative organisms, with the important exception that it was inert against 6-APA. However, 6-APA was less readily inactivated than penicillin G by the other penicillinases tested (Table 5).

Identification of penicillinases. The penicillinases of all the numerous Gram-negative organisms tested were identified as β-lactamases. These bacteria (from human sources) were tested for amidase activity but none was found.

Table 5. *Inactivation of different penicillins by penicillinases of various organisms*

Preparation	Ratio inactivated relative to penicillin G (all at 0·15 mM substrate concentration)				
	Penicillin G	Ampicillin	Methicillin	BRL 1621	6-APA
Escherichia coli CVR 400	1	0·04	< 0·001	0·002	0·04
E. coli	1	0·08	< 0·001	0·001	0·06
Proteus mirabilis	1	0·09	< 0·001	0·003	0·04
Aerobacter aerogenes	1	0·05	< 0·001	0·001	0·03
Staphylococcus aureus	1	1·25	0·002	0·003	0·05
Standard β-lactamase (*Bacillus cereus* 10 units/ml.)	1	1·05	0·13	0·2	0·11
Standard amidase (from coliform organism)	1	0·04	0·002	0·002	0*

* No detectable inactivation

DISCUSSION

It has been shown that, by serial subculture of three *Escherichia coli* strains (CV, C3, K28) in the presence of penicillin G or ampicillin, variants resistant to both these penicillins could be selected. Resistant variants invariably produced a penicillinase, but apart from the production of this enzyme, no difference could be found between highly ampicillin-resistant variants and their parent strains. The resistance of the

variants selected was stable after repeated subculture on antibiotic-free media. Of the strains tested, resistant variants were most easily selected from the standard laboratory strain *E. coli* cv. Minute amounts of penicillinase activity were detected in the parent strain of this organism; penicillinase production in the parent strain and its resistant variants was non-inducible. The penicillinase activities of cell-free filtrates of resistant variants of *E. coli* strain cv were proportional to their degree of resistance (Fig. 1; Table 2). Although there was complete cross-resistance between ampicillin-resistant and penicillin G-resistant variants, these variants were no less susceptible to BRL 1621 than was the parent strain (Table 1). It was also found that compound BRL 1621 was resistant to the penicillinase produced by the variants (Table 5). We suggest, therefore, that increased resistance to ampicillin and to penicillin G was due to the ability of the variants to produce greater amounts of penicillinase.

The degree of ampicillin resistance of several naturally resistant Gram-negative organisms was also found to be proportional to their penicillinase activities (Table 2). However, resistance in some Gram-negative organisms was not associated with penicillinase production (Table 4). Penicillinase production by the penicillin-resistant variants of the Gram-negative organisms investigated was small as compared with that by Gram-positive organisms such as the penicillin-resistant staphylococci. The possibility that in resistant variants there was a substantial quantity of bound intracellular penicillinase, not detected by normal testing, was excluded by studying bacteria disrupted by ultrasonic treatment and showing that the bulk of penicillinase was extracellular. It seems, therefore, that in these Gram-negative bacteria a high degree of resistance was conferred by the synthesis of relatively small amounts of penicillinase.

Chemical analysis of cell walls of Gram-negative bacteria has shown marked differences from Gram-positive organisms (Salton, 1953; Cummins & Harris, 1956). Weidel & Primosigh (1957, 1958) suggested the presence in the Gram-negative cell wall of a layer of lipoprotein lying superficial to the lipopolysaccharide-mucopeptide layer and accounting for 80 % of the wall by weight. Since it is believed that the site of action of penicillin is at the cell membrane (Cooper, 1955), where cell wall synthesis is also thought to occur (Crawthorn & Hunter, 1958), we suggest that the complex lipid-containing Gram-negative cell wall may form a barrier to the penetration of penicillins. In Gram-positive bacteria, where the cell wall is free from lipid, no such barriers exist. Therefore, penicillin resistance can be more directly related to penicillinase activity on a quantitative basis and relatively much greater amounts of the enzyme are required to confer increased resistance to penicillin (Table 2) than in Gram-negative bacteria.

Kirby (1945) and Luria (1946) suggested that the finding of high values of the minimum inhibitory concentration of antibiotic when using a large inoculum indicates that resistance is due to penicillinase activity. We found that this inoculum effect was much less pronounced with the ampicillin-resistant variants of *Escherichia coli* strain cv than with a strain of *Staphylococcus aureus* (Table 3), and therefore in Gram-negative organisms tests based on inoculum size cannot be relied upon to distinguish between penicillin resistance due to penicillinase production and that from other causes. Again, the explanation for this difference is probably that diffusion of penicillin through the Gram-negative wall was slowed so that the need

for a large inoculum to destroy penicillin and allow initiation of growth was obviated.

The demonstration of a minute amount of penicillinase activity in the parent *Escherichia coli* strain cv suggests that resistance was due to small increases in the ability of the variants to produce penicillinase spontaneously in a manner similar to that described by Pollock (1957) for a strain of *Bacillus cereus*. It is understandable that attempts to demonstrate a similar effect in other Gram-positive bacteria might be difficult, since the degree of increased resistance might be expected to be very small. For example, there has been great difficulty in isolating penicillinase-producing variants from penicillin-sensitive staphylococci *in vitro*. Barber (1957) was able to demonstrate the slow evolution of strains producing traces of penicillinase by growing staphylococci for long periods in low concentrations of penicillin, although the presence of penicillinase in the parent penicillin-sensitive strains could not be detected.

The finding that ampicillin was more resistant than penicillin G to the action of the β-lactamase produced by Gram-negative bacteria is relevant to an explanation for the greater activity of ampicillin against these organisms. However, other factors also determine the activity of a particular penicillin against Gram-negative bacteria, because compound BRL 1621, which was resistant to the action of the β-lactamase produced by Gram-negative bacteria, was considerably less active than were ampicillin or penicillin G against sensitive organisms. The ability of the penicillin to penetrate the Gram-negative cell wall and then to combine with its specific receptor site are additional factors which must be taken into consideration.

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The Steroid Growth-Requirement of *Mycoplasma mycoides*

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SUMMARY

Several steroids closely related structurally to cholesterol were tested for growth-promoting activity for a strain of *Mycoplasma mycoides* (V5) and a *Mycoplasma* sp. isolated from a goat (GY). Cholestanol and lathosterol promoted growth of both strains. Cholestenone, cholest-5-en-3-one, 7-dehydrocholesterol and progesterone inhibited cholesterol-promoted growth; cholestanone was almost inactive either as growth promotor or as growth inhibitor. Cholesterol amounted to 4–5 % of the dry weight of the water-washed organisms, or about 20 % of the total lipids, of either strain grown in the presence of cholesterol. Cholesterol esters or transformations to other steroids were not detected in lipid extracts of the V5 strain grown in the presence of cholesterol-4-^{14}C. The GY strain was grown in the presence of cholesterol-4-^{14}C of known specific activity, the lipid extract diluted with a known amount of unlabelled cholesterol, and cholesterol isolated and purified. Its specific activity was very close to the value expected if no sterol transformations had occurred. When grown in the presence of cholestanol, the GY strain incorporated this sterol without desaturation.

INTRODUCTION

Certain species of the genus *Mycoplasma* differ from the Eubacteriales in possessing a steroid growth requirement. This requirement was first demonstrated by Edward & Fitzgerald (1951), who found that cholesterol, cholestanol or stigmasterol were effective, and that the acetate, stearate or oleate esters of cholesterol were not. With some Mycoplasma strains of human and avian origin, it was shown by Smith & Lynn (1958) that β-sitosterol and cholestanol promoted better growth than did cholesterol, while ergosterol and stigmasterol were less effective. Cholesterol esters of saturated fatty acids of chain-length C_4–C_{12} promoted growth less effectively than free cholesterol. These authors considered that the 3β-hydroxy group, either free or esterified, was essential. With *Mycoplasma laidlawii*, however, Butler & Knight (1960) found that cholestanone, cholest-1-en-3-one and cholest-4-en-3-one all had nearly that same growth-promoting effect as cholesterol; cholesta-1,4-dien-3-one and cholesta-1,4,6-trien-3-one inhibited completely the cholesterol-promoted growth of *M. laidlawii* in a serum-free medium and of other Mycoplasma species in a medium which contained serum.

The lipid composition of several Mycoplasma strains, including two without a steroid requirement for growth, was examined by Lynn & Smith (1960). They concluded that those strains which did not require steroid for growth did not contain any when grown in its absence. The steroid-requiring strains had a cholesterol

content in free and esterified forms of from 0·7 to 0·9 % of their dry weight. In one strain examined, there were esters of volatile and non-volatile fatty acids in about equimolar proportions; in another strain the esters were almost entirely those of volatile fatty acids. After sonic disintegration of the organisms, cholesterol was found in the insoluble and in the soluble fractions; the cholesterol esters were concentrated in the insoluble fraction, and the free cholesterol in the soluble fraction. Smith (1959, 1960) described both cholesterol ester-hydrolysing and ester-synthesizing activities in strains having a steroid growth requirement, and suggested that the function of these enzyme activities might be to mediate the transfer of volatile fatty acids across the membrane which encompasses the organisms.

Rothblat & Smith (1961) examined the non-saponifiable lipid fraction. Non-fermentative steroid-requiring strains incorporated cholesterol unchanged or as an ester. With a fermentative steroid-requiring strain grown in the presence of glucose and cholesterol, about 80 % of the incorporated steroid was in the form of the β-D-glucoside. Morowitz *et al.* (1962) found that the lipids of a strain of *Mycoplasma gallisepticum* contained about 6 % cholesterol esters and 13 % cholesterol.

Mycoplasma mycoides requires a steroid for growth (Edward & Fitzgerald, 1951; Rodwell, 1956); a full study of this requirement has not yet been made. When viable particles were incubated in a medium deficient in cholesterol and a defatted serum protein fraction (Fraction C), the viable count decreased rapidly. Loss of viability did not occur when another essential nutrient required for cytoplasmic synthesis (uracil) was also omitted, or when chloramphenicol was added. This suggested that a deficiency of lipid precursors caused a type of unbalanced growth in which there was impairment of the synthesis of the limiting membrane, but not of cytoplasm (Rodwell & Abbot, 1961).

METHODS

Organisms. The strain V5 of *Mycoplasma mycoides* of this laboratory was isolated from a case of bovine pleuropneumonia in 1936. Strain GY was isolated from a case of peritonitis in a goat (Laws, 1956). The GY strain, although incapable of causing pleuro-pneumonia in cattle, is related serologically and biochemically to *M. mycoides* Its nutritional requirements are like that of the V5 strain of *M. mycoides* (Rodwell, 1960).

Media. Medium B1 was as described for medium B by Rodwell & Abbot (1961) except that the concentration of defatted bovine serum albumin (Fraction V) was decreased to 0·2 g./l., and riboflavin added to the autoclaved medium as a sterile filtered solution. Riboflavin underwent partial destruction when autoclaved in this slightly alkaline medium. For medium B2, the concentrations of palmitate and oleate were doubled and that of defatted bovine serum albumin (Fraction V) increased to 0·8 g./l. Steroids were added as aqueous dispersions in the concentrations indicated. Cholesterol-4-^{14}C was added as a fine stream of a solution in ethanol to the stirred medium at 60° before the medium was autoclaved; the volume of solution used did not exceed 1 % (v/v).

Medium BVF-OS was as described by Turner, Campbell & Dick (1935) but was supplemented with glycerol (0·003 M), glucose (0·02 M) and oleate (2×10^{-5} M) and adjusted to pH 7·9 (Dr P. Plackett; personal communication).

Growth tests. Duplicate tubes containing 5 ml. amounts of medium were seeded with about 10^7 viable particles suspended in 0·1 ml. 0·4M-sucrose solution containing 0·01 M-phosphate buffer (pH 7·5). Growth was estimated turbidimetrically at 660 mμ at intervals during static incubation at 37°. The optical density values recorded are in most instances after 24 and 65 hr. incubation for the GY and V5 strains, respectively, when the turbidities were approaching the maximum values.

Steroids. The following steroids were prepared. (*i*) Cholesterol dibromide (Fieser, 1953). (*ii*) Cholesterol; m.p. 149·3–150°; $\alpha_D = -39{\cdot}5°$, $c = 1{\cdot}0$ in $CHCl_3$, by reduction of (*i*) in accordance with Fieser (1953). (*iii*) 5α, 6β-dibromocholestane-3-one, by chromic acid oxidation of (*i*) (Fieser, 1953). (*iv*) Cholest-5-en-3-one, m.p. 118–121°, by reduction of (*iii*) (Fieser, 1953). (*v*) Cholest-4-en-3-one (cholestenone) m.p. 79·5°–80·5°, by isomerization of (*iv*) with oxalic acid (Fieser, 1953). (*vi*) Cholestane-3-one (cholestanone) m.p. 129·7°, by chromic acid oxidation of cholestanol (Barton & Cox, 1948). (*vii*) Cholesteryl palmitate (m.p. 77–79°) by the method of Swell & Treadwell (1955). (*viii*) Cholesteryl oleate, m.p. 42–45°, by heating cholesterol with oleic acid (Page & Rudy, 1930).

Other steroids were obtained from the California Corporation for Biochemical Research: cholestanol (m.p. 141·5–142·0°), C Grade; cholest-7-en-3β-ol (lathosterol, A Grade ($\alpha_D = +4{\cdot}25°$, $c = 2{\cdot}0$ in $CHCl_3$ at 19°)); cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), B Grade (m.p. 143–144°; $\alpha_D = -116°$, $c = 1{\cdot}0$ in $CHCl_3$ at 24°); stigmasterol, C Grade (m.p. 162–166°); ergosterol, C Grade (m.p. 145–155°); progesterone, C Grade; cortisone, C Grade. The Radiochemical Centre, Amersham, Buckinghamshire, England, supplied cholesterol-4-^{14}C (specific activity 33·8 μc./μmole).

Chemical procedures. Three methods were used for the extraction of steroids from the organisms. (i) The method of Folch, Lees & Sloane-Stanley (1957) which uses chloroform + methanol (2 + 1 by vol.). (ii) Extraction of aqueous suspensions using about 25 vol. of acetone + ethanol (1 + 1 by vol.) mixture; the suspension was added to most of the solvent, the mixture brought to the boil, cooled, made to volume with solvent mixture, then filtered. (iii) Dry extraction of lyophilized material by refluxing with successive portions of ethanol + ether (3 + 1 by vol.) mixture.

The conditions for the saponification of extracts, digitonin precipitation and colorimetric estimation of cholesterol by the Liebermann–Burchard reaction were as described by Sperry & Webb (1950). To conserve materials, the amounts of extracts and reagents were decreased so that the final reaction volume was 0·45 ml. Optical densities were determined with a Beckmann model DU spectrophotometer equipped with a Lowry and Bessey attachment for 1 cm. light-path microcuvettes. Total 3β-hydroxy sterol was determined by the anthrone method on the washed digitonide precipitates as described by Vahouny, Mayer, Roe & Treadwell (1960).

Systems used for the paper chromatography of steroids were as follows. (i) System A (acetic acid + liquid paraffin B.P.) of Michalec & Strasek (1960); (ii) System B (acetic acid + chloroform + liquid paraffin B.P. 80 + 15 + 5 by vol.) of Michalec & Strasek (1960); (iii) n-propanol + methanol + water (15 + 8 + 3 by vol.), saturated with liquid paraffin B.P. (Kodicek & Ashby, 1954); (iv) acetic acid + water (84 + 16 by vol.) saturated with liquid paraffin B.P. (Peereboom, Copius & Roos, 1960); (v) n-propanol + water (60 + 40 by vol.) saturated with kerosene (Martin & Bush, 1955). The paper used for systems (i) to (iv) was Whatman No. 3 impregnated with

liquid paraffin B.P., for system (v) it was Whatman No. 1 impregnated with kerosene. The solutions of steroids or of lipid extracts in chloroform were applied in a narrow band about 1 cm. long at the origin of the papers. The papers for systems (i)–(iv) were irrigated by the ascending method for 3–6 hr. at room temperature, and for system (v) by the descending method for 24 hr. at 37°. Steroids were located by spraying with an ethanolic solution of either phosphotungstic or phosphomolybdic acid (Martin, 1957).

Radioactive cholesterol was purified first by digitonin-precipitation followed by further purification through the dibromide derivative as described for amounts of the order of 1 mg. by Kabara & McLaughlin (1961). The conditions for bromination were modified slightly, bromine being added as a solution in acetic acid and the reaction being allowed to proceed for 1 hr. at 30°.

Determination of radioactivity. A thin end-window Geiger counter was used. In some experiments, solutions of lipid extracts in ethanol were plated for counting on polythene planchets; in others, the solvent was ethanol + acetone (1 + 1 by vol.) mixture, and the solutions were then plated on aluminium planchets. In either case, a disc of lens tissue was used to obtain uniform distribution, and a drop of a 1% (w/v) aqueous solution of polyvinyl alcohol to attach the discs to the planchets. Samples were plated in duplicate at infinite thinness, except for the counts of radioactivity in the growth medium or in the supernatant fluid after growth. Self-absorption corrections were applied for these, by counting a known amount of ^{14}C cholesterol added to the medium. When a more accurate count was desired, as in the determination of the specific activities, samples were plated in triplicate and corresponding replicate planchets counted in rotation. A count of a standard source was interposed between each set, and counting proceeded until a minimum of 5000 counts was obtained from each sample.

RESULTS

Effects of steroids on growth

Cholesterol purified through the dibromide derivative had about the same growth-promoting activity for *Mycoplasma mycoides* strain V5 as had an unpurified sample. The growth responses of strain V5 to cholesterol in media B1 and B2, and of strain GY in medium B2, are shown in Fig. 1. The concentration required for growth of the V5 strain was greater in medium B2 than in medium B1. Growth of the V5 strain in either medium was abnormal in that the turbidity increased at an exponential rate until about half of the maximum value had been reached, and thereafter increased more slowly. The organisms were highly filamentous and remained so during continued incubation, particularly in medium B2. The GY strain grew in medium B2 with a doubling time of about 3 hr., and continued at about this rate until the turbidity approached the maximum value.

Tests were made of the growth-promoting activity of steroids in concentrations ranging from $2\cdot5 \times 10^{-6}$ M to 10^{-4} M, for strain V5 in medium B1 (Fig. 1*a*) and for strain GY in medium B2 (Fig. 1*b*). The only steroids which, for either strain, replaced cholesterol at all effectively were cholestanol and lathosterol. Dark-field examination with a light microscope revealed no differences in the morphology of the organisms when grown in the presence of these sterols or of cholesterol. The

GY strain grew at a lesser rate with lathosterol than with cholesterol; with strain V5, growth in medium B1 was inhibited by concentrations of lathosterol greater than 2×10^{-5}M (Fig. 1). 7-Dehydrocholesterol promoted no growth of strain V5, and slight growth of strain GY, cholestanone slight growth of both strains, cholestanone and cholest-5-en-3-one no growth of either strain. Stigmasterol and ergosterol promoted slight growth of strain V5, progesterone and cortisone no growth. These last four steroids were not tested for growth of strain GY.

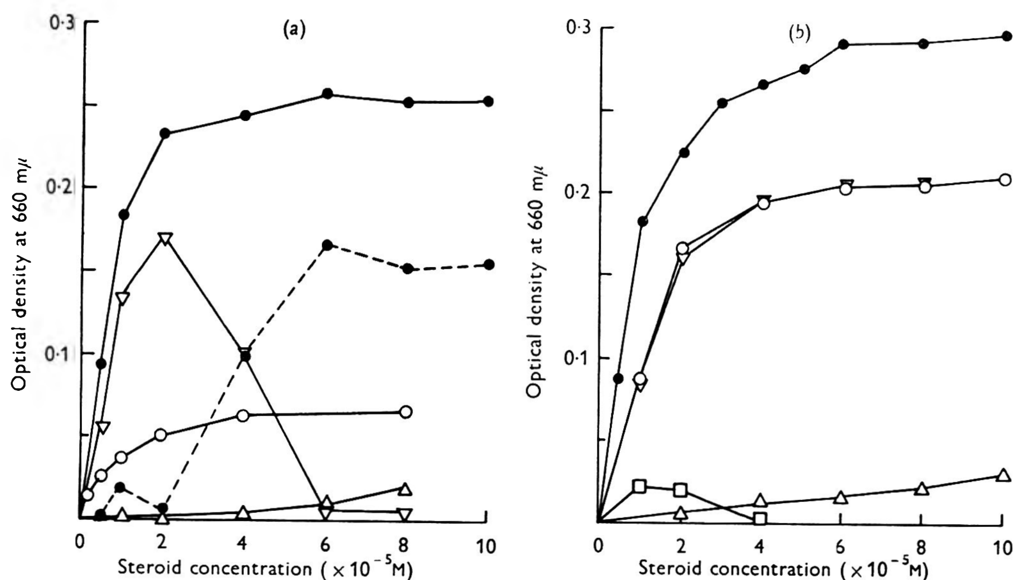

Fig. 1. Growth-promoting activity of steroids for Mycoplasma strains V5 and GY.
(*a*) Strain V5; —— medium B1; – – –, medium B2. 65 hr. incubation.
(*b*) Strain GY; medium B2. ●, cholesterol; ○, cholestanol; △, cholestanone; ▽, lathosterol; □, 7-dehydro-cholesterol 24 hr. incubation, except for lathosterol 40 hr. incubation.

The same steroids were tested for their effects on cholesterol-promoted growth of strains V5 and GY in media B1 and B2 containing 10^{-5}M and 4×10^{-5}M cholesterol respectively. The results of some of these tests are illustrated in Fig. 2*a* for strain V5 and Fig. 2*b* for strain GY. Cholestenone and cholest-5-en-3-one (not included in Fig. 2) were about equally potent inhibitors of the cholesterol-promoted growth of both strains; 7-dehydrocholesterol was less potent. The effect of 7-dehydrocholesterol was tested also on the growth of strain V5 and medium B1 containing 5×10^{-5}M-cholesterol. The concentration ($\times 10^{-5}$M) resulting in a 50 % decrease of growth increased from 0·7 for a cholesterol concentration of 10^{-5}M to 2·3 for one of 5×10^{-5}M. Cholestanone, lathosterol and cholestanol were either inactive, or caused only partial inhibition of growth at the highest concentration tested. Progesterone and cortisone inhibited growth of the V5 strain, the concentrations ($\times 10^{-5}$M) resulting in a 50 % decrease being 2·6 and 7·2, respectively. Stigmasterol and ergosterol caused partial growth inhibition of strain V5 at the highest concentration tested (8×10^{-5}M). These last four steroids were not tested for their effect on the cholesterol-promoted growth of strain GY.

The inhibitory concentrations of steroids for the GY strain were almost constant during the exponential growth phase, and increased when the incubation period was prolonged: e.g. the concentrations of cholestenone required for 50 % decrease of growth of strain GY were 1·4 to 1·6 $\times 10^{-5}$ M for an incubation period of 16–24 hr. and 2·7 $\times 10^{-5}$ M for one of 40 hr. The results illustrated in Fig. 2*b* for strain GY are for an incubation period of 24 hr.

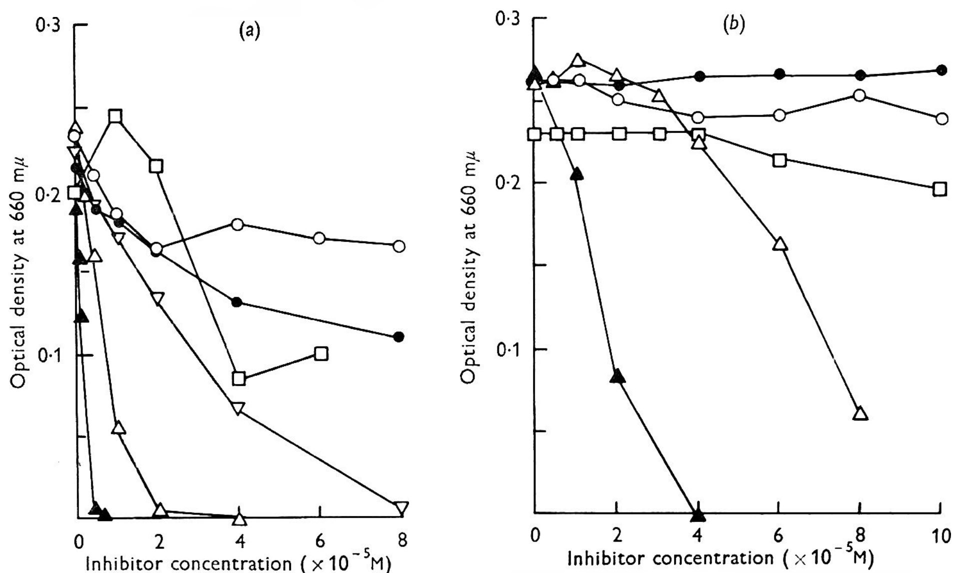

Fig. 2. Inhibition of cholesterol-promoted growth of Mycoplasma strains V5 and GY by steroids.
(*a*) Strain V5; medium B1 cholesterol 10^{-5}M. 65 hr. incubation.
(*b*) Strain GY; medium B2 cholesterol 4×10^{-5}M. ●, cholestanol; □, lathosterol; △, 7-dehydrocholesterol; ○, cholestanone; ▲, cholestenone; ▽, progesterone. 24 hr. incubation.

Steroid composition of organisms

In a lipid extract (Folch method) of strain V5 grown in supplemented BVF + OS medium, the digitonin-precipitable sterol was determined, directly and after saponification, by the Liebermann–Burchard reaction. Digitonin-precipitable sterol (estimated as cholesterol) represented 19 % of the dry weight of the total lipid extract; the same value was obtained after saponification. It was therefore concluded that, if esters were present, their amount must have been small relative to the amount of free cholesterol. The same lipid extract was also examined for the presence of cholesterol esters by paper chromatography in systems (i) and (ii) (see Methods). When large amounts of extract were chromatographed, faint bands which migrated in the region of long-chain fatty acid esters were detected; these may have been derived from contamination by medium constituents. Esters were not detected in chromatograms of lipid extracts of strain V5 grown in medium B1 + cholesterol. The amount of Liebermann–Burchard positive material, estimated as cholesterol, was about 4 % of the water-washed residue.

Incorporation of ^{14}C *labelled cholesterol*

The object of these experiments was to determine whether sterol esters had been formed in smaller amounts or whether steroid transformations had occurred.

Radioautography of paper chromatograms

Organisms from 100 ml. culture of strain v 5 in medium B 1 containing 2×10^{-5} M unlabelled cholesterol and 2 μc. cholesterol-4-^{14}C were harvested before maximum turbidity had been reached and while the culture still exhibited a pronounced 'swirl'. The organisms were washed with water, lyophilized and lipid extracts were prepared from the lyophilized residue as described in Table 1. The assessment of radioactivity in the fractions showed (Table 1) that 84 % of the cholesterol supplied in the medium was incorporated, and that all of this was in lipid extract 1.

Table 1. *Incorporation of cholesterol*-4-^{14}C *by Mycoplasma strain* v 5

The organisms from 100 ml. culture in medium B 1 containing 2×10^{-5} M unlabelled cholesterol and 2 μc. cholesterol-4-^{14}C were washed twice with 15 ml. water and lyophilized (yield 7·1 mg.). The lyophilized residue was thrice refluxed for 30 min. with 2·5, 1·0 and 1·0 ml. ethanol+ether (1+1) mixture and the extracts combined (lipid extract 1). This residue was then refluxed with 2 ml. ethanol+ether (1+1) mixture for 1 hr. (lipid extract 2). The weight of the defatted residue was 5·6 mg.

Fraction	Radioactivity counts/min. ($\times 10^{-3}$)
Medium	156
Culture supernatant fluid	27
Water-washed residue	131
Lipid extract 1	133
Lipid extract 2	0·2

Two ml. of lipid extract 1 were evaporated to dryness and dissolved in 50μl. chloroform. Samples of this solution containing radioactivity equivalent to 3,300 counts/min. and 8·4 μg. cholesterol (estimated colorimetrically) were chromatographed with a marker solution containing 10 μg. cholesterol and 16 μg. cholesterol palmitate in each of the five solvent systems described (see Methods). The marker solution alone and in admixture with a solution of ^{14}C-labelled cholesterol containing radioactivity equivalent to 1450 counts/min. were included on each chromatogram. Radiograms were prepared from each of the chromatograms by exposure to X-ray film for 5 and 21 days. The radiograms showed that the radioactive areas on all of the chromatograms coincided with the cholesterol areas. All five solvent systems separated cholesterol and cholesterol palmitate. Since radioactivity equivalent to about 25 counts/min./cm.^{2} paper should be detectable after exposure for 3 weeks, it was concluded that < 1 % of the cholesterol was esterified or transformed to other steroids separable in any of the solvent systems used. If no transformations had occurred, the cholesterol content calculated from the specific activity of the cholesterol supplied in the medium would be 4·2 %; the cholesterol content determined by the Liebermann–Burchard reaction was 4·2 %.

Isotope dilution

Cholesterol-4-^{14}C was diluted with non-radioactive cholesterol and the mixture purified by the digitonin precipitation and dibromide procedure. The specific activity was 34·6 counts/min./μg. cholesterol. Strain GY was grown in 100 ml. of

medium B to which purified radioactive cholesterol had been added to 5×10^{-5}M. The organisms were harvested at the end of the exponential growth phase, washed with water, lyophilized, and a lipid extract was made from the lyophilized residue as described in Table 2. The radioactivity determinations (Table 2) showed that 48 % of the cholesterol had been incorporated. Only 90 % of the radioactivity incorporated was recovered in the lipid extract; but, since very little remained in the residue after extraction, the discrepancy may be attributed to experimental error.

Table 2. ^{14}C-*cholesterol incorporation by Mycoplasma strain* GY

Organisms from 100 ml. culture in medium B2 containing 5×10^{-5}M-^{14}C-cholesterol (specific activity 34·6 count/min./μg. cholesterol) were washed once with 12 ml. water and lyophilized (yield 17·7 mg.). The lyophilized residue was refluxed with successive portions of ethanol + ether (3 + 1) mixture and the extracts combined.

Fraction	Radioactivity counts/min. ($\times 10^{-3}$)
Medium	61·0
Culture supernatant fluid	32·4
Water-washed residue	29·0
Lipid extract	26·0
Defatted residue	*c.* 0·2

An amount of the lipid extract which contained radioactivity equivalent to $17 \cdot 7 \times 10^3$ counts/min. (or 0·438 mg. cholesterol if the specific activity were unchanged) was diluted with 2·0 mg. purified non-radioactive cholesterol, and the cholesterol isolated from the mixture, as before. The specific activity of the isolated sample was 6·1 counts/min/μg. cholesterol. If no sterol transformations had occurred, the specific activity after dilution would be expected to be 6·2 counts/min./μg. cholesterol. The difference found is within the experimental error of the specific activity determinations. The experiment therefore did not detect any sterol transformations. The cholesterol content calculated from the specific activity of the cholesterol supplied and the amount of radioactivity incorporated was 4·7 % of the water-washed residue.

Cholestanol incorporation

Strain GY was grown in 200 ml. medium B2 in which the cholesterol was replaced by cholestanol at 6×10^{-5}M. The organisms were washed once with water and resuspended in 1 ml. water; the yield of water-washed organisms was 34·9 mg. Sterols were extracted from the aqueous suspension with ethanol + acetone (1 + 1), and suitable samples of extract taken for precipitation of sterol digitonides. Total 3β-hydroxy sterols as determined by the anthrone method amounted to 4·8 % of the water-washed organisms. Liebermann–Burchard positive sterols (as cholesterol) amounted to about 0·1 % of the water-washed residue, but the colour obtained did not match that of the cholesterol standards, and was no doubt due largely, if not entirely, to interfering substances. It was concluded, therefore, that within the limits of detection cholestanol was incorporated without desaturation.

Cholesterol exchange

The object of this experiment was to see whether cholesterol in the organisms exchanged with cholesterol in the medium during growth, and whether it could be displaced from the organisms by the growth-inhibitory steroid cholestenone. To replicate tubes of medium B2 were added: (*a*) cholesterol (4×10^{-5}M); (*b*) no steroid; (*c*) cholesterol + cholestenone (4×10^{-5}M each); (*d*) cholestenone (4×10^{-5}M). The tubes were inoculated with a suspension of organisms of strain GY which had been grown in a medium containing radioactive cholesterol (see Table 3). Immediately, and after 1, 3, 5·75 hr. incubation at 37°, one tube of each set was chilled, centrifuged and the radioactivity in the pellet was determined. Ater 5·75 incubation, the turbidity increased by factors of 2·8 and 1·7 in (*a*) and (*b*), respectively, but remained almost stationary in (*c*) and decreased slightly in (*d*). There was a loss of radioactivity from the sedimentable fraction, most of which occurred during the 1st hr. of incubation. The extent of this loss was similar under all conditions, about 75 % of the radioactivity in the inoculum being present in the sedimentable fraction after 5·75 hr. incubation (Table 3). The reason for the loss of radioactivity from the organisms is not known; it might have been due to lysis of some of the organisms in the inoculum. Complete equilibration with cholesterol in the medium in (*a*) would have decreased the radioactivity in the sedimentable fraction to 9 % of that in the inoculum. It seems likely that cholesterol does not turn over during growth, and that once incorporated it cannot be displaced from the organisms by cholestenone.

Table 3. *Cholesterol exchange between the organisms of Mycoplasma strain* GY *and medium during incubation at* 37°

Organisms for the inoculum were grown in 25 ml. medium B2 containing 1 μc. cholesterol-4-^{14}C and 4×10^{-5}M nonradioactive cholesterol. Organisms were harvested while in the exponential growth phase, resuspended in 5 ml. sucrose + phosphate solution, and 0·25 ml. of suspension (containing radioactivity equivalent to $2{\cdot}3 \times 10^{3}$ counts/min.) was used to inoculate replicate tubes containing 4·75 ml. of medium B2 with steroid additions as indicated. After incubation at 37° for the periods indicated, tubes were chilled, centrifuged, and the radioactivity in the pellets determined.

	Hr. at 37°			
	0	1	3	5·75
Steroids (4×10^{-5}M) added to medium	% of initial radioactivity in pellet			
(*a*) Cholesterol	96	84	78	73
(*b*) None	98	85	80	75
(*c*) Cholesterol + cholest-4-en-3-one	102	80	79	77
(*d*) Cholest-4-en-3-one	95	80	75	71

DISCUSSION

The results of the growth tests were qualitatively the same for the two strains examined; there were some quantitative differences. These are more likely to have been due to the differences in the lipid composition of media B1 and B2 than to differences in the sterol requirements of the two strains. The concentration of cholesterol required for growth of strain V5 was greater in medium B2 than in medium B1. Other experiments have shown that this requirement for higher

concentrations of cholesterol by strain v 5 in medium B 2 was related to the increased fatty acid concentration rather than to that of defatted bovine serum albumin. The concentration of cholesterol required for optimum growth of strain v 5 in a medium of undefined composition decreased from 5×10^{-5}M to 10^{-5}M when Fraction C was defatted with ethanol + ether mixture (Rodwell, 1956)—(defatted Fraction C was used in the work described in this paper). Under conditions where a higher cholesterol concentration was required, either lipid associations formed with other medium constituents might have rendered some cholesterol unavailable to the organism, or cholesterol may have had a protective function (as suggested earlier, Rodwell, 1956) as well as being required as an essential metabolite.

Since the series of steroids examined was limited, not many conclusions can be drawn which relate structure to activity. Cholestanol, and lathosterol (which has a double bond in the 7 rather than the 5 position) promoted growth; these sterols also had growth-inhibitory properties when tested in the more sensitive system (strain v 5 in medium B 1 containing a lower concentration of cholesterol). The introduction of a second double bond, as in 7-dehydrocholesterol, almost abolished growth-promoting activity and conferred potent growth-inhibitory properties. Cholestanone was almost inactive as a growth promoter or a growth inhibitor; but cholestenone and cholest-5-en-3-one were potent inhibitors of cholesterol-promoted growth. These results support the conclusion of Smith & Lynn (1958) that the 3β-hydroxy group is essential for growth-promoting activity, and differ from those obtained by Butler & Knight (1960), who found that cholestanone, cholest-1-en-3-one and cholestenone promoted the growth of *Mycoplasma laidlawii* (strains A and B). However, the function of steroids in the nutrition of *M. laidlawii* is not clear. Steroids do not seem to be an essential nutritional requirement for this species since they can be replaced for growth in a partly defined medium by bovine serum albumin (Butler & Knight, 1960). It is possible that, with this species, steroids may function primarily in a protective capacity. On the other hand, Rothblat & Smith (1961) showed that their strains which did not require sterol for growth incorporated cholesterol when supplied with it. Cholesterol esters were not formed under the growth conditions described in this paper; it is possible that they might be formed under other growth conditions. But they do not appear to be essential constituents of *M. mycoides*.

It is concluded that, with the strains of Mycoplasma examined, cholesterol is incorporated without chemical modification or combination. It would be difficult to exclude the possibility that this is the result of an irreversible, but unnecessary, adsorption. It seems more likely that cholesterol forms part of the structure of the lipid membrane system. Certain other steroids, structurally closely related to cholesterol, can also be incorporated unchanged, and fulfil the same function. In a recent paper Smith (1962) reported that cholestanol and ergosterol were incorporated by a Mycoplasma organism without transformation to other steroids. Certain other steroids inhibit growth, either by interfering with the incorporation of cholesterol, or by themselves being incorporated into a membrane which, as a consequence, cannot function properly.

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The Accumulation of Nucleotides by *Escherichia coli* Strain 26–26

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SUMMARY

Escherichia coli strain 26–26 (a mutant requiring lysine for growth) releases into the medium diaminopimelic acid, lipomucoprotein and nucleotides, including flavins, when grown with suboptimal concentrations of lysine. Cytidine diphosphate glycerol, cytidine diphosphate ribitol and a uridine-linked mucopeptide containing N-acetylmuramic acid, glutamic acid, *meso*diaminopimelic acid and alanine were identified among the nucleotides extracted from the medium. Similar uridine diphosphate-linked mucopeptides were isolated from extracts made from bacteria at various stages of growth. In addition, uridine diphosphate-linked mucopeptides were isolated from bacterial extracts which were found to contain muramic acid and lysine but no diaminopimelic acid. The possible role of these compounds as precursors of cell wall structures is discussed.

INTRODUCTION

Escherichia coli strain 26–26 is a lysine-requiring mutant derived from *Escherichia coli* 9637 (Davis, 1952), lacking the enzyme 2,6-diaminopimelate carboxylase (Dewey & Work, 1952). When growth is limited by suboptimal amounts of lysine this mutant accumulates both diaminopimelic acid and a lipomucoprotein in the growth medium and Meadow (1958) suggested that these compounds might be cell wall precursors. The work described in the present paper was carried out to discover whether any other possible precursors of cell wall material accumulated at the same time. A preliminary report has already appeared (Lilly, 1962).

Cummins & Harris (1956) showed that cell walls of a large number of Gram-positive bacteria contained a residue which was resistant to trypsin and ribonuclease and after acid hydrolysis released glucosamine, muramic acid, glutamic acid, alanine and either lysine or diaminopimelic acid. Detailed analysis of cell wall preparations of a number of Gram-positive bacteria has led to the current view that the rigid backbone is composed of a mucopeptide containing muramic acid, glucosamine and certain amino acids (Salton, 1962). The cell walls of Gram-negative bacteria are apparently more complex in nature than those of Gram-positive bacteria but it has been possible to show that *Escherichia coli* strain B cell wall preparations contain a mucopeptide component forming part of the rigid layer (Weidel, Frank & Martin, 1960; Martin & Frank, 1962). Mandelstam (1962) has shown that the cell wall mucopeptides from a number of different Gram-negative bacteria are similar in composition and all contain both diaminopimelic acid and lysine.

Since the initial report by Park (1952) of the accumulation of nucleotides linked to amino acids and amino sugars in penicillin-treated *Staphylococcus aureus*, similar

compounds have been found in a number of organisms. Strominger (1959) showed that the sequence of one of the nucleotides from *S. aureus* is UDP-N-Acmur-L-ala-D-glu-L-lys-D-ala-D-ala and Strominger, Scott & Threnn (1959) have isolated from a DAP-requiring mutant of *Escherichia coli* a nucleotide which differs only in that *meso*diaminopimelic acid replaces lysine. Several nucleotide-linked peptides have been found in other strains of *E. coli* (Smith, 1959; Tomasz & Borek, 1960; Comb, Chin & Roseman, 1961). The accumulation of nucleotide-linked peptides in the presence of penicillin and the similarity between their amino acid composition and that of the cell wall mucopeptide of Gram-positive organisms led Park & Strominger (1957) to conclude that these uridine nucleotide derivatives were precursors of the cell wall mucopeptide.

Cytidine diphosphate derivatives were detected in extracts of *Lactobacillus arabinosus* by Baddiley & Mathias (1954) and subsequently these were identified as cytidine diphosphate glycerol (Baddiley, Buchanan, Mathias & Sanderson, 1956) and cytidine diphosphate ribitol (Baddiley, Buchanan, Carss & Mathias, 1956). Clarke, Glover & Mathias (1959) have shown the presence of these cytidine compounds in some other strains of lactobacilli, *Bacillus subtilis* and *Staphylococcus aureus*. Saukkonen (1961) showed that there was a large increase in the amount of CDP ribitol in *S. aureus* 209P in the presence of penicillin. Baddiley, Buchanan & Carss (1958) investigated the cell walls of *B. subtilis* and *L. arabinosus* and found that ribitol phosphate was present and accounted for nearly all the phosphorus present in the cell walls. The name teichoic acid was given first to the ribitol-containing polymers in the cell wall (Armstrong, Baddiley, Buchanan & Carss, 1958) but was extended to include glycerophosphate polymers when it was found that they were present in the cell walls of other species (Armstrong *et al.* 1959). There is also some evidence that intracellular glycerol teichoic acids occur in several species of lactobacilli (Baddiley & Davison, 1961). It seems reasonable to regard the cytidine nucleotide derivatives, CDP ribitol and CDP glycerol, as precursors of these teichoic acids.

MATERIALS AND METHODS

Organism. Escherichia coli strain 26–26 (lys$^-$) was originally isolated by Dr B. Davis from the wild type strain *E. coli* ACTC 9637. It was maintained by monthly subculture at 37° on slopes of minimal medium agar containing 2×10^{-4} M-lysine and stored at 4°.

Media. The minimal medium (Meadow, Hoare & Work, 1957) contained: K_2HPO_4, 7 g.; KH_2PO_4, 3 g.; sodium citrate, 0·5 g.; $MgSO_4.7H_2O$, 0·1 g.; $(NH_4)_2SO_4$, 1 g.; water to 1 l.; pH was 7·0. Sterile glucose was added after autoclaving at 10 lb/in.^{2} for 10 min. to give a final concentration of 0·5 % (w/v). Minimal medium agar contained 2 % (w/v) New Zealand agar. Lysine hydrochloride was added to the medium before autoclaving to give the required final concentration for different experiments.

Reversion to lysine independence was tested by subculture into minimal medium with and without lysine, followed by incubation for 18 hr. In later experiments the detection of the enzyme 2,6-diaminopimelate carboxylyase was used as a test for reversion.

Growth of bacteria. The organism was subcultured into universal bottles containing

10 ml. of minimal medium supplemented with 2×10^{-4} M-lysine and incubated for 16 hr. These cultures were used to inoculate 5 l. of medium contained in a 10 l. aspirator. All incubations were carried out at 37°. A high degree of aeration was maintained by bubbling moist air at 37° through the medium at about 5 l./min.

Bacteria were harvested by centrifugation at 10,000 *g* for 10 min. at 2° in a M.S.E. Angle 17 refrigerated centrifuge (Measuring and Scientific Equipment, London).

Measurement of dry weights of bacteria. A standard curve relating optical density to dry weight was constructed with measured dilutions of a suspension of the organism. Measurements of optical density were made in a Unicam SP 600 spectrophotometer (Unicam Intruments Ltd., Cambridge) at 670 mμ using 1 cm. cells. An optical density of 1·0 corresponded to 0·490 mg. dry weight bacteria/ml.

Preparation of nucleotide extracts from bacteria. Washed bacterial cells were extracted with 10% (w/v) trichloracetic acid (10 ml./g. dry weight bacteria) for 30 min. at 0°. The cell debris was removed by centrifugation at 10,000 *g* for 5 min. at 0° and then washed with 5 ml. cold trichloracetic acid and recentrifuged. The supernatants were pooled and rapidly extracted with 10 ml. portions of ether until the extract was above pH 4. Any remaining ether was removed by warming the extract which was then brought to pH 7·0 by the addition of potassium hydroxide.

Preparation of nucleotide extracts from the growth medium. Norit PC III charcoal was prepared for use by washing with 1 N-hydrochloric acid followed by water and ethanol + ammonia (0·88) + water (50:5:45, v/v) and finally thoroughly washed with water and dried. The charcoal (2 g./l. of medium) was added to the cell-free growth medium and stirred overnight at 2°. An equal weight of Hyflo Supercel (John-Manville and Co. Ltd., London) was then added and the charcoal collected by filtration through a Buchner funnel precoated with Hyflo Supercel. After washing with water the nucleotides were eluted from the charcoal by percolation with ethanol + ammonia (0·88) + water (50:2:48, v/v) at room temperature. The effluent was collected until the absorption at 260 mμ fell to below 0·5 and was then concentrated under reduced pressure. All measurements of optical density in the u.v. range were carried out in a Uvispek spectrophotometer H. 700 (Hilger & Watts Ltd., London) using 1 cm. cells.

Nucleotide separation. Two elution systems were used for separation of nucleotides by ion-exchange chromatography. Elution with formic acid was carried out as described by Hurlbert, Schmitz, Brumm & Potter (1954) using a Dowex-1 × 10 column (0·9 × 12·5 cm.) and a 250 ml. mixing chamber. Fractions (5 ml.) were collected at a rate of 0·3 ml./min. The fractions containing each nucleotide peak were combined and freeze-dried.

The other elution system was a modification of the concave chloride gradient described by Pontis & Blumsom (1958) using a column (0·9 × 50 cm.) of Dowex-1 × 2 (200–400 mesh). The mixing chamber initially contained 1·8 l. 0·0002 N-HCl and the reservoir 1 l. 0·15 M-calcium chloride in 0·01 N-HCl, giving a ratio of the cross-sectional areas of the reservoir to mixing chamber of 0·55. Fractions (5 ml.) were collected at a rate of 0·5 ml./min. The fractions containing each nucleotide peak were pooled, concentrated and the calcium chloride removed as described by Pontis & Blumsom (1958).

Each nucleotide fraction was examined by chromatography on Whatman No. 1 paper. The solvents used were: *iso*butyric acid + ammonia (0·88) + water (66:1:33,

v/v); propanol + ammonia (0·88) + water (6:3:1, v/v); ethanol + 1 M-ammonium acetate, pH 7·5 (7:3, v/v). The u.v.-absorbing spots were cut out, eluted with 3 ml. 0·01 N-HCl and the absorption spectrum determined, using as a blank the eluate from a paper of similar area and R_F value from a blank track. Other tracks were sprayed with either the periodate-Schiff reagent (Baddiley, Buchanan, Handschumacher & Prescott, 1956) or the phosphate reagents of Hanes & Isherwood (1949).

Hydrolysates of the eluates were examined for amino acids and amino sugars by chromatography on Whatman No. 4 paper in the following solvents: phenol + ammonia (0·88) + water (80:0·3:20, v/v); butanol + acetic acid + water (63:10:27, v/v); butanol + pyridine + water (6:4:3, v/v) and methanol + pyridine + 10 N-HCl + water (80:10:2·5:17·5, v/v). Amino acids were detected with ninhydrin and estimated by the method described by Mandelstam & Rogers (1959). Amino sugars were detected by the method of Partridge (1948).

Estimation of nucleotides and flavins. The concentration of nucleotides in solution was determined by measurement of the absorption at 260 mμ. The values for the molar absorption coefficients and absorption ratios were those quoted in 'Ultraviolet absorption spectra of 5′-nucleotides' (Pabst, 1956). A molar absorption coefficient of 10^4 was used to calculate the concentration of mixed nucleotides in solution.

The concentration of flavins in the growth medium was obtained by measurement of the absorption at 430 mμ. After separation by column chromatography, riboflavin and flavin mononucleotide (FMN) were measured at 445 mμ and flavin adenine dinucleotide (FAD) at 450 mμ. The concentrations were calculated using the molar absorption coefficients obtained by Whitby (1953).

Chemical estimations. The following methods of estimation were used: diaminopimelic acid (Work, 1957); protein (Lowry, Rosebrough, Farr & Randall, 1951); total and acid-labile phosphate (Chen, Toribara & Warner, 1956); ribose (Hurlbert *et al.* 1954); *N*-acetyl-hexosamines (Strominger, 1957); and hexosamines (Rondle & Morgan, 1955). The conversion factor obtained by Crumpton (1959) was used to calculate muramic acid concentrations from the hexosamine reactions.

Estimation of lysine concentration in the growth medium. At the lysine concentrations used in the growth medium chemical methods of estimation were not sufficiently sensitive. For certain experiments 2 μc./l. of universally labelled lysine hydrochloride (supplied by The Radiochemical Centre, Amersham) was added to the growth medium. Samples (0·1 ml.) were taken in duplicate at various times during growth and counted at infinite thinness on 1 cm.^{2} planchets with a Tracerlab windowless gas-flow counter SC 16 (Tracerlab Inc., Boston 10, Mass., U.S.A.). The lysine concentration was obtained by comparing the counts/min. for the sample with the counts/min. for a sample taken at the start of the experiment when the lysine concentration was known.

Detection of 2,6-diaminopimelate carboxylyase. (Modified from the method described by Antia, Hoare & Work, 1957.) Washed cells from 5 ml. of the culture were suspended in 0·3 ml. 0·1 M-sodium phosphate buffer, pH 6·8 containing 100 μg/ml. of cetyl trimethyl ammonium bromide. After addition of 0·1 ml. of 2×10^{-4} M-pyridoxal phosphate and 0·1 ml. of 0·025 M-*meso*diaminopimelic acid, the mixture was incubated at 37° for 16 hr. and a 10 μl. sample of the reaction mixture was

chromatographed on Whatman No. 1 paper in the methanol + pyridine + HCl + water solvent for 3–4 hr. The paper was developed with ninhydrin. A control without diaminopimelic acid was run at the same time. The appearance of lysine on the chromatogram showed that the enzyme was present in the cell extract.

RESULTS

Effect of lysine on growth

Meadow *et al.* (1957) showed that maximal growth of *Escherichia coli* 26–26 was obtained in minimal medium containing 2×10^{-4} M-lysine and this has been confirmed for the growth conditions used in these experiments (Fig. 1). Preliminary experiments showed that after 18 hr. growth the maximum values for diaminopimelic acid (DAP) and lipomucoprotein (estimated as protein) released into the growth medium were obtained with an initial lysine concentration of 1×10^{-4} M.

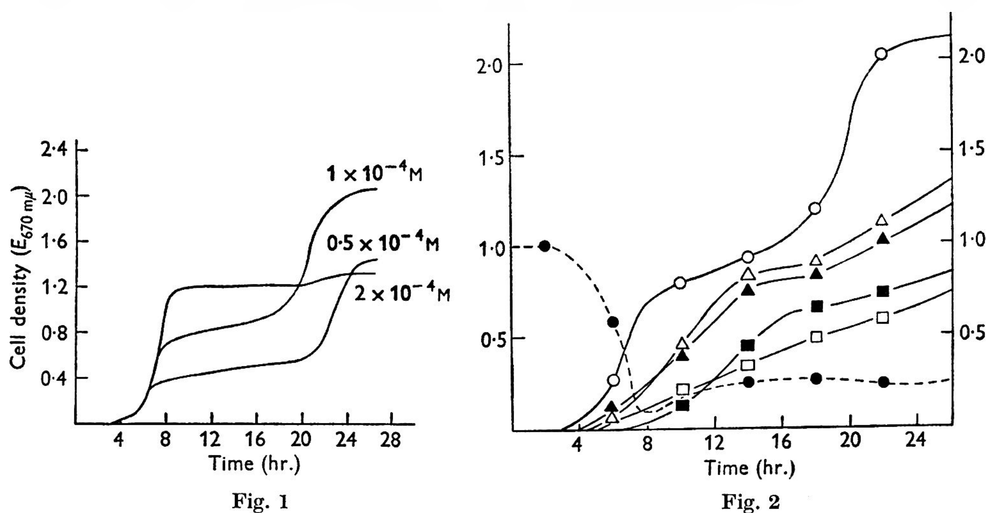

Fig. 1 Fig. 2

Fig. 1. Effect of lysine concentration on the growth of the lysine-requiring mutant *Escherichia coli* strain 26-26.

Fig. 2. Accumulation of extracellular materials during growth of *Escherichia coli* 26-26 in medium containing 1×10^{-4}M-lysine. Measurements were made at 30 min. intervals. ○—○, Cell density (E_{670} mμ); ●---●, lysine concentration in medium (M × 10^4); △—△, nucleotides (E_{260} mμ); ▲—▲, protein (mg./ml. × 10); □—□, flavins (E_{430} mμ × 10); ■—■, diaminopimelic acid (mg./ml.).

The relation of the growth phase of the organism to the release of compounds into the medium and to the utilization of lysine was investigated by growing the organism in media in which the initial lysine concentrations were: 0·5, 1·0 and $2 \cdot 0 \times 10^{-4}$M. Samples were removed at 30 min. intervals and examined for cell density, DAP, lipomucoprotein, nucleotides and flavins. ^{14}C lysine was used in these experiments and the decrease of free lysine in the medium followed by the decrease in radioactivity of the medium samples. The results for the experiment using 1×10^{-4} M-lysine are shown in Fig. 2.

It can be seen from Fig. 2 that the growth was diphasic. The end of the first growth phase corresponded to the exhaustion of lysine in the medium. The second growth phase after 16 hr. appeared to be due to reversion of the culture to lysine

independence. Tests for reversion by inoculation into minimal media were variable and mostly negative but 2,6-diaminopimelate carboxylyase could be detected in culture samples removed during the second growth phase. It was concluded that the increase of cell density in the later stages of the experiment was due, at least in part, to reversion of the mutant. Nucleotides, lipomucoprotein and flavins were released slowly into the medium as soon as the cell density increased. When growth was no longer logarithmic DAP accumulated in the medium at about 0·15 mg./mg. dry weight bacteria/hr. and nucleotides and lipomucoprotein were released at a more rapid rate. The radioactive lysine did not completely disappear from the medium and at the end of the first growth phase there was an increase in the amount of labelled lysine in the medium. By radioautography it has been possible to show that most of the lysine was present as the free amino acid. The remaining ^{14}C lysine may have been present in the lipomucoprotein in solution in the medium.

The growth curves for experiments using 0·5 and $2·0 \times 10^{-4}$ M-lysine are shown in Fig. 1. The growth of bacteria in medium containing $0·5 \times 10^{-4}$ M-lysine was also diphasic. At the end of the first growth phase when all the lysine had been utilized nucleotides, lipomucoprotein. DAP and flavins were released into the medium in the same way as for bacteria grown in 1×10^{-4} M-lysine. The rate of accumulation of these compounds in the medium was about half that which occurred for cultures grown in 1×10^{-4} M-lysine. When bacteria were grown in 2×10^{-4} M-lysine which allows maximal growth under these conditions, only small amounts of these compounds were found in the medium at the end of growth.

Separation and identification of extracellular nucleotides

Nucleotides were removed with charcoal from 5 l. of medium initially containing 1×10^{-4} M-lysine after 26 hr. growth. After elution from charcoal the extract was

Table 1. *Paper chromatography of the cytidine derivatives from peaks II and III and their hydrolysates*

	Reaction with				
	IO_4^- Schiff reagent	phosphate ester reagent	R_F (observed)	R_F* (Baddiley)	Compound
Peak II cytidine derivative	Blue (fast)	+	0·31	0·33	CDPribitol
(Hydrolysate)	Blue (slow)	−	0·77 (0·76)†	0·87	Anhydroribitol
	−	−	(0·66)†	0·76	Ribitol
	Yellow (fast)	+	0·36	0·39	Ribitol 3-phosphate
	Blue (slow)	Trace	0·32	0·34	Ribitol 1-phosphate
	Blue (slow)	+	0·23 (0·21)†	0·20	CMP
Peak III cytidine derivative	Reddish-blue (fast)	+	0·31	0·31	CDPglycerol
(Hydrolysate)	Reddish-blue (fast)	+	0·32	0·35	α-glycerophosphate
	Blue (slow)	+	0·21 (0·20)†	0·18	CMP
	−	−	−	0·74	Glycerol

* R_F values reported by Baddiley, Buchanan, Mathias & Sanderson (1956) and Baddiley, Buchanan, Carss & Mathias (1956).

† R_F values of known marker compounds.

Solvent: propanol + ammonia + water.

fractionated on a Dowex-1 ion-exchange column using the formic acid elution gradient. Fig. 3 shows a typical nucleotide separation. Each peak was concentrated and examined by paper chromatography. Peak I contained CMP, AMP and traces of NAD. Peak IV contained UMP and traces of CDP. Two cytidine derivatives were present in peaks II and III, which also contained GMP. The cytidine derivatives were examined further. Both compounds and their products on hydrolysis in

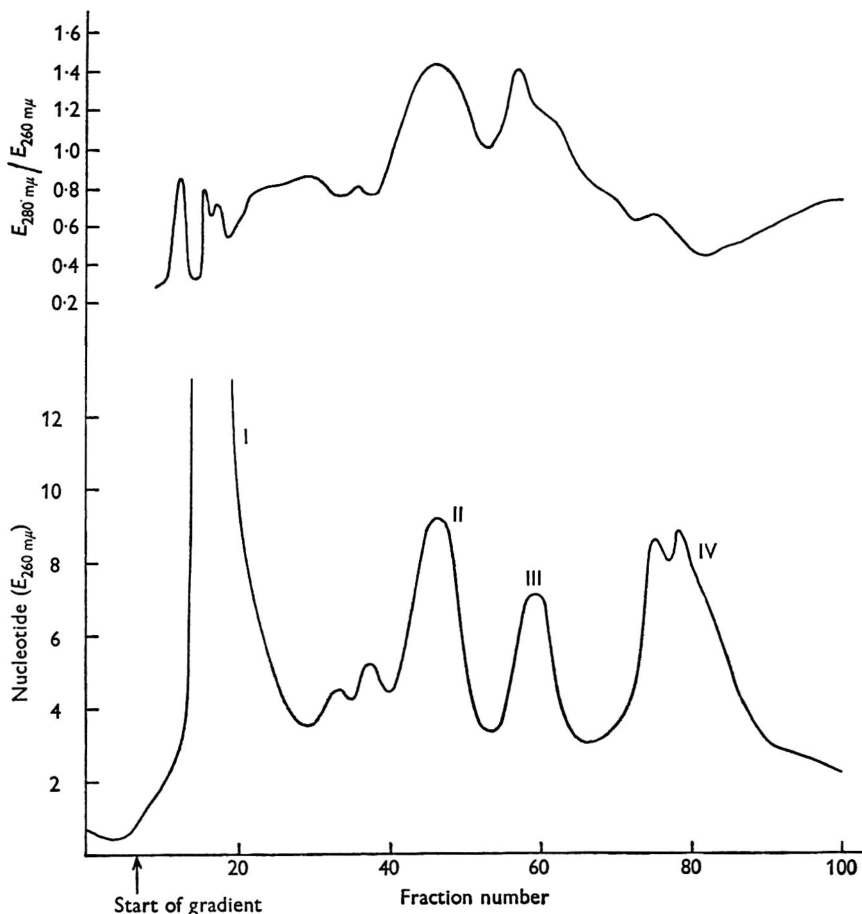

Fig. 3. Separation of extracellular nucleotides in an extract from medium initially containing 1×10^{-4}M-lysine after 26 hr. growth. Nucleotides were eluted from Dowex-1 × 10 using a convex formic acid elution gradient.

N-hydrochloric acid at 100° for 1–2 hr. were identified by paper chromatography in propanol + ammonia + water. The results are given in Table 1. It was concluded that peaks II and III contained cytidine diphosphate ribitol and cytidine diphosphate glycerol respectively.

A similar extract was examined by column chromatography on Dowex-1 using the chloride concave elution gradient. Apart from those compounds already identified a further major peak was obtained. On paper chromatography this compound had an R_{UMP} value of 0·9 in ethanol + ammonium acetate and an R_{AMP} value of 0·27

in *iso*butyric acid + ammonia + water. It contained 0·88 mole ribose, 0·99 mole acid-labile phosphate and 0·85 mole hexosamine (muramic acid) per mole of uridine base. After prolonged acid hydrolysis and chromatography in butanol + acetic acid + water and butanol + pyridine + water, alanine, glutamic acid and diaminopimelic acid were detected. It was concluded that this compound was UDP-N-Acmur-(ala, glu, DAP) and was probably identical to that found in peak G after column chromatography of cell extracts (Fig. 4).

Separation and identification of intracellular nucleotides

In an attempt to explain the extracellular accumulation of nucleotides, extracts were also made from bacteria at different stages of growth in media containing various concentrations of lysine. In a typical experiment 6 l. of growth medium initially containing 2×10^{-4} M-lysine were harvested after $9\frac{1}{2}$ hr. growth when the optical density at 670 mμ was 1·15 (0·56 mg. dry weight bacteria/ml.). A cold trichloracetic acid extract of the bacteria was investigated by column chromatography on Dowex-1 using the chloride concave elution gradient (Fig. 4).

The nucleotide components from each peak were separated by paper chromatography in *iso*butyric acid + ammonia + water and ethanol + ammonium acetate, eluted from the paper and the purine or pyrimidine base identified by measurement of the u.v. absorption spectrum. Some of the components could be identified without

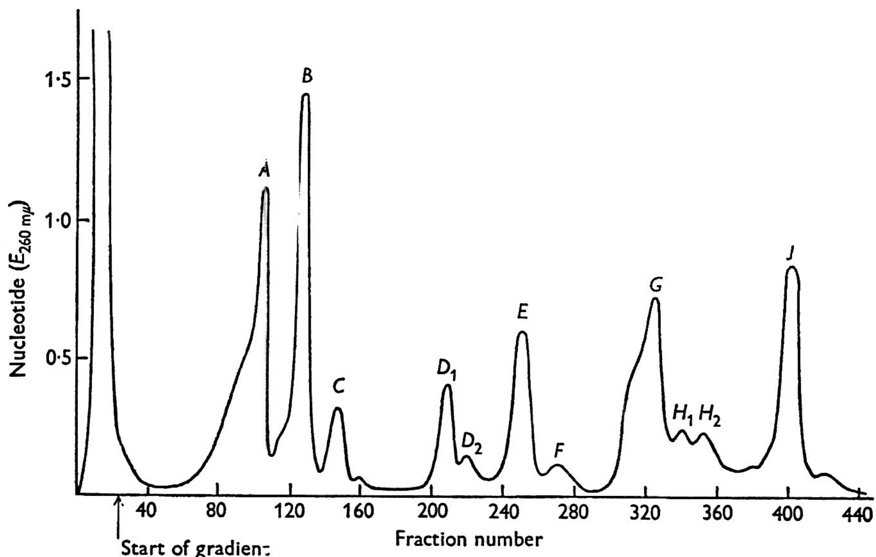

Fig. 4. Separation of intracellular nucleotides in an extract from bacteria harvested after $9\frac{1}{2}$ hr. from 6 l. of medium initially containing 2×10^{-4}M-lysine. Nucleotides were eluted from Dowex-1 × 2 with a concave calcium chloride and hydrochloric acid gradient.

further analysis (Table 2). Two of the peaks were very close together and did not separate well. These are labelled in Fig. 4 as D_1 and D_2. Similar considerations apply to peaks H_1 and H_2. When the nucleotides from all the peaks had been examined by paper chromatography it was found that peaks *A*, *E* and *G* contained two compo-

nents and these are described in Tables 2 and 3 as A_1 A_2, E_1 E_2 and G_1 G_2. A total of 12 nucleotide derivatives were examined. Peaks *B* and *C* contained only AMP and UMP respectively. D_2 was GMP and E_1 was ADP. Peak *F* was identified as FMN by its absorption peaks at 373 and 445 mμ. H_2 was similarly identified as FAD by its absorption peaks at 375 and 450 mμ and this was confirmed by paper chromatography. A_2 was identified as NAD by the presence of an absorption peak at 327 mμ in 0·8 M-KCN. None of these compounds or G_2 and H_1 which contained uridine and guanine respectively gave any ninhydrin reacting material after hydrolysis.

Table 2. *Paper chromatography and absorption spectra of intracellular nucleotide components*

Component no.	Solvents: Ethanol + ammonium acetate R_{UMP}	Solvents: *Iso*butyric acid + ammonia + water R_{AMP}	Purine or pyridine base (from absorption spectra)
A_1	—	0·47	Uridine
A_2	—	0·74	Adenine
B	0·75	0·98	Adenine
C	0·95	0·55	Uridine
D_1	0·13	0·58	Uridine
D_2	0·46	0·28	Guanine
E_1	0·20	0·73	Adenine
E_2	0·68	0·97	Uridine
G_1	0·80	0·29	Uridine
G_2	—	0·47	Uridine
H_1	—	0·20	Guanine
J	0·14	0·50	Uridine

Table 3. *Analysis of amino acid-linked uridine nucleotides from bacterial extracts*

Component	Uridine	N-acetyl muramic acid	Total phos-phate	glu	*meso*-DAP	ala	ser	lys	asp
A_1	+	+	+	+	+	+	0	0	0
D_1	1·0	0·86	1·93	1·0	0	0	1·1	0	0
E_2	+	+	+	+	0	0	+	+	0
G_1	1·0	0·91 (0·94)*	0·99†	1·0	0·88	2·75	0	0	0
J	+	+	+	+	0	+	+	+	+

* Estimated after hydrolysis as free hexosamine.
† Acid-labile phosphate.
+, Compound detected qualitatively on chromatogram.
Quantitative estimations expressed as molar ratios of uridine.

The remaining five components are listed separately in Table 3. These compounds were hydrolysed and the products examined by paper chromatography. They all contained uridine, muramic acid and several amino acids. Where possible quantitative estimations were made on both the purified components and their hydrolysates. It was concluded that the composition of the uridine-linked peptides from bacterial extracts was as follows: A_1, UDP-N-Acmur-(glu, *meso*DAP, ala); D_1, UDP-N-Acmur-(glu, ser); E_2, UDP-N-Acmur-(glu, ser, lys); G_1, UDP-N-Acmur-(glu, *meso*DAP, 3 ala); J, UDP-N-Acmur-(glu, ala, ser, lys, asp).

The flavin content of bacteria and growth medium

During the first growth phase and for a long time after the lysine had been exhausted, flavin compounds accumulated in the medium and gave it a faint yellow colour. The flavin compounds were completely removed from the medium by the procedure used for nucleotide extraction. The extract was fractionated by column chromatography using the method of Yagi, Okuda & Matsuoka (1955) (Fig. 5). The flavin peaks were identified by comparison with riboflavin and FMN and confirmed by paper chromatography (Crammer, 1948). The molar proportions of extracellular flavins were 31 % riboflavin, 33 % FMN and 36 % FAD. No allowance has been made for degradation during the extraction procedure.

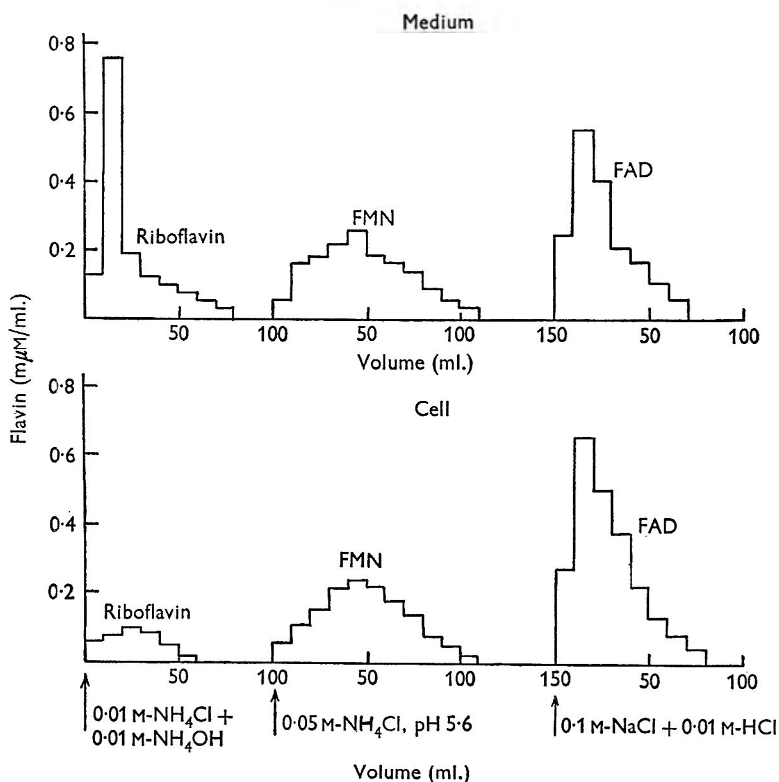

Fig. 5. Elution of flavins in bacterial and medium extracts from Dowex-1 × 2 using the elution system of Yagi, Okuda & Matsuoka (1955).

The flavin content of extracts from bacteria at different stages of growth was investigated to determine whether the appearance of flavins in the medium was a reflection of changes inside the bacteria. There was no significant difference in the total amounts or the proportions of each flavin in any of the extracts examined. A typical flavin separation by column chromatography is shown in Fig. 5. The molar proportions in this bacterial extract were 7 % riboflavin, 37 % FMN and 57 % FAD.

DISCUSSION

Among the different nucleotides isolated either from whole organisms of *Escherichia coli* 26–26 or from the growth medium with suboptimal concentrations of lysine were a number of uridine-linked peptides. These appear to fall into two groups. Representative of the first group is component G_1 which has been identified as UDP-N-Acmur-(glu, *meso*DAP, 3 ala) and is probably identical to the UDP-linked mucopeptide isolated from *E. coli* by Strominger *et al.* (1959) with the sequence: UDP-N-Acmur-ala-glu-*meso*DAP-ala-ala. Component A_1 also contained glutamic acid, *meso*diaminopimelic acid and alanine. It is probably of similar structure to component G_1 except that it contains fewer alanine residues. The amount of this material was so small that the number of alanine residues and their optical configurations could not be determined. This would be of considerable interest in view of the conflicting findings of different workers on the number of D-alanine residues in peptides of this type (Pelzer, 1962).

The remaining three uridine nucleotide-linked peptides appear to be members of a second group of these compounds. From the amino acid composition of the three components isolated it is possible to suggest the following tentative sequence for component *J*: UDP-N-Acmur-(ser, glu)-lys-(asp, ala). Similar compounds containing lysine and aspartic acid have been isolated from *Staphylococcus aureus* strain H (Ito, Ishimoto & Saito, 1959) and are believed to be precursors of cell-wall material in that organism (Ishimoto, Saito & Ito, 1958). If the uridine-linked mucopeptides are all precursors of cell-wall material this would suggest that the cell wall of *E. coli* 26-26 contains both diaminopimelic acid and lysine mucopeptide components.

Only one of the UDP-linked mucopeptides could be demonstrated among the compounds released into the growth medium i.e. UDP-N-Acmur-(glu, *meso*DAP, 3 ala). Non-specific leakage of nucleotides from the bacterial cells only occurs after a prolonged period in the stationary phase and then is due probably to lysis. The relative amounts of nucleoside monophosphates inside and outside the bacterial cells also indicate that under normal conditions general leakage does not occur. Thus it seems likely that the organism retains the UDP-linked mucopeptides until the peptide part is complete. It may then pass through the cytoplasmic membrane to be polymerized into cell wall material.

The isolation of CDP ribitol and CDP glycerol from the growth medium led us to consider the possibility that glycerol and ribitol teichoic acids might be present in the cell wall or cytoplasmic membrane of *Escherichia coli* 26–26. Armstrong *et al.* (1959) had already reported the presence of traces of glycerol in cell walls of *Escherichia coli* B and we have been able to isolate from cell wall preparations of *Escherichia coli* 26-26 an ethanol-insoluble polymer containing ribitol (Lilly, 1962). It is possible therefore that these two cytidine derivatives are precursors of teichoic acids present in the cell wall or cytoplasmic membrane of *Escherichia coli* 26-26. It is interesting that CDP ribitol and CDP glycerol were isolated only from the growth medium and not from bacterial extracts. This would indicate that the intracellular concentrations are very low although Shaw (1962) has reported that these nucleotides are particularly unstable under the conditions used for extraction.

The accumulation of flavins in the growth medium seemed to be independent of the release of other nucleotides and recent work by Wilson & Pardee (1962) has

shown that in *Escherichia coli* B flavin synthesis is in excess of cell requirements during growth and continues at the same rate for some time when growth is stopped. During this time flavins appear in the growth medium. The proportions of intracellular riboflavin, FMN and FAD for *Escherichia coli* 26-26 were similar to those obtained by Wilson & Pardee (1962) but the ratio of FAD to FMN in the growth medium was higher. This may be due to the different method used for extraction of the flavins or to the particular growth conditions of *Escherichia coli* 26-26 under lysine limitation.

Thus it has been possible to isolate from *Escherichia coli* 26-26 a number of nucleotides which are believed to be precursors of cell wall material. From these results it seems likely that the cell wall of this organism contains a complex mucopeptide structure containing both lysine and diaminopimelic acid and also ribitol or glycerol teichoic acids. The relationships between these nucleotides and the composition of the cell wall of this organism is being investigated.

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Investigations on the Action of the Iron-Containing Growth Factors, Sideramines; and Iron-Containing Antibiotics, Sideromycins

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SUMMARY

The formation of catalase activity by an Arthrobacter strain JG-9 is dependent upon the addition of exogenous haemin, or the iron-containing growth factor ferrichrome. The iron-containing antibiotic ferrimycin A inhibited the synthesis of catalase in bacterial suspensions supplemented with ferrichrome, but did not measurably alter catalase formation in suspensions supplemented with haemin. This suggests that ferrichrome is necessary for haemin (catalase) synthesis, and that ferrimycin A acts by blocking this step. Cell-free extracts of *Rhodopseudomonas spheroides* were able to synthesize haemin when incubated with an oxidizable substrate, protoporphyrin IX and iron supplied as ferrichrome.

INTRODUCTION

During the last 10 years a series of naturally occurring iron-binding compounds possessing potent biological activity has been discovered (Hesseltine *et al.* 1952; Neilands, 1952; Lochhead, Burton & Thexton, 1952; Bickel *et al.* 1960). The only feature common to all these compounds is that the iron-binding structure is in each case a secondary hydroxamic acid (Emery & Neilands, 1959; Bickel *et al.* 1960). Biologically the compounds seem to fall into two groups: growth factors and antibiotics. One biological characteristic common to all the compounds is that they are extremely potent: e.g. the growth factor ferrichrome supports half maximal growth of Arthrobacter JG-9 at a concentration of 0·3 mμg./ml., and the antibiotic ferrimycin A is ten times as active as penicillin on a weight basis against *Bacillus subtilis*.

Bickel and co-workers have observed that with certain bacteria these iron-containing antibiotics and iron-containing growth factors are mutually antagonistic (Bickel *et al.* 1960; Zähner, Hütter & Bachmann, 1960). They proposed the generic name siderochrome to include all naturally occurring iron-binding hydroxamic acids. Those compounds which have antibiotic activity they called sideromycins, and those capable of antagonizing sideromycins were classed as sideramines (Bickel *et al.* 1960). The distinction between these two types of iron-binding compounds becomes less clear when they are examined for growth-factor activity against certain bacteria. *Arthrobacter terregens*, Arthrobacter JG-9, and *Arthrobacter flavescens* are representative organisms which have a growth-factor requirement satisfied by haemin or a naturally occurring hydroxamic acid (Burnham & Neilands,

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1961), and with these organisms the sideromycins are about as effective as sideramines in promoting growth (author's unpublished results). It seems likely that such micro-organisms have lost not only the ability to synthesize sideramines, but have also lost much of their specificity for the siderochrome necessary to replace the natural sideramine. Under these circumstances, the loss of specificity would have survival value. Recent work on the metabolic function of ferrichrome provided evidence that this compound directly influences the ability of Arthrobacter JG-9 to incorporate iron into protoporphyrin (Burnham, 1962). In the present paper additional evidence for the participation of ferrichrome in the synthesis of haemin is presented, and a possible site of action of the sideromycins is suggested.

The design of the experiments was based on the observations of Zähner *et al.* (1960) that the antibiotic action of the sideromycins could be countered by the sideramines. Instead of using growth inhibition to measure antagonism, however, the ability of the test bacteria to synthesize catalase in the presence of sideramines and sideromycins was studied. Ferrichrome was chosen as the representative sideramine, and ferrimycin A was chosen as representative sideromycin. The test micro-organism was Arthrobacter JG-9, whose growth-factor requirement can be satisfied by ferrichrome or by ferrimycin A. However, the organism does display specificity to the extent that, when grown in the presence of one of these compounds, it will not respond to the other for a period of several hours, and for a short time the compounds are antagonistic in the classical manner. Since the antagonistic period is short, and since the growth rate is slow, it is generally not possible to observe an effect upon growth.

Previously it was shown that the amount of catalase in the organism, measured as catalase activity/mg. dry wt., provided a very sensitive index to the state of ferrichrome nutrition of Arthrobacter JG-9 (Burnham & Neilands, 1961). In ferrichrome-deficient organisms, the catalase content decreased before growth was measurably affected, and the synthesis of new catalase was apparent before growth recommenced after providing growth factor. Similarly, with organisms treated with ferrichrome and ferrimycin A, it was possible to measure an effect on catalase synthesis under conditions where growth was not measurably affected.

METHODS

Organisms. Arthrobacter strain JG-9, a ferrichrome-requiring bacterium, was originally obtained from Dr H. A. Barker, and has been described previously (Burnham & Neilands, 1961; Greenberg & Barker, 1962). The photosynthetic bacterium *Rhodopseudomonas spheroides*, and a streptomycin-resistant variant of *Staphylococcus aureus*, strain SG 511 var, which requires haemin for growth, were both obtained from Dr June Lascelles (Oxford). These organisms were grown under conditions described previously by Lascelles (1956*a*, *b*).

Chemicals. Ferrichrome was obtained from iron-deficient *Ustilago sphaerogena* fermentations as described previously by Neilands (1952). Ferrimycin A was generously donated by Dr E. Vischer (Ciba Ltd., Basle). Protohaemin IX was purchased from Schuchardt and Co. (GmbH, Munich, Germany) and was recrystallized three times from glacial acetic acid before use.

Determination of haemin. Haemin was measured by the very sensitive nitrate reductase assay with *Staphylococus aureus*, as developed by Lascelles (1956*b*).

Assay of catalase activity. The catalase activity of washed cell suspensions was measured by the colorimetric titanium sulphate method described by Weil-Malherbe & Schade (1948).

RESULTS

Antagonism experiments

The organism Arthrobacter JG-9 was grown for about 48 hr. in the usual medium (Burnham & Neilands, 1961) at a ferrichrome concentration of 1 mμg./ml. Under these conditions, growth was limited and the catalase activity of the organisms very low. The organisms were harvested by centrifugation and divided into 6 portions for subsequent treatments. Ferrichrome, ferrimycin A and haemin were added singly and in admixtures to these suspensions.

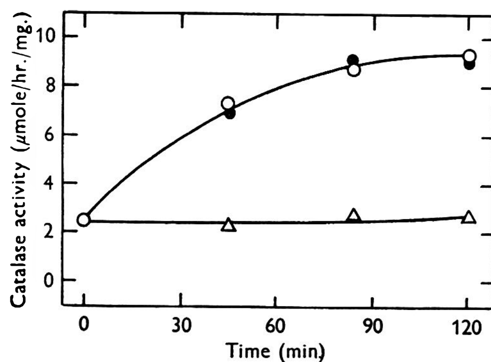

Fig. 1

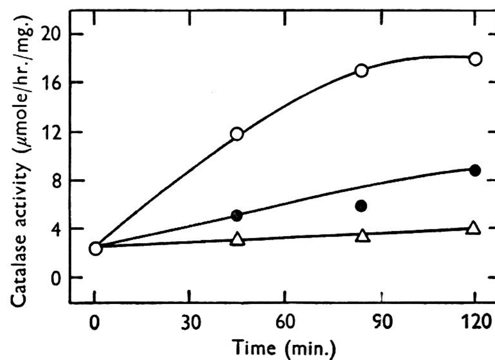

Fig. 2

Fig. 1. Increase in catalase activity after adding haemin and ferrimycin A to a ferrichrome-deficient culture of Arthrobacter JG-9. The organism was grown in basal medium + ferrichrome to 1 mμg./ml. After 48 hr. at 30° the organisms were harvested by centrifugation and resuspended at a concentration equiv. 0·5 mg. dry wt. organism/ml. in media as follows: ○, basal medium + 100 mμg. haemin/ml.; ●, basal medium + 100 mμg. haemin/ml. + 200 mμg. ferrimycin A/ml.; △, basal medium + 200 mμg. ferrimycin A/ml.

Fig. 2. Increase in catalase activity after adding ferrichrome and ferrimycin A to a ferrichrome-deficient culture of Arthrobacter JG-9. The organism was grown in basal medium + ferrichrome to 1 mμg./ml. After 48 hr. at 30° the organisms were harvested by centrifugation and resuspended at a concentration equiv. 0·5 mg. dry wt. organism/ml. in media as follows: ○, basal medium + 100 mμg. ferrichrome/ml.; ●, basal medium + 100 mμg. ferrichrome/ml. + 200 mμg. ferrimycin A/ml.; △, basal medium + 200 mμg. ferrimycin A/ml.

When haemin was supplied to ferrichrome-deficient organisms, the catalase activity increased (Fig. 1). This response to haemin resembled the response to ferrichrome reported previously, and it is believed that the increased catalase activity resulted from *de novo* synthesis of the enzyme (Burnham & Neilands, 1961). When haemin and ferrimycin A were simultaneously supplied to the organisms, the increase in catalase activity paralleled that obtained when haemin was added alone.

When ferrichrome was supplied to deficient Arthrobacter JG-9, catalase activity increased several fold (Fig. 2). However, when ferrimycin A and ferrichrome were added to deficient organisms simultaneously, the increase in catalase activity was markedly decreased. The control experiment with haemin and ferrimycin A indicated that ferrimycin A was not simply inhibiting catalase activity, nor was it

inhibiting apoenzyme formation or combination of apoenzyme and haemin to form active enzyme. These results, therefore, strongly suggest that ferrimycin A functions as an antibiotic by antagonizing the participation of ferrichrome (sideramines) in the synthesis of haemin.

Synthesis of haemin by cell-free extracts of Rhodopseudomonas spheroides

The evidence that ferrichrome can participate in the biosynthesis of haemin was further examined in cell-free extracts of the photosynthetic bacterium *Rhodopseudomonas spheroides*. Resting suspensions of *R. spheroides*, incubated anaerobically in the light with δ-aminolaevulate, iron citrate and an oxidizable substrate, have been shown to synthesize haemin and excrete it into the suspending medium (Lascelles, 1956*b*). In the present investigation, haemin formation was followed when iron was supplied as ferrichrome by using the nitrate reductase assay for haemin (Lascelles, 1956*b*). Results obtained using this assay were confirmed with ^{59}Fe. The growth and preparation of cell-free extracts of *R. spheroides* was as described by Lascelles (1956*a*). The incubation mixture contained (μmole/ml.): succinate 100; tris buffer (pH 7·8) 50; cell-extract and test compounds to 1·0 ml. Incubation was at 37° for 2 hr. under an atmosphere of N_2. The reaction was

Table 1. *Haemin formation by* Rhodopseudomonas spheroides *sonic extract*

Rhodopseudomonas spheroides sonic extract 0·3 ml. (equiv. 24 mg. protein/ml.), succinate 100 μmole, tris buffer (pH 7·8) 50 μmole, + protoporphyrin and ferrichrome as indicated; water to 1·0 ml.; incubated at 37° for 2 hr. under N_2.

Tube	Addition to incubation mixture: Protoporphyrin 10^{-3} M	Addition to incubation mixture: Ferrichrome 10^{-3} M	Haemin formed (mμmole/ml.)	CPM/mg. haemin*
1	0·05 ml.	—	0·36	—
2	—	0·05 ml.	0·0	638
3	0·05 ml.	0·05 ml.	0·30	4,820
4	0·075 ml.	0·05 ml.	0·54	5,750
5	0·1 ml.	0·05 ml.	0·84	9,100

* Corrected for background.

stopped by dilution with ice water and samples removed for haemin assay with *Staphylococcus aureus*. Carrier haemin was added to the remainder of each sample, and haemin was crystallized three times. Radioactivity was measured in a scintillation counter.

Ferrichrome-^{59}Fe was prepared by mixing 1 ml. of 2×10^{-3} M iron-free ferrichrome with 0·7 ml. of 2×10^{-3} M-$^{59}FeCl_3$ adjusted to about pH 7·0 with M-KOH, and the volume was taken to 2·0 ml. The slight excess of ferrichrome insured that all of the ^{59}Fe added to the system was bound by ferrichrome.

An oxidizable substrate was required for haemin formation, presumably for reduction of the ferric iron (ferrichrome) by the extract. It is generally recognized that iron is incorporated into protoporphyrin only in the ferrous state (Paul, 1960). The haemin-forming system was dependent upon protoporphyrin and iron, and the iron could be supplied as ferrichrome. This was shown by assay with the mutant

strain of *Staphylococcus aureus* and by incorporation of ^{59}Fe. Ferrichrome could serve as the sole source of iron in this system (Table 1), though iron citrate was also effective.

DISCUSSION

Zähner *et al.* (1960) with *Bacillus subtilis* and Burnham (unpublished results) with *Arthrobacter citreus* found that haemin did not counter the action of ferrimycin A upon the growth of these organisms. If sideramines function only in the synthesis of haemin, and if sideromycins block only that reaction, then haemin should reverse the action of sideromycins on growth. Since this is not the case, one must consider additional functions for the sideramines, or, alternatively, additional sites of action for the sideromycins.

It is a pleasure to acknowledge my gratitude to Dr June Lascelles for her stimulating interest and for encouragement and patience during this work. Thanks are also due to Dr E. Vischer of Ciba Ltd., Basle, and Dr H. Zähner of the Eidg. Technischen Hochschule, Zürich, for their interest and for generous gifts of siderochromes. The skilful technical assistance of Miss Lynda Butler is gratefully acknowledged. The author was a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research during this work, which was aided by a grant from this Fund.

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Some Effects of Respiration Inhibitors and *o*-Coumaric Acid on the Inhibition of Sporulation in *Venturia inaequalis*

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SUMMARY

The non-competitive inhibitors fluoroacetate and arsenite inhibited sporulation and respiration of *Venturia inaequalis* (Cke.) Wint. to a greater extent than malonate. They also restricted growth. *o*-Coumaric acid acted similarly to the non-competitive inhibitors but, while not inhibiting growth, it caused changes in the colour of the mycelium, suggesting an altered metabolism. Arsenite increased α-ketoglutarate in the medium and to some extent pyruvate, while *o*-coumaric acid produced only a slight increase in the concentration of pyruvate. No detectable amounts of succinic acid resulted from the presence of malonate. Injection of *o*-coumaric acid into scab-infected apple shoots decreased the incidence of disease; malonate had the opposite effect and was strongly phytotoxic.

INTRODUCTION

The production of conidia by phytopathogenic fungi is often essential to the local spread of disease. *Venturia inaequalis*, the causal fungus of the scab disease of apple, produces large numbers of conidia after establishment of ascospore infection early in the season. Restriction of sporulation after infection, whether by host resistance or by application of antisporulants, could therefore be important in controlling the disease. Kirkham (1957*b*, *c*) outlined the significance of phenolic host metabolites in resistance, and Kirkham & Flood (1956) showed the activity of certain analogues of caffeic acid, including *o*-coumaric acid, as inhibitors of the sporulation of *V. inaequalis*. In the present paper the activity of *o*-coumaric acid is further investigated and compared with the effects of respiration inhibitors.

METHODS

Culture methods. A single conidial isolate of *Venturia inaequalis* (Cke.) Wint. (clone E1; Kirkham, 1957*a*) was used. Cultures were grown on filter-paper cylinders standing in 10% (w/v) malt-extract solutions; sporulation was estimated turbidimetrically, the Spekker drum readings recorded being directly proportional to the number of conidia produced (Kirkham, 1956). There were six replicate cultures in each treatment and analyses of variance were made on the data. In sporulation tests the paper cylinder cultures were transferred at the end of the log phase of growth to media containing the inhibitors. For biochemical tests, the paper cylinder cultures were each rinsed aseptically in 100 ml. sterile distilled water towards the end of the log phase of growth and for the last 3 days of growth transferred

to a simple medium (glucose, 1·5 g.; $MgSO_4.7H_2O$, 0·05 g.; KH_2PO_4, 0·15 g.; 100 ml. trace element solution; Kirkham, 1957*a*) containing the inhibitors. The pathogen was also grown in surface culture in Roux bottles, each containing 100 ml. 10 % (w/v) malt-extract solution to provide material for chemical analysis and measurement of respiration. Inhibitors were added during the log phase of growth. The pH values of media containing *o*-coumaric acid, malonic acid and transaconitic acid were adjusted with 2N-NaOH before use.

The *in vivo* test on shoots of Cox's Orange Pippin was conducted as described by Kirkham (1957*c*).

Analysis of culture filtrates for acids from the paper cylinder method. Twelve tubes were used for the analysis; the paper cylinders from each tube were removed and the culture medium bulked. Each paper cylinder was cut in half, the retaining ring removed and the papers unrolled; they were then transferred to beakers and covered with water. After gentle agitation for 10 min. the fluid was decanted and the process repeated twice. The eluates and the bulked culture medium were combined, centrifuged to remove spores, poured into 4 vol. of boiling ethanol, held at the boil for 5 min., then rapidly cooled and kept at 1° overnight. Precipitated material was separated by centrifugation and the ethanol removed by distillation under reduced pressure in a nitrogen atmosphere at 30–35°. After bringing it to pH 7–8, the aqueous residue (about 10 ml.) was extracted continuously with light petroleum (60–80°) for 6 hr. After removal of traces of light petroleum in a stream of nitrogen, the aqueous phase was shaken for 3 hr. with 1 g. deactivated charcoal (Schramm & Primosigh, 1943) to remove pigments. The charcoal was separated by filtration and the filtrate and washings made to 50 ml. A sample of this solution was used for determining nitrogen (micro-Kjeldahl) and potassium (flame photometry). These figures enabled the size of the cation exchange column to be calculated. The remainder of the solution was brought to pH 2–2·5 with 8N-acetic acid (Harris, Tigane & Hanes, 1961) and passed through a column of Dowex 50 × 4 (100–200 mesh) in the hydrogen form. The eluate and washings were added to a column (9 × 0·6 cm. diam.) of Dowex 1 × 8 (200–400 mesh) in the acetate form and the column washed until the eluate was free from sugar (Molisch test). The analysis of the absorbed acids was completed by gradient elution (Hulme & Wooltorton, 1958).

Keto acid analysis of culture filtrates from Roux bottles. Two Roux bottle cultures were used for each treatment. After centrifugation and filtration through Celite as described above, metaphosphoric acid (100 ml. of a solution to give a final concentration of 0·6M-HPO_3) was added to the clear filtrate, which was kept at 0° until analysed. For the analysis, dinitrophenylhydrazine (20 ml. of a 1 % solution in 5N-H_2SO_4) was added; the mixture was brought to room temperature and allowed to stand for 45 min. Dinitrophenylhydrazones of the keto acids were isolated by the method of Isherwood & Niavis (1956) and then separated on a paper chromatogram and determined individually by the method of Isherwood & Cruickshank (1954).

Measurement of respiration. Mycelium was removed from individual Roux bottles by filtration through muslin, washed with water to remove spores and culture medium, sucked dry on filter paper in a sintered-glass funnel, blotted for further drying, macerated for 5–10 min. in 0·05M-KH_2PO_4, and the fluid adjusted to pH 6·0 with 2N-NaOH. The extract was made up to be equivalent to about 50 mg (wet wt.)

mycelium/2·2 ml. The inhibitors were used at pH 6·0, except for the higher concentrations of *o*-coumarate and arsenite, which came out of solution below about pH 6·5 and had to be prepared immediately before use. Respiration was measured in a standard Warburg apparatus by the direct method, the inhibitors being added from the side arms of the flasks after a steady respiration rate had been attained.

RESULTS

Effects of inhibitors on growth and sporulation

Table 1 shows that malonate inhibited the sporulation of the pathogen. The degree of inhibition was dependent on the initial pH value of the transfer medium, being greater at pH 6·2 than at pH 4·2. In no case was the mycelial growth decreased in rate or amount.

Table 1. *Inhibition by malonic acid of sporulation of* Venturia inaequalis *clone E*1 *grown in malt-extract solution*

Concentration of inhibitor (M)	Initial pH value	Sporulation*	Level of significance
—	4·2	0·661	
0·05	4·2	0·632	
0·1	4·2	0·402	$P < 0{\cdot}01$
—	5·0	0·263	
0·04	5·0	0·281	
0·1	5·0	0·076	$P < 0{\cdot}001$
—	6·2	0·456	
0·0125	6·2	0·315	
0·025	6·2	0·196	
0·05	6·2	0·145	$P < 0{\cdot}001$
0·1	6·2	0·018	

* Spekker drum reading of conidial suspension (directly proportional to the number of conidia).

The effect of malonate injection on inoculated shoots of Cox's Orange Pippin was also investigated. In a preliminary trial malonic acid (0·1 M at pH 5·0) damaged the immature leaves when injected through the petioles below the shoot tips. Interveinal scorch and distortion gradually increased during the 5 days after injection until the leaves were all severely damaged. The concentration of the acid was therefore decreased to 0·05 M in the main trial, where inoculated shoots were injected after the 36 hr. infection period (9 replicate shoots/treatment). Some slight damage again resulted but was insufficient to interfere with the expression of disease symptoms. Figure 1 shows that the incidence of disease was increased in the malonate treatment. In contrast with this result, the injection of similar host material with *o*-coumaric acid (0·1 M at pH 7), after infection with *Venturia inaequalis* clone E 1, caused a considerable degree of inhibition of the pathogen (Fig. 2) and was in no way damaging to the host.

Table 2 shows that arsenite and fluoroacetate were no more active as antisporulants than the competitive inhibitors (transaconitate, malonate; Table 1) and that where sporulation was markedly depressed mycelial growth was inhibited. Similarly,

the respiration rate of mycelium was decreased much more by these inhibitors than by malonate. *o*-Coumarate, however, while having no effect on the rate and amount of growth, strongly inhibited both respiration and sporulation.

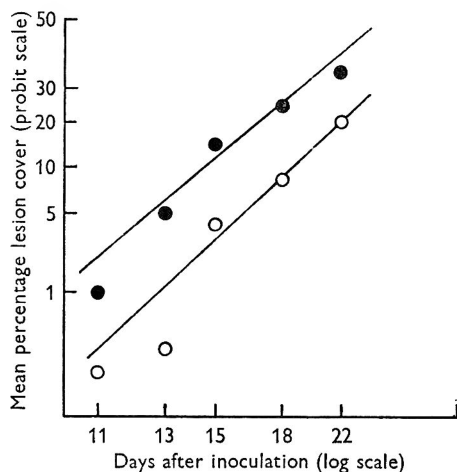

Fig. 1

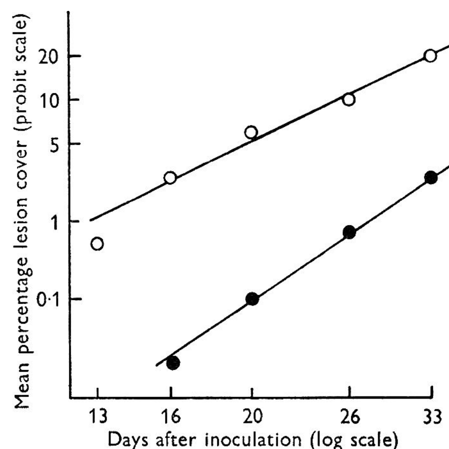

Fig. 2

Fig. 1. Stimulation of *Venturia inaequalis* clone E1 on the second fully expanded leaves of Cox shoots after injection of malonate below shoot tip. ○, Control; ●, sodium malonate.

Fig. 2. Inhibition of *Venturia inaequalis* clone E1 on Cox leaves after injection of *o*-coumarate. ○, Control; ●, sodium *o*-coumarate.

Table 2. *Inhibition of* Venturia inaequalis *clone E*1, *grown in* 10% *malt-extract solution at* pH 6·2, *by respiration inhibitors and* o-*coumaric acid*

Inhibitor	Concentration (M)	Sporulation*	Growth† category	Inhibition of respiration‡ (%)
—	—	0·788	3	—
Na arsenite	0·005	0·472	3	50
	0·01	0·270	2	50
	0·1	0·001	0	—
Na fluoroacetate	0·0005	0·568	2	40
	0·001	0·280	1	40
	0·01	0·000	0	—
*trans*Aconitic acid	0·05	0·129	3	—
	0·1	0·055	3	—
o-Coumarie acid	0·01	0·227	3	—
	0·05	0·024	3	21
	0·1	0·020	3	92
Malonic acid	0·1	—	—	20

* Difference significant: $P < 0{\cdot}001$.

† 0 = none or traces; 1 = thin and inhibited; 2 = normal but suboptimal; 3 = optimal.

‡ Inhibition of oxygen uptake by mycelium from Roux bottle cultures; inhibition of carbon dioxide output was similar.

Analysis of culture filtrates for acids

The filtrates from two Roux bottle cultures were used for analysis in each case. Adding arsenite to these cultures, 22 days after inoculation, greatly increased pyruvate and α-ketoglutarate concentrations as assessed at the end of the incubation period 15 days later (Table 3). Adding *o*-coumarate, however, only slightly increased the pyruvate concentration, and in similar cultures grown in the presence of malonate (0·1 M) no succinic acid was detected. Adding malonate or *o*-coumarate to Roux bottle cultures or to washed paper-cylinder cultures resulted in acid profiles which differed from the control; but most of the compounds produced are as yet unidentified and were present only in very small amounts. Thus, except for α-ketoglutarate, there occurred no large amounts of those acids which would have been expected if a Krebs cycle were functioning. Oxaloacetic acid was detected in trace amounts in all the culture filtrates but not in the original medium.

Table 3. *Production of keto acids by* Venturia inaequalis *in the presence of sporulation inhibitors added to the cultures during the log phase of growth*

Inhibitor	Pyruvate/200 ml. culture fluid (mg.)	α-Keto-glutarate/200 ml. culture fluid (mg.)
Control	0·72	1·8
Na arsenite (0·01 M)	5·5	4·1
o-Coumarie acid (0·1 M)	1·4	1·9

DISCUSSION

Inhibition of sporulation of *Venturia inaequalis* by phenolic metabolites of the host (particularly depsides) may be important in the host relations of the pathogen. Structural analogues of caffeic acid, the active part of the chlorogenic acid molecule, have shown greatly increased activity, *in vitro* and *in vivo*; of these analogues *o*-coumaric acid has so far proved to be the most active antisporulant agent. An understanding of the mechanism of this action might provide clues to the nature of host resistance and to the type of chemical structure of potential value as an eradicant fungicide.

It seemed possible that the energy requirement for sporulation might be supplied by a respiration mechanism such as the Krebs cycle and that known inhibitors of this cycle might also inhibit sporulation. At about this time Behal (1959) published the results of a study of the effect of respiration inhibitors on the sporulation of *Aspergillus niger*. His results agree with ours, but in addition we have analysed the culture filtrates in the hope of detecting the expected accumulation of metabolites just before the inhibited stage of the metabolism.

Tables 1 and 2 show that the known competitive inhibitors were less active as antisporulant agents than the known non-competitive inhibitors and that the latter also restrict the growth of the pathogen. The activity of malonate is interesting, since it is generally agreed that malonate is most active at a low pH value, where it is predominantly undissociated, and in this form it penetrates plant tissue more readily than in the ionized form. Perhaps with *Venturia inaequalis* either the

ionized and unionized forms of malonic acid penetrate the mycelium with equal ease or the inhibitory action occurs extracellularly and the ionized form is active (Turner & Hanly, 1947). Succinate never accumulated with malonate inhibition. In fact no more than traces of it were detected in any of the culture filtrates. Assuming that malonate reached the site of the reaction under our experimental conditions and inhibited succinic dehydrogenase, the absence of increased quantities of succinate would suggest that succinic dehydrogenase is not active in *V. inaequalis*. Barinova (1960) concluded that succinic dehydrogenase did not participate in the metabolism of *Rhizopus nigricans* or *Aspergillus niger* growing in glucose media, but in the presence of malonic acid (0·005–0·06 M) succinic acid production increased. However, because fumaric acid production also increased, Barinova suggested that the two processes of acid production were independent rather than linked as they would be in a Krebs cycle. When arsenite was used as an inhibitor, pyruvate and α-ketoglutarate were found in increased quantities in the culture filtrates (Table 3). It would appear, therefore, that oxidative decarboxylation of pyruvate and α-ketoglutarate plays a part in the metabolism of *V. inaequalis*. Walker, Hall & Hopton (1951) described the production of pyruvate by *A. niger* as well as an unknown keto acid and a non-acidic carbonyl compound. Apart from pyruvate and α-ketoglutarate, our culture filtrates all contained traces of oxaloacetate, but no other detectable carbonyl compounds. *o*-Coumarate, although inhibitory of sporulation, showed no similarity in action to any of the respiration inhibitors used, either *in vitro* or *in vivo*. Pyruvate production increased slightly (Table 3), but there was no increase in α-ketoglutarate. Examination of the acid profile showed a number of minor differences from that produced by malonate, but neither profile differed strikingly from that of the control. In spite of the difficulty of obtaining satisfactory acid profiles, due to the production by *V. inaequalis* of many mostly unknown acids, each in a small quantity, it is clear that the mechanism of action of *o*-coumarate bears little resemblance to that of the respiration inhibitors tested. It appears therefore that *o*-coumarate either has a different effect on the tricarboxylic acid cycle or that the mechanism by which it inhibits sporulation is not concerned directly with this cycle. Since it is still uncertain whether a tricarboxylic acid cycle operates in *V. inaequalis*, it is possible that the action of the respiration inhibitors in decreasing sporulation might be unconnected with their known action on respiration. Further comment must await the results of investigations of the course of respiration in the fungus.

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Morphology of Actinophage Φ 17

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SUMMARY

The negative staining technique was used to study the morphology of actinophage Φ17 which infects *Streptomyces chrysomallus* strain s17. It was found that the particle does not possess the characteristic tail of the bacteriophages, and that its capsid is built of distinct subunits.

INTRODUCTION

Some streptomycete phages have already been described (see references in Bacq & Dierickx, 1962); they comprise a head of about 800 Å diameter and a tail up to 2800 Å long. Mach (1962) observed a substructure in the tail of a *Streptomyces olivaceus* phage; this consisted of discs 45 to 50 Å thick, but was not contractile. The present paper deals with observations made on the phage Φ17 which infects strains of streptomyces from the *S. griseus* group (Welsch, Corbaz & Ettlinger, 1957). The normal host strain used for its isolation and propagation is an actinomycin C-producing *S. chrysomallus* s17 (Welsch, Minon & Schönfeld, 1955).

METHODS

A concentrated and highly purified preparation of phage Φ17 was obtained using methods described elsewhere (Bacq & Dierickx, 1962). The material examined contained 10^{13} plaque-forming units (pfu)/ml. and was prepared for electron microscopic observation by the negative staining technique according to Brenner & Horne (1959).

RESULTS

Electron micrographs revealed particles of 625 ± 30 Å average diameter (60 measurements) and of hexagonal outline (Pl. 1, fig. 1). No appendage in the form of a tail structure was visible. Actinophage Φ17 is normally stable in phosphate buffer (pH 6·5, ionic strength 0·1) but was found to be very sensitive to dialysis against ammonium acetate buffer or distilled water and only empty capsids (Lwoff, Anderson & Jacob, 1959) were seen after dialysis. This preparation had been kept frozen ($-80°$); this did not modify its infectivity but increased its sensitivity towards further treatments. The complete particles (Pl. 1, fig. 1) were observed when the preparation was fixed with 1 % (w/v) formaldehyde in phosphate buffer for 20 min. and dialysed overnight against distilled water before negative staining. The

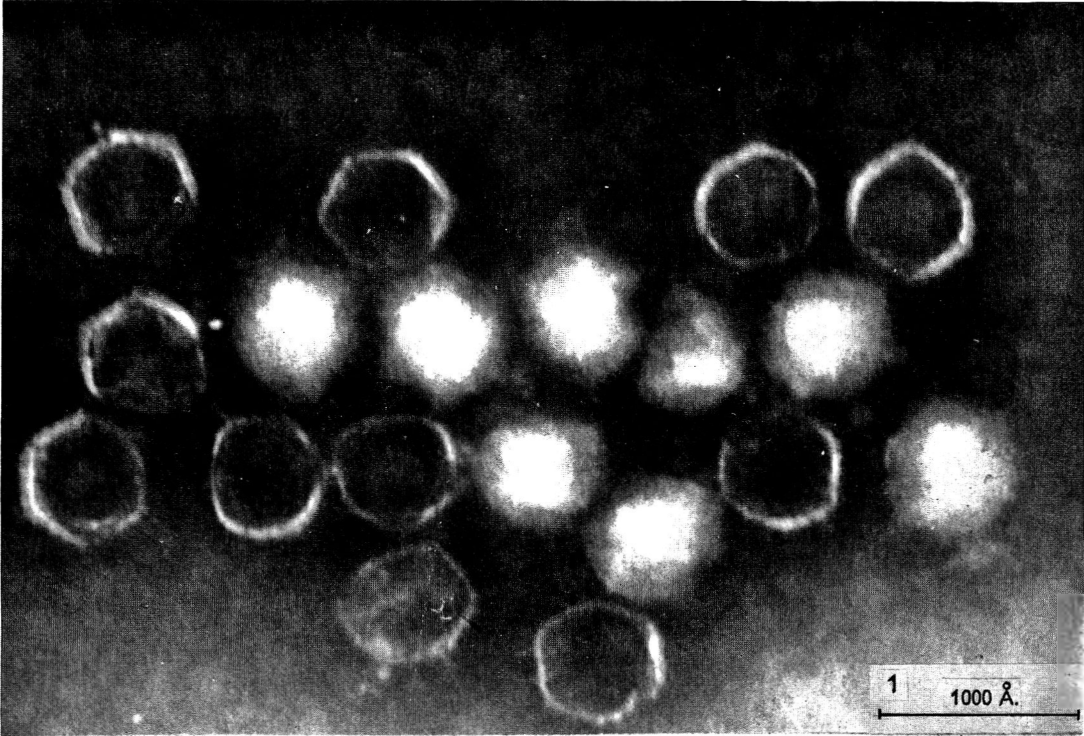
percentage of empty capsids was in good agreement with the degree of inactivation calculated for the initial suspension from the measurement of the absorption at 260 mμ per infective particle (Bacq & Dierickx, 1962). The empty capsids kept the polygonal shape of the intact particles; measurements on electron micrographs indicate an average diameter of 595 ± 25 Å. The crude lysate when examined by the same methods revealed particles of identical morphology; hence the tailless character is probably not an artifact due to the preparation procedures. Empty capsids prepared by dialysis against distilled water appeared to be composed of subunits or capsomeres (Pl. 1, fig. 2). It was not possible to determine the precise number of capsomeres forming the shell. When the phage was lyophilized and resuspended in distilled water before examination, the empty capsids seemed to aggregate by means of short projections borne by the capsomeres (Pl. 2, fig. 3). These projections may be like those observed in electron micrographs of ΦX 174 bacteriophage by Tromans & Horne (1961). The capsomeres have a centre-to-centre spacing of 70 Å. As estimated from the pictures of the empty particles, the protein shell is 20–35 Å thick. These small dimensions of the surface components and a close packing would explain that the subunits are difficult to resolve on the entire particle because the phosphotungstate could not penetrate between them.

Sometimes, when specimens were mixed with the stain at pH 4·5 without previous dialysis, an isolated structure of hexagonal outline was observed (Pl. 2, fig. 4). The external diameter is estimated at 105 –110 Å, with a central hole of a diameter of 35 Å. Thin fibres of 15 Å diameter and up to 140 Å long are attached to the hexagonal structure. A tentative interpretation of the isolated hexagonal plate is that it forms a type of fixation plate as observed in other phages (Brenner *et al.* 1959; Bradley & Kay, 1960). The arrow (Pl. 2, fig. 3) points to a ring-like design on the capsid, the diameter of which is slightly larger than the isolated hexagonal component, and which could be its site of localization on the complete particle. It remains to be seen whether these two structural features are related to an adsorption mechanism. From Pl. 2, figs. 3, 4, the plate structure appears to be located most probably on a vertex of the polygonal head outline.

DISCUSSION

The position of the plate structure raises the question of the three-dimensional shape of the virus particle, for in the case of T2 phage the idea has been put forward that the various symmetries of the head and tail components are related (Horne & Wildy, 1961). The plate structure being hexagonal, a vertex of the particle is characterized by a sixfold axis of symmetry; consequently the shape of the total phage should be that of an hexagonal bipyramidal prism, but less elongated than in the T2 phage head, to give a more hexagonal profile. Pictures of empty capsids as those shown in Pl. 2, fig. 6 (*a*, *b*), have been interpreted in other instances (Bradley & Kay, 1960) as derived from icosahedral capsids, but this type of symmetry seems unlikely in the case of Φ17.

In conclusion, Φ17 is different from actinophages hitherto described. It can be associated with other tailless phage particles, such as bacteriophage P22 studied by Anderson (1961), to form a new morphological group. The interesting feature in Φ17 is the substructure of its capsid, which suggests that it is built of small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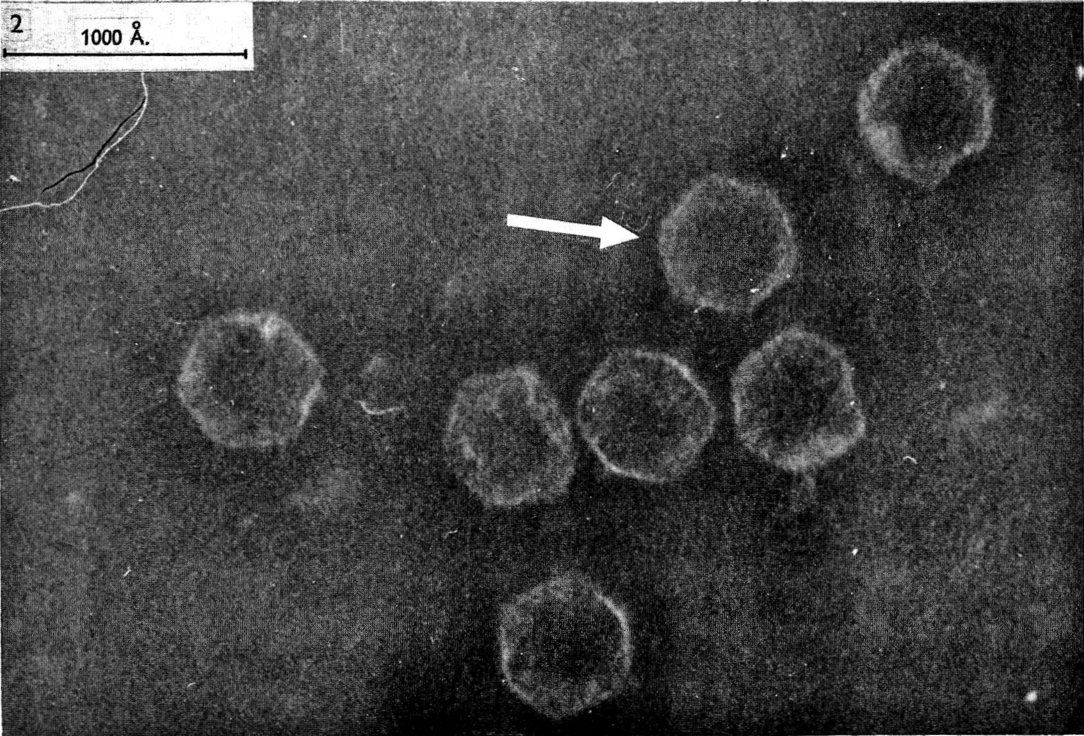
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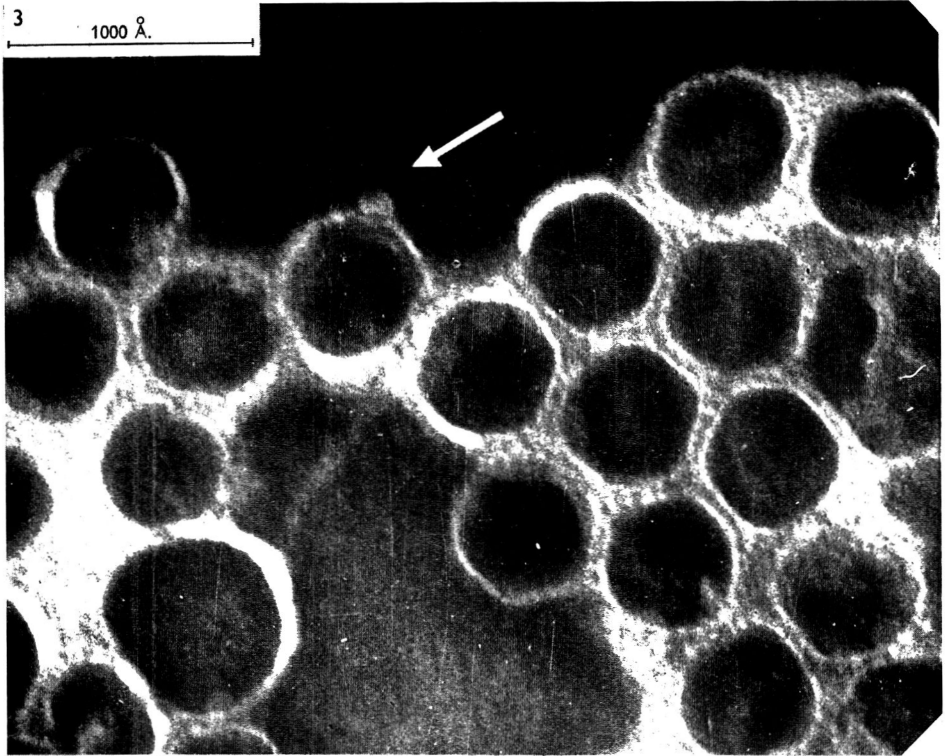

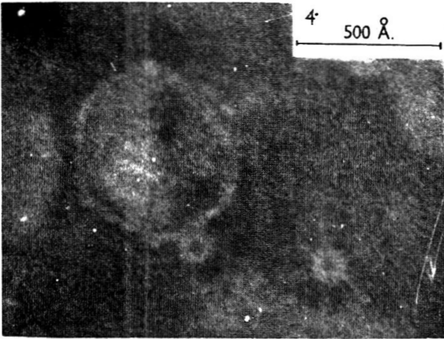

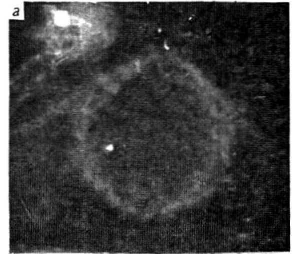

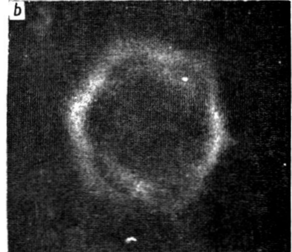

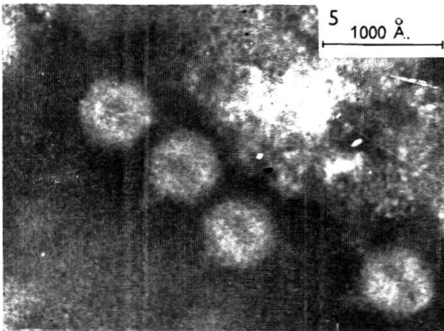

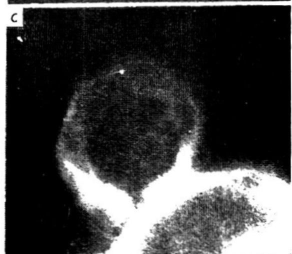

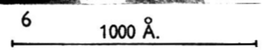

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subunits according to symmetry patterns described in other viruses (Horne & Wildy, 1961). The capsomeres or subunits are probably of very small dimensions and different from those observed on coliphage T5 by Bradley & Kay (1960).

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EXPLANATION OF PLATES

Electron micrographs of negatively stained preparations of actinophage Φ 17.

Plate 1.

Fig. 1. Particles fixed with 1% (w/v) formaldehyde. ×115,000.

Fig. 2. Empty shells obtained by dialysis against distilled water. ×202,500.

Plate 2

Fig. 3. Shells of lyophilized phages. ×300,000.

Fig. 4. Isolated hollow structure. ×360,000.

Fig. 5. Particles fixed with 1% (w/v) formaldehyde, showing a fixation structure. ×187,500.

Fig. 6. (*a*, *b*, *c*). Different aspects of empty shells. ×300,000.

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Serological Relationships and Some Properties of Tobacco Necrosis Virus Strains

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SUMMARY

Isolates from seven virus stocks called tobacco necrosis were serologically related, but fell into two groups (serotypes) showing widely different degrees of serological relationship. Serotype A contained the five closely related strains A, B, C (Dutch cucumber necrosis virus), F and S (bean stipple-streak virus); and serotype D strains D and E. Strains A and F, isolated in Britain and the U.S.A. respectively, are possibly identical, but the others could be distinguished by the kinds of lesions caused in French bean, and C by the symptoms it causes in young cucumber plants. The virus particles of all strains were hexagonal in outline and of the same width. They had the same absorption spectrum and sedimentation constants, and all except strain B crystallized into rhombic plates. Only strain D failed to aid the multiplication of the satellite virus. It is proposed to restrict the name tobacco necrosis to viruses serologicaly related to what seems the commonest strain, namely, A.

INTRODUCTION

Tobacco necrosis was the name given by Smith & Bald (1935) to a virus disease of tobacco seedlings in which the lower leaves developed necrotic streaks along the midrib, while the upper ones remained symptomless and virus-free. The roots of infected plants contained virus as also did the roots of many healthy-looking plants. Bawden (1941) found that the symptoms of tobacco necrosis could be caused by serologically unrelated viruses and Bawden & Pirie (1942, 1945) described the purification and properties of strains of these viruses. They did not propose any new names for the serologically unrelated viruses, but distinguished different virus cultures by the place or host from which they came. All the cultures caused necrotic local lesions only in tobacco, French bean and all other hosts to which they were inoculated, but since then three viruses have been described under the name tobacco necrosis that caused systemic diseases, namely stipple-streak of bean (Bawden & Van der Want, 1949), Augusta disease of tulip (Kassanis, 1949) and cucumber necrosis (Van Koot & Van Dorst, 1955; McKeen, 1959). Our work was done to compare the properties and to find the serological relationship between a range of these viruses.

METHODS

Tobacco necrosis viruses were derived from several sources and will be specified with a small capital letter for the sake of brevity. They will thus be referred to as e.g., TNVA or strain A. Strain A was isolated from a naturally infected tobacco root (Kassanis, 1960; Kassanis & Nixon, 1961). Strain B is the major infective component

of the Rothamsted TNV culture (Bawden & Pirie, 1942; Kassanis & Nixon, 1961). Strain D came from the leaves of a normal looking *Datura taiula* plant. Strain S is from the stock culture of bean stipple-streak virus, supplied by Dr D. Noordam. Strain C is an isolate of cucumber necrosis virus from Holland supplied by Dr Van Dorst. Strains E and F came from cultures of TNV supplied to us by Dr D. Teakle from U.S.A. Satellite virus was obtained as before (Kassanis, 1962). Each strain was derived from a single lesion after a series of single-lesion isolations. The serial transfers from single lesions were usually done on French bean (*Phaseolus vulgaris* L., var. Prince) but occasionally on *Nicotiana tabacum* L., var. Xanthi. Strain S was isolated from the young leaves of a succession of systemically infected French beans before it was inoculated to tobacco in which several serial single-lesion transfers were made and each one was tested in French bean to ensure that it became systemic. After the last transfer, inoculum from a single lesion was rubbed over as many bean leaves as possible, and these leaves supplied the inoculum for inoculating a large number of tobacco plants. The tobacco sap was stored as 2 ml. samples at $-20°$ and used throughout the entire work. These precautions were taken to ensure the purity of the strains because leaves of apparently healthy plants often contain TNV (Kassanis & Nixon, 1961; Babos & Kassanis, 1963*b*).

The viruses were purified as before (Kassanis & Nixon, 1961), except that they were salted out with ammonium sulphate at 30% saturation before the two cycles of high and low-speed centrifugation. The precipitation with ammonium sulphate was omitted when strain B was purified as it denatured much of the virus. The virus preparations in water were kept at 4°. The purified preparations of all strains were colourless except those of strain B which were light brown. Concentrations were estimated by optical density at 260 mμ using a Unicam SP 500 spectrophotometer.

For infectivity tests the primary leaves of French bean were inoculated when not quite fully expanded and before any trifoliate leaves had expanded. All experimental plants were kept in an insect-proof glasshouse at an average temperature of 20°. Inoculations were made by rubbing the upper surfaces of leaves with the forefinger wet with inoculum. To increase the number of lesions, 'Celite' (a diatomaceous earth) was mixed with the inoculum.

Antisera were prepared by injecting rabbits intravenously with two doses of 3 mg. of purified virus, at an interval of a week. The rabbits were bled 10 days after the second injection. The antisera were stored at 4° with 0·5% phenol added. Six antisera were prepared, two against strain D and one each against A, B, F and S strains. None reacted with sap from uninfected plants.

Serological tests were made either by precipitation tests in narrow tubes or by double gel-diffusion precipitation in 0·5% 'Ionagar' (or purified Difco-Bacto agar) dissolved in different buffers. Reservoirs were cut with a cork borer 0·6 cm. diameter and the distance between reservoirs was usually 0·2 cm.

Sedimentation constants were determined with a Spinco Model E ultracentrifuge.

Mr R. D. Woods did the electron microscopy on purified virus preparations by methods previously described (Kassanis & Nixon, 1961).

RESULTS

Symptoms in French beans

Of a range of commonly grown experimental plants tested to find a differential host for the strains, French bean was the best. Except strain S, which caused no obvious local lesions and became systemic, all strains caused only necrotic local lesions, but these differed enough in colour and size, and in the time they took to become visible, to permit reliable identification. The small, round, dark reddish brown necrotic local lesions produced by strain A within 2 days of inoculation gradually spread along the veins giving a network of red-brown necrotic tissue with almost transparent interveinal areas. They increased in size with time and, when few, reached 4 cm. in diameter before the leaf abscissed. The lesions produced by strain B took about 3 days to appear. They were dull red-brown and spread along the veins like those caused by strain A, the interveinal areas becoming transparent. They, too, increased in size until the leaf abscissed (Pl. 1, fig. 1). Strains C and F produced lesions similar to those caused by A, but C could be distinguished by the systemic infection caused in very young cucumber plants.

Strain D was isolated from the leaves of a normal-looking *Datura tatula* L. plant. When it was first inoculated on to French bean minute necrotic lesions appeared 3 days after inoculation, and remained small. Transfers from these lesions to further beans gave lesions of different sizes and some increased in size considerably in one direction only (Pl. 1, fig. 4). Inoculation to bean from any part of such an outgrown lesion gave only one kind of lesion. The minute lesions appeared several times during our work and their formation seemed dependent on the physiological condition of the bean plants. Inoculations from the same purified preparation often gave large lesions in one plant and small in another growing in the same pot, but occasionally the two kinds of lesion appeared on the same leaf. This strain differs from the rest in that its lesions remained discrete and did not spread along the veins. The reddish brown lesions, which almost always remained circular in outline, grew to about 1 cm. in diameter after about 2 weeks and then seemed to consist of concentric rings (Pl. 1, fig. 3).

Strain E formed lesions intermediate between those formed by strains A and D. Two days after inoculation they appeared as distinct dark reddish brown spots which became irregular in outline and eventually reached the smallest adjacent veins. They continued to increase, but did not spread along the larger veins like those caused by strain A or B (Pl. 1, fig. 2).

The culture of bean stipple-streak virus supplied by Dr D. Noordam produced only necrotic local lesions in French bean during the winter, but when temperature increased a few plants became systemically infected. The rarity of systemic infections was found to be because the culture contained satellite virus which interfered with the multiplication of the stipple-streak virus. Strain S isolated from this culture, and freed from the satellite virus, infected French bean systemically throughout the year, although the severity and kind of symptoms varied with the season. During spring and winter the symptoms were very severe; the inoculated leaves showed veinal necrosis with occasional interveinal green or yellow mottling. The mottled leaves became stiff and brittle with their edges often curled downwards. Long streaks formed on the petioles and often on the stems. The necrosis extended

to the growing point and to the young trifoliate leaves, killing the whole plant. During summer the leaves were severely mottled but less necrotic, shoots proliferated extensively and some plants fruited although both their flowers and pods showed lesions (Pl. 1, fig. 5). The virus concentration was high in all infected tissues.

Serological relationships

Tube precipitation tests. The six antisera were titrated against six of the isolates, using the virus at no more than 0·02 mg./ml. to avoid antigen excess at the higher dilutions of antiserum. All six viruses were serologically related but the antiserum titres against the heterologous viruses showed that the six fell into two groups (Table 1). Cross-absorption tests showed that strains within each group also differed serologically from each other, although to a lesser extent than between groups.

Table 1. *The reciprocals of precipitation titres of tobacco necrosis virus antisera against homologous and heterologous antigens*

Test antigen	Antiserum A	F	S	B	D1	D2
A	1024	4096	4096	256	32	32
F	1024	4096	4096	256	32	32
S	1024	4096	4096	256	4	32
B	1024	4096	4096	256	4	32
D	32	256	256	8	256	512
E	32	256	256	8	128	256

A series of two-fold dilutions of antiserum were incubated with equal volume of 0·02 mg./ml. of purified virus. The titres were recorded after standing for 5 hr. at 37 ° and overnight at room temperature.

For absorption the mixture of antiserum and heterologous virus at optimal proportions was incubated for 5 hr. at 37° and then kept overnight at 4° before the resulting precipitate was removed by low-speed centrifugation. When the absorbed antisera were titrated, the unabsorbed were also tested as controls. Antisera to strains A, B, S and D were absorbed with some of the strains selected to provide the maximum information on antigenic composition. Each absorbed antiserum was tested against all strains except C. Antisera against strains A, B, F and S had few antibodies reacting with strains D or E, and *vice versa*. After an antiserum was absorbed with a strain belonging to the other group, its titre against the homologous antigen was unchanged. Using the nomenclature proposed by Kassanis (1961) the viruses in the two groups are serotypes, and those within each group strains of one another.

When an antiserum was absorbed by a member of the same group most or all antibodies were removed. Such tests showed that strains A and F were indistinguishable in antigenic composition, and their similarities in other properties imply that they may well be identical. The inability of any other strain to absorb completely the antibodies in antisera prepared against strains A, B or D indicates that they have specific antigenic determinants. Absorption of antiserum to strain S with strain B showed an antigen common to strains A, F and S, which was not found in strain B, whereas absorption of antiserum to strain B with strain S showed an antigen common to A, F and B, which was not possessed by strain S.

The two antisera prepared against strain D differed in antibody composition. All cross-reacting antibodies were removed from both antisera when absorbed by strain E. Antiserum D1 contained some antibodies that reacted only with strains A and F of the other group, as indicated by absorption with strain B or S, whereas antiserum D2 still reacted with strain B after absorption with strain A or S (Table 2). From these results the minimum antigenic composition of each strain can be derived. Strains A and F appeared identical, and more closely related to strains B and S than strain B to strain S. Strains A and B are equally related to strains D and E, but strain S is less so. The close relationship between strains D and E indicates that these two strains form a separate serotype with a large proportion of specific antigenic determinants.

Table 2. *The reciprocals of precipitation titres of absorbed antisera to strains of tobacco necrosis virus*

Antiserum	Antigen used for absorption	Antigen used for testing					
		A	F	S	B	D	E
A	S	8*	8	0	0	0	0
	D	1024	—	1024	1024	0	0
S	A	0	0	0	0	0	0
	B	16	16	16	0	0	0
	D	4098	4096	4096	4096	0	0
B	A	0	0	0	4	0	0
	S	16	16	0	16	0	0
	D	256	256	256	256	0	0
D1	A	0	0	0	0	256	128
	B	16	16	0	0	256	128
	S	16	16	0	0	256	128
	E	0	0	0	0	32	0
D2	A	0	0	0	16	512	256
	S	8	8	0	32	512	256
	B	0	0	0	0	512	256
	E	0	0	0	0	64	0

* The figures are reciprocals of precipitation titres of absorbed antisera. The sign 0 signifies absence of precipitation at the lowest dilutions tested, which were 1/8, 1/8, 1/2, 1/4, and 1/2 for antisera against strains A, S, B, D1 and D2 respectively. Titres of unabsorbed antisera are shown in Table 1.

In addition to the cucumber necrosis virus found in Holland by Van Koot & Van Dorst (1955) and designated here as strain C, there is the Canadian cucumber necrosis virus which McKeen (1959) showed to be serologically unrelated to tobacco necrosis virus but included in the tobacco necrosis group because of certain similarities. Using an inoculum provided by Dr McKeen, we found this virus unrelated serologically to either of our serotypes and the lesions produced in French beans distinctly differed from any described here.

Diffusion precipitation tests in agar-gel. Titration of TNV antiserum in agar-gel against homologous or heterologous antigen showed that at several antigen-antibody (Ag/Ab) ratios, irrespective of buffer, pH and ionic concentration, two and sometimes three, precipitation lines were formed, especially when the distance between reservoirs was 0·9 cm. When the distance was 0·2 cm., often there was only

one line that split at either end, probably because at these points the reactants travelled longer distances to meet at optimal proportions. The phenomenon was not caused by contamination of the antigen and antiserum with a different strain, because such large contamination as indicated by the intensity of the lines could easily have been detected by infectivity tests. Neither sap from healthy plants, nor the material produced by subjecting large quantities of such sap to TNV precipitation procedure, reacted with any antiserum either in gel-diffusion tests or in tube precipitation tests. The double line might have been caused by the breakage of the virus into fragments whose size, antigenic composition and diffusion coefficient determined the position of the precipitation line. This is suggested by the fact that sometimes the two lines crossed each other. These fragments must have similar electrophoretic mobilities in agar because they were not separated by immunoelectrophoresis. Two or more lines in an apparently homogeneous antigen reacting with its homologous antiserum have been reported with other antigens and these are discussed by Crowle (1960).

All strains, except strain B, produced clearly visible precipitation lines with their homologous or heterologous antisera in different buffers and Ag/Ab ratios. Of the several buffers in which agar was dissolved (tris, phosphate, borate, and veronal) borate gave the best line definition and sensitivity at pH 7 to 8·3 at wide Ag/Ab ratios. Line formation with strain B against any antiserum was erratic and often no line formed irrespective of the conditions of the test. This strain also differed from the rest in flattening when air-dried on collodion film and breaking into fragments when stained with sodium phosphotungstate for electron microscopy. Incubation with an equal volume of 2 % formaldehyde for 30 min. stabilized the particles (Kassanis & Nixon, 1961). This treatment also allowed strain B to form precipitation lines in any medium with any of the antisera tested. All experiments involving this strain were therefore done with formaldehyde-treated material. Precipitation in gel was much less sensitive than precipitation in tubes. The minimum concentration of virus that produced a line with homologous antiserum at dilutions 1/5 to 1/20 was between 0·05 and 0·1 mg./ml., as against 0·004 mg./ml. in the tube precipitation test.

To investigate relationship between strains, a single undiluted antiserum was placed in the central reservoir and the strains at appropriate concentrations were arranged in all possible combinations in the surrounding reservoirs. The antigenic relationships between the strains suggested by this method were the same as that given by the tube precipitation method. Spurs were formed by those strains which had an antigen not shared by the strain in the neighbouring reservoir. The spurs were deflected from the parent line when strains in each serotype were compared, and continued the course of the parent line without deflection when strains from the two serotypes were compared. When strains from the two serotypes were compared in neighbouring reservoirs against the mixed antiserum of the two, the lines crossed apparently without deflection. It is therefore worth noting that the crossing of the lines of two antigens tested against mixed antiserum does not necessarily signify complete lack of relationship.

Similar results were obtained in immunoelectrophoretic tests using the technique described by Hirschfeld (1960). As the strains had different electrophoretic mobilities in agar-gel crosses or spurs were easily formed when a mixture of two strains

was run and tested against antiserum. This method confirmed the findings of the double diffusion method and also checked the purity of the preparations. When virus preparations of strain A were run at 6 mg./ml. and tested against an antiserum to strain D a single line was produced. This test had a sensitivity of about 0·025 mg./ml. All strains, except B, moved towards the negative pole at speeds depending on the buffer used. Under the same conditions strain B was either stationary or moved towards the positive pole. As strain B has greater electrophoretic mobility in liquid medium than the other strains, the immunoelectrophoretic results indicate that the effect of electroosmosis was greater than that of electrophoresis.

Some properties

Crystallization. When concentrated virus solutions were sedimented in the ultracentrifuge the pellets of strains A, F and S sometimes became insoluble in water; suspensions of the pellets showed a strong sheen and consisted of numerous small flat rhombic crystals of various thicknesses (Pl. 1, fig. 6), which usually dissolved overnight at 4°. With some preparations, however, not all the crystals dissolved; these were centrifuged at low speed and dissolved in 0·067 M-phosphate buffer pH 7.

Virus preparations in distilled water left at 4° for several months sometimes crystallized, especially strains A, F and D, but never preparations of strain B. All strains, except B, were easily crystallized by adding saturated ammonium sulphate solution drop-wise until a slight turbidity appeared. The solution was then left at 4° overnight or longer for the crystals to separate. These crystals were also rhombic plates of variable thickness; the small angle made by the sides of the plates was 79°, as recorded by Bawden & Pirie (1942) for a strain found in the roots of a potato plant which was called potato culture (Pl. 1, fig. 7).

Electron microscopy. Kassanis & Nixon (1961) reported that particles of strain A had a diameter about 5 mμ larger than strain B when shadowed or negatively stained with sodium phosphotungstate. We found no significant difference in size between any of the strains, including A and B. The negatively stained particles were hexagonal in outline and their diameter ranged from 25·9 to 26·7 mμ. A second examination of strain A by the same methods gave a diameter of 30 mμ. The condition of the virus or the way the specimens are prepared for electron microscopy may influence the apparent size of the particles. Whatever the real size may be, it seems unlikely that there is any consistent difference in size between strains. Strain B was fixed with an equal volume of 2 % formalin before staining, to preserve the integrity of the particles (Kassanis & Nixon, 1961). There was no evidence that formalin fixing alters the size of the particles; strain S had the same diameter fixed or unfixed.

Ultraviolet light absorption. The u.v. absorption spectrum of all strains was the same, with a maximum at 260–262 mμ and a minimum at 242–244 mμ. The ratio of maximum to minimum differed between preparations from 1·2 to 1·7, the most frequent being 1·3, irrespective of strain used. The ratio of absorbancies at 260 and 280 mμ was 1·5.

The virus concentrations of several purified preparations were found by estimating dry weight or total nitrogen, from which the specific extinction coefficient at 260 mμ was determined. The values were somewhat variable, but no systematic deviation

from the mean was observed for any strain. The average extinction coefficient was 5·5 per mg./ml. Phenol-extracted nucleic acid in 0·067 M-phosphate buffer, pH 7 gave a maximum at 260 mμ and a minimum at 240 mμ. The maximum to minimum ratio ranged from 1·7 to 2·4 and the 260/280 ratio was 1·5.

Sedimentation. Sedimentation constants for several TNV isolates have been reported (Pirie, Smith, Spooner & McClement, 1938; Price & Wyckoff, 1939; Bawden & Pirie, 1942; Kassanis & Nixon, 1961). The values obtained ranged between 112 S and 130 S. Strains D, E and S, never previously examined, were centrifuged at two concentrations in distilled water. The sedimentation constants obtained were between 116 and 120 S.

Interactions with satellite virus

Satellite virus multiplies in the presence of TNV strains A and B, but not in mixed infections with several other viruses (Kassanis & Nixon, 1961; Kassanis, 1962). The concentration of satellite virus in the presence of all strains was compared in tests using French bean leaves as the host. The inoculated leaves were usually detached and placed on a wet towel in trays covered with polythene sheet, as this gave greater virus concentrations, especially with strain D. Five days after inoculation, the sap was extracted and the individual virus content estimated serologically. Satellite virus is not serologically related to either of the two TNV serotypes.

Table 3. *The concentration of tobacco necrosis virus (TNV) and satellite virus in French beans in single and double infections determined by serological precipitation tests*

	TNV alone		TNV + satellite virus	
Strains	Homologous antiserum	Antiserum to satellite virus	Homologous antiserum	Antiserum to satellite virus
A	32*	0	4	16
B	8	0	8	128
S	64	0	8	32
D	16	0	16	0
E	8	0	4	8

* Reciprocals of virus dilution end points in precipitation tests determined by incubating serial two-fold dilutions of heat-clarified sap with the indicated antiserum diluted 1/50. Strains F and C behaved as strain A. The satellite virus did not multiply when inoculated alone.

Only strain D failed to induce the multiplication of satellite virus (Table 3). In this and other experiments, strains B and S were usually more effective than strains A, F and C. All these strains have major antigens in common. Strain E, although not closely related to the other strains, also assisted the multiplication of the satellite virus, but usually less than the others. Satellite virus multiplied at the expense of the assisting viruses, decreasing their lesion-size as well as their concentration. At certain ratios of the concentration of the satellite and assisting viruses the lesion number is also decreased (Kassanis, 1962) (Pl. 1, fig. 2). The ability of these strains to induce the multiplication of satellite virus is not related to their antigenic composition.

DISCUSSION

All the TNV isolates we have studied were serologically related. They fall into two groups, or serotypes, each containing distinguishable strains. Serotype A contains strains A, B, C, F and S; and serotype D strains D and E. No serological differences were detected between A, F and C, but the last could be distinguished because it became systemic in young cucumber plants. Apart from the serological differences, the strains can be identified by the symptoms they cause on French bean and by some other properties, e.g. strain B fails to crystallize, loses its infectivity faster than the others and breaks up when stained with sodium phosphotungstate, unless fixed with formalin. The thermal inactivation rates and rates at which they multiply in the plants are also useful in distinguishing between the different strains (Babos & Kassanis, 1963*a*, *b*).

Our strains do not include all the kinds of tobacco necrosis viruses described by Bawden & Pirie (1942), for we have found only one crystal type and they reported several. The crystals formed by six of our strains resemble those of the potato culture, which they found to be serologically related to some that crystallized in different ways. From an old preparation of the potato culture given to us by Mr N. W. Pirie we obtained a virus that in every way resembles our strain A. The cultures called Princeton, Potato and Tobacco VI by Bawden & Pirie therefore are probably closely related serologically to our strain A. Of the cultures they reported as serologically unrelated to the potato culture, Tobacco I and II no longer exist and we have encountered nothing like them. Hence they may well be quite unrelated to any of our strains. The Rothamsted culture has been maintained continuously since 1942 and from it Kassanis & Nixon (1961) isolated both the strain called B and the satellite virus. Some of the properties Bawden & Pirie (1942) reported for the infective component of the Rothamsted culture suggest that it was strain B, even though they reported it to be serologically unrelated to the potato culture, which we find is serologically related to strain B. One likely reason for the discrepancy is that the purified preparations of the Rothamsted culture Bawden and Pirie used as antigen had been treated in ways that largely inactivated strain B and they possibly contained only the satellite virus which is antigenically distinct from strain A.

The name tobacco necrosis virus is now largely meaningless, for it is used, not only for viruses that cause the disease first given this name, but also for viruses that cause systemic diseases. We propose that it should now be restricted and defined in terms of serological relationships. There is reason to think that the original cultures used by Smith & Bald (1935) and most of those studied by Bawden & Pirie (1942) contained viruses serologically related to those we have studied and we suggest that the name should now be used only for viruses serologically related to them. This means excluding the viruses called Tobacco I and II by Bawden & Pirie (1942), should they be rediscovered. The virus causing cucumber necrosis in Canada (McKeen, 1959), which is not serologically related to our types, would also be excluded but the one that causes cucumber necrosis in Holland would be included in the group.

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EXPLANATION OF PLATE

Fig. 1. French bean leaf inoculated with strain A (right half-leaf) and strain B (left half-leaf), three days after inoculation.

Fig. 2. French bean leaf inoculated with strain E (left half-leaf) and strain E with satellite virus (right half-leaf), 5 days after inoculation. Fewer and smaller lesions were caused by the mixed inoculum although the two halves were inoculated with the same concentration of strain E.

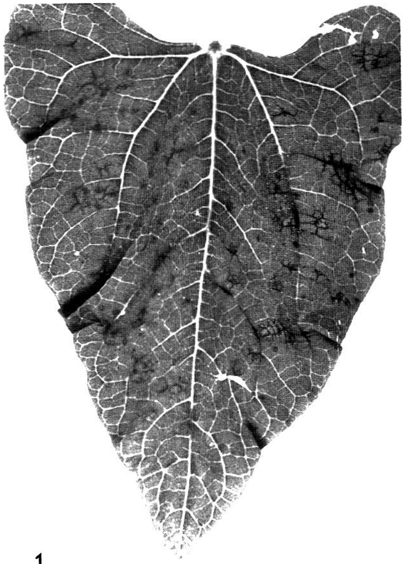
Fig. 3. French bean leaf inoculated with strain D, 2 weeks after inoculation.

Fig. 4. Half French bean leaf showing atypical lesions of strain D, 3 weeks after inoculation.

Fig. 5. French bean plant systemically infected with strain S (stipple-streak), 3 weeks after inoculating the primary leaves.

Fig. 6. A carbon replica of a small crystal of strain A formed during ultracentrifugation. × 36,000 approx.

Fig. 7. Crystals of strain A formed in the presence of ammonium sulphate. × 150.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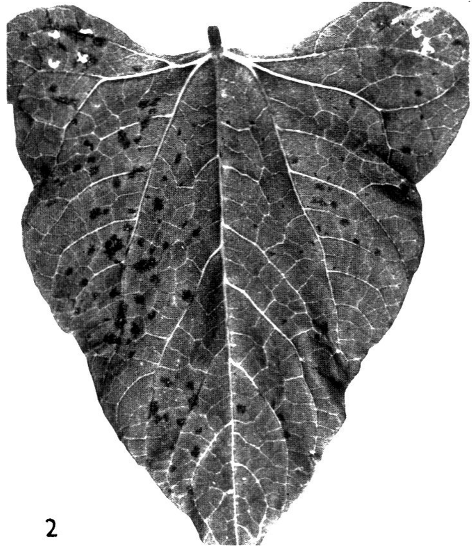
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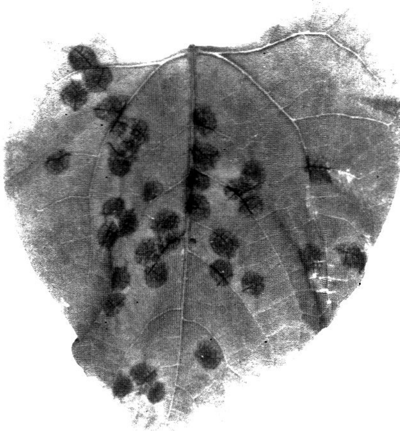
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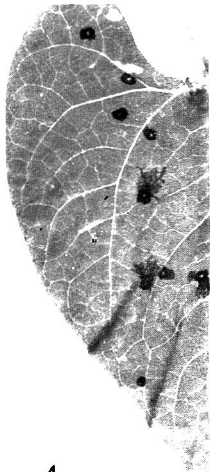
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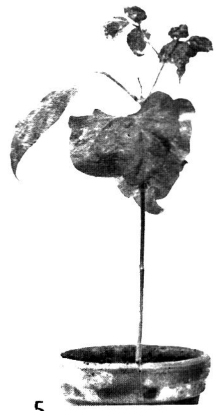
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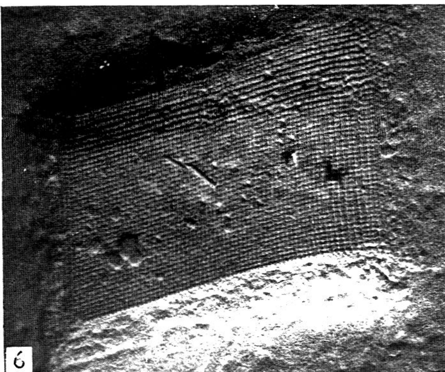
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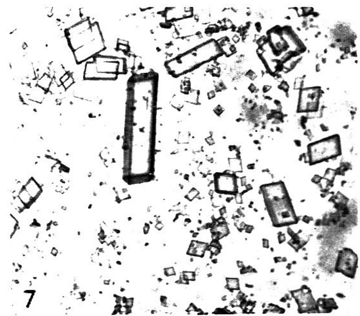
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P. BABOS AND B. KASSANIS

(*Facing p.* 144)

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The Specificity of Trypanosomal Antigens by Immunofluorescence

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SUMMARY

The immunofluorescent method was applied to a strain of *Trypanosoma brucei* and of *Trypanosoma vivax* by using the direct antibody technique. Conjugated antibodies to the soluble antigens of trypanosomes reacted specifically with the homologous species only; antibodies to the bound antigens reacted with both species.

INTRODUCTION

Serological investigations of trypanosomes are considerably limited by the relatively small amounts of antigens available for test. In addition, the heterogeneity of populations of organisms or of antigens derived from them may remain undetected unless the tests are specifically designed to characterize the antigens of individual organisms. The use of the fluorescent labelled antibody technique, therefore, seemed particularly appropriate for the study of the immunological behaviour of trypanosomes, because the precise location of the antigens can be determined on individual organisms as they occur in the blood of infected animals. Fife & Muschel (1959) described a diagnostic test for antibody to *Trypanosoma cruzi* in human sera, treating suspensions of formalin-killed *T. cruzi* obtained from cultures with human sera and subsequently with anti-human fluorescent rabbit antibody. In the present work the antigenic character of trypanosomes was studied by allowing specified fluorescent conjugated antisera to react directly with the trypanosomes.

METHODS

Organisms. Two species of trypanosomes were used: (1) *Trypanosoma brucei* 427, a strain isolated from a cow in East Africa; (2) *T. vivax*, a strain originally isolated from a sheep in West Africa and later adapted to rats and mice (Desowitz & Watson, 1951, 1953). Both strains were stored at −70° in glycerinated suspension in capillaries (Cunningham & Vickerman, 1962) and when required for tests were maintained in mice by rapid passage.

Antisera. Antisera to the exoantigens (anti-EX) and to the bound antigens (anti-TH) of both species of trypanosomes were obtained from white rats after multiple intramuscular injections of the antigens mixed with potash alum solution as adjuvant (Weitz, 1960). Groups of immunized rats were bled from the heart about 5–10 days after the last injection of antigen and the sera from the bleedings of each group of rats were pooled and stored at −15° until required.

Fractionation of antisera. After testing for potency and quality by precipitin

test (Weitz, 1960, 1962*a*), the antisera were fractionated by chromatography on DEAE (diethylaminoethyl)-cellulose. The sera were dialysed against 0·01 M-phosphate buffer (pH 7·5) before fractionation and the eluates concentrated by pressure dialysis to a content of 2·5 % protein and again dialysed. Analysis by starch gel electrophoresis indicated that the fractions used for conjugation contained only the β- and γ-globulin components of rat serum.

Conjugation of globulin fractions. Volumes containing 2·5 % protein ranging from 1–5 ml. were coupled with fluorescent dyes by the method described by Riggs *et al.* (1958) with the modifications detailed by Goldstein, Slizys & Chase (1961). The solutions maintained in an ice bath and constantly stirred with a magnetic stirrer were adjusted to pH 9·0–9·2 with cold M-carbonate bicarbonate solution. Crystalline fluorescein isothiocyanate (FICT)* or tetramethylrhodamine isothiocyanate (RICT)* were added slowly at the rate of 0·025 mg./mg. protein. The coupling was allowed to occur for 1 hr. with FICT and for 4–6 hr. with RICT (see Smith, Carski & Griffin, 1962). At the end of this period the solutions of conjugates were adjusted to pH 7·2–7·6 with N-HCl.

Removal of unconjugated fluorescent material. The conjugates were dialysed in the cold overnight against a solution of 0·01 M-phosphate buffer in saline (pH 7·2) and were then passed through a Sephadex-G25 column, taking only the first dyed effluent fraction. A half volume of acetone-dried rabbit liver as a fine powder was added and shaken mechanically for 18–24 hr. in the cold. The liver powder was then centrifuged down and the clear supernatant fluid removed. Some loss of protein was noted on some occasions; all the conjugates were restored to a final concentration of 2·5 % protein and stored at -15^{c}.

Preparation of smears. Mice or rats, infected for 2–3 days and containing large numbers of trypanosomes in the blood, were stunned and blood obtained from the sectioned axilla and immediately mixed in 4–5 volumes of Alsever solution (dextrose 4·66 g., sodium chloride 1·05 g., sodium citrate 2·0 g., distilled water 200 ml., pH 6·1). Blood smears on microscope slides were prepared as soon as practicable after the mixing in Alsever's solution and were allowed to dry in air. As soon as the slides were dry they were fixed in methanol for 1 min., shaken dry and dried in air on a warm plate.

Staining of blood smears. Comparisons between different treatments of the same preparation were all made on the same slide on which several small areas were separated by painting with nail varnish, a procedure which prevented the different reagents from mixing with each other, and which allowed the microscopic examination of the differently treated areas of one slide without altering the illumination, thus ensuring a correct assessment of the different intensities of staining or fluorescence.

The fluorescent antibody, used neat or diluted in phosphate buffered saline, was applied to the appropriate section of the slide, covering the whole area of the section. After 10 min. at room temperature, the conjugate was removed by suction with a small capillary pipette and the sections washed with phosphate buffered saline, first by carefully squirting each section with a pipette and then by covering the whole slide for 1 min.; the buffer solution was then shaken off and the smears dried

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in air on a warm plate. In this way the possibility of contaminating sections with the different reagents during washing was minimized.

Examination of specimens. A Reichert fluorescence illuminator was used, consisting of an Osram HBO-200 W. mercury vapour lamp, with adjustable condenser and field diaphragm. The smears were examined in a darkened room with dark-ground illumination; a control smear stained with acridine orange was used to adjust the apparatus initially. Various primary filters were used, singly or combined, including BG 12/3 mm., UG 1/1·5 mm. and UG 1/2·5 mm. (Schott & Genossen) and various ocular barrier filter combinations, including a Wratten 2B, pale green-yellow filter and orange-yellow filter OG 1/1·5 mm., all with ultraviolet-stopping glass GG 9/1 mm.

Owing to the very rapid loss of fluorescence of the smears under illumination, photographs were taken with a fast 35 mm. Kodak TRI-X film (ASA 400) giving about 3–4 min. exposures with a BG 12/3 mm. primary filter and UG 1 barrier filter under 1/12 in. oil-immersion objective and $\times 6$ ocular. Colour transparencies were obtained with Kodak high-speed daylight Ektachrome film (ASA 160) by using the same objectives and ocular with a UG 1/2·5 mm. or UG 1/1·5 mm. primary filter and a pale green-yellow barrier filter and allowing 10 min. exposures.

RESULTS AND DISCUSSION

Non-specific fluorescence of trypanosomes. Many preparations of trypanosomes showed non-specific fluorescence of various intensities, and no results were accepted unless control preparations fluoresced either not at all, or only minimally. The controls consisted of portions of the smears left untreated and portions which had been treated with conjugated antisera to rabbit serum protein, which should not react with the preparations derived from mouse or rat. Other controls were treated with normal rat serum or normal rabbit sera similarly conjugated with fluorescent dyes. Unless the control preparations showed no more than a low-intensity fluorescence, which appeared unavoidable under the conditions of the test, the results were rejected.

No single factor proved to be mainly responsible for the production of non-specific fluorescence of trypanosomes. The best results were obtained when the diluted infected blood was smeared without delay and when the smears were fixed immediately after the films were dry. Suspensions of trypanosomes obtained by centrifugation usually yielded non-specifically stained specimens, and thus smears of diluted whole blood were mostly used. Washing the preparations for prolonged periods was found unnecessary and gave poor results. It thus seemed that any step which favoured the proper preservation of the trypanosomes was useful in avoiding non-specific fluorescence.

Specific fluorescence. The results of treating blood smears of *Trypanosoma brucei* and *T. vivax* with anti-EX and anti-TH are illustrated in Pl. 1, figs. 1–8. The specificity of the anti-exoantigen is shown by the well-marked staining of homologous species only (Pl. 1, figs. 1, 2, 5, 6). The anti-TH sera on the other hand stained the smears of both species equally (Pl. 1, figs. 3, 4, 7, 8). These findings were confirmed by making mixed smears of the two species and treating them with antisera conjugated with different dyes. When a mixture of *T. brucei* and *T. vivax* was treated

with 'brucei' anti-EX conjugated with FITC and subsequently with 'vivax' anti-EX conjugated with RITC, the respective organisms were stained green and red, showing that only the homologous species was stained by the homologous antiserum. When the mixed organisms were similarly treated with two differently conjugated anti-TH of different colour, both species of trypanosomes appeared greenish yellow as a result of the double staining with fluorescein and rhodamine. No difference was noted in the localization of the antigens with the different antisera.

These results are consistent with the properties of exoantigen as determined by other methods. Thus, mice immunized with the exoantigen of one species are protected against infection with the homologous species but not with a heterologous species (Weitz, 1962*b*). Similarly, anti-exoantigen sera precipitate only the homologous exoantigen, whereas anti-TH precipitating sera cross-react with the heterologous species (Weitz, 1962*a*). The results confirm the distinctive characters of the soluble antigens found in the sera of infected animals (exoantigen) and those which are derived from the disruption of the trypanosome (bound antigens). The exoantigens determine the small differences in antigenic structure of different species of trypanosome. Moreover, antigenic variation appears to affect the exoantigen more than other antigenic components in the organisms. For immunization of animals against infections with trypanosomes it is evident that the bound antigens are therefore more likely to promote antibodies effective against more species of trypanosomes and possibly their variants than the antibodies provoked solely by exoantigens. Further investigations are being made to confirm these results with a variety of strains and species of trypanosomes.

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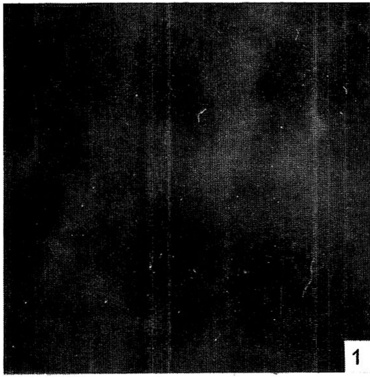

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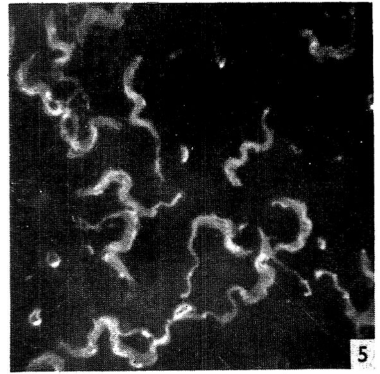
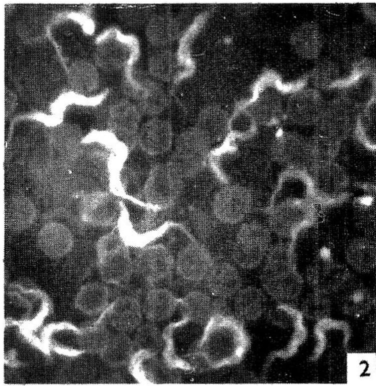


5

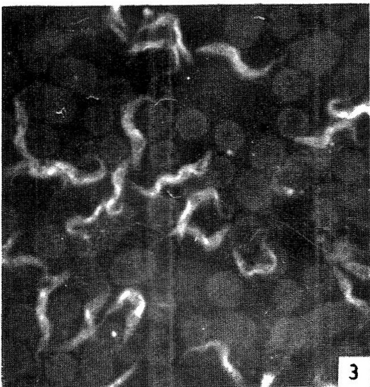
Anti-brucei exoantigen



2

6

Anti-vivax exoantigen



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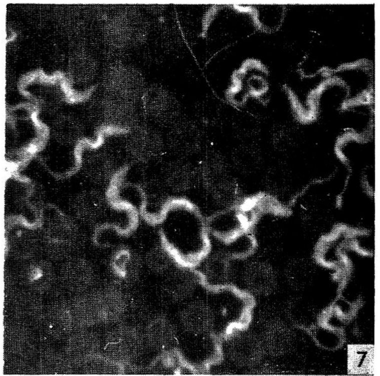

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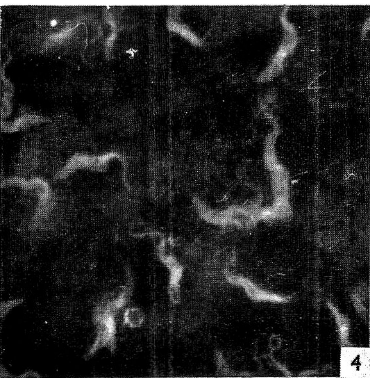
Anti-brucei bound antigen



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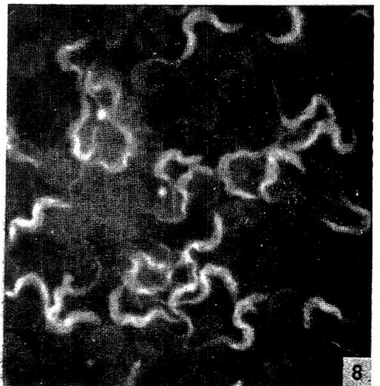

8

Anti-vivax bound antigen

Trypanosoma vivax

Trypanosoma brucei

EXPLANATION OF PLATE

Figs. 1–4 *Trypanosoma vivax*, Figs. 5–8 *Trypanosoma brucei*, treated with fluorescent antibody. All photographs were taken with a 3 min. exposure, developed and printed together under the same conditions. The intensities of fluorescence are comparable for each species of trypanosome since the photographs were made from the same slide without alteration of the illumination, with a BG 12/3 mm. exciter filter and OG 1 barrier filter and examined under 1/12 in. oil immersion objective with a ×6 ocular.

Figs. 1, 5. Treated with fluorescent antibody to *T. brucei* exoantigen (anti-EX).

Figs. 2, 6. Treated with fluorescent antibody to *T. vivax* exoantigen (anti-EX).

Figs. 3, 7. Treated with fluorescent antibody to *T. brucei* bound antigens (anti-TH).

Figs. 4, 8. Treated with fluorescent antibody to *T. vivax* bound antigens (anti-TH).

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its thirty-seventh General Meeting at the Royal Institution, London, on Monday, Tuesday and Wednesday, 8, 9 *and* 10 *April,* 1963. *The following communications were made:*

ORIGINAL PAPERS

Electron Microscopy of Membrane Prepared from *Candida utilis*. By C. GARCIA MENDOZA and J. R. VILLANUEVA (*Instituto 'Jaime Ferran' de Microbiologia, Velazquez* 138, *Madrid*, 6)

Several studies in recent years have helped to elucidate the structure of the cell envelope of yeasts. Conway & Downey ((1950), *Biochem. J.* 47, 347) produced evidence of the existence in intact yeast cells of an 'outer metabolic region', freely permeable to small molecules and without osmotic properties, which they considered to be a true cell wall. Bartholomew & Levin ((1955), *J. gen. Microbiol.* 12, 473) obtained pictures that gave confirmation of the double membrane nature of the yeast cell wall reported by earlier workers, such as Northcote & Horne ((1952), *Biochem. J.* 51, 232), and gave some indication of the presence of additional very thin membranes in the envelope. Robinow & Murray ((1953), *Exp. Cell Res.* 4. 390) demonstrated the cytoplasmic membrane of yeast by a staining method and observation with the light microscope. In their pictures the cytoplasmic membrane adhered to the protoplasm and shrank away from the cell wall on plasmolysis.

In an attempt to discover the construction of the cell, and in particular the structure of the cell membrane, we have used enzymic techniques. By controlled treatment with 'strepzyme' (Garcia Mendoza, C. & Villanueva, J. R. (1962), *Nature, Lond.* 195. 1326) protoplasts have been obtained from which isolation of nuclear, ribosomal and mitochondrial components may be possible. Lysis occurs when a suspension of protoplasts of *Candida utilis* in 0·8 M-mannitol plus 0·1 M-phosphate buffer pH 6·8 is diluted with distilled water. Microscopic observation of the resulting suspension reveals granules of various sizes and empty vesicles. By differential centrifugation a dark yellow mass of membrane-like bodies is precipitated. The present paper will present results of phase contrast and electron microscopic studies of the membranes. Preliminary observations indicate that the membranes are very fragile and readily destroyed by small changes in the environment or very brief exposure to sonic vibration. Electron microscopy has not revealed the presence of any organized structure on their surfaces. Their chemical composition and immunological behaviour is being studied.

Plasma Enzyme Levels in Mice Infected with Various Viruses. By B. W. J. MAHY, C. W. PARR and K. E. K. ROWSON (*Departments of Cancer Research and Biochemistry, The London Hospital Medical College, University of London*)

Transplantable tumours and virus preparations derived from mouse tissues are often contaminated with a virus, first described by Riley, V. *et al.* ((1960), *Science*, 132, 545), that causes no apparent pathological changes in the infected animal, but induces marked elevations in the levels of several plasma enzymes, including lactic dehydrogenase (LDH) (Plagemann, P. G. W., Watanabe, M. & Swim, H. E. (1962), *Proc. Soc. exp. Biol., N.Y.* 111, 749). Our own studies have yielded similar findings. Viruses can be freed from contamination with this enzyme-elevating virus by passage through rats or tissue culture. The Moloney leukaemogenic virus and polyoma virus propagated in mouse embryo tissue

culture have no action on the plasma LDH activity until the appearance of gross neoplastic lesions (Adams, D. H., Rowson, K. E. K. & Salaman, M. H. (1961), *Brit. J. Cancer*, 15, 860). Contrary to this finding it was recently reported that a number of oncogenic and non-oncogenic viruses induce a rapid significant rise in the serum LDH levels of infected mice (Wenner, C. E. *et al.* (1962), *Virology*, 18, 486). In the present study the plasma levels of lactic dehydrogenase (LDH), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), phosphoglucose isomerase (PGI) and alkaline phenyl phosphatase (AlkP) were measured in mice after infection with a number of viruses. Vaccinia, polyoma, influenza A and lymphocytic choriomeningitis (LCM) viruses caused no significant alterations in the plasma enzyme levels up to 10 days after infection, even though the influenza A and LCM viruses killed the inoculated animals within this period. Encephalomyocarditis virus induced a rapid rise in plasma GOT activity and a later rise in LDH and PGI. The rise in GOT was much greater than that occurring in Riley infection, and was associated with demonstrable myocardial damage. The increases in LDH and PGI activity were similar to those seen after Riley infection—in which no pathological lesions are visible.

Viral Infections in Thymectomized Animals. By P. Denys, Jun., R. Leyten, M. Vandeputte and P. De Somer (*Virus Department, Rega Institute, University of Louvain, Belgium*)

The high susceptibility of newborn mice and rats to certain viruses, inoculated within the first 24 hr. after birth, may be due to an incomplete development of the immunological potential at this period. To test this hypothesis, we have inoculated such viruses into animals that had been thymectomized at birth and which, considering the interference with the development of immunity produced by thymectomy, might be expected to retain their susceptibility for a longer period. NMRI mice and inbred Wistar rats, thymectomized within 24 hr. after birth, grow normally for the first 3 weeks. Differences in growth between thymectomized and sham-operated animals set in at the age of 4 weeks. Only suckling rats, 1–8 days old, are susceptible to herpes virus. Such animals die on the 6th day after subcutaneous inoculation. Similarly, young mice are much more susceptible to herpes virus than adult mice.

Rats that have been thymectomized at birth and inoculated with herpes virus on the 14th day do not die but fail to gain weight normally; and the difference between their weight and that of controls gradually increases. Five weeks after infection the mean weight of such rats was 55 g. At the same age all four control groups (infected and sham-operated, infected normal, uninfected thymectomized and uninfected normal), had a mean weight of 85 g. The animals were killed at the age of 7 weeks to measure the serum antibodies against herpes. The average titre was 1/30 in thymectomized rats and 1/480 in sham-operated and normal animals.

Thymectomy also increased the susceptibility of mice to herpes virus. When inoculated at the age of 1 month, 72 % of the thymectomized died within 11 days after infection, whereas death occurred in only 33 % of the controls. Mice inoculated with Coxsackie B_5 virus within 24 hr. after birth die on the 4th–5th day. Mice infected more than 3 days after birth recover from this infection. In contrast, mice that had been thymectomized at birth and inoculated with Coxsackie B_5 virus at 3 days had a 30 % mortality. The growth of survivors was markedly retarded; at the age of 3 weeks their mean weight was 4·5 g. whereas that of the control groups was 9·5 g. The animals of this experiment were killed at the age of 3 weeks and histopathological examinations were carried out.

Hitherto, attempts to produce disease with APC_{16} virus in common laboratory animals have failed. However, thymectomized mice inoculated with APC virus grew more slowly than the controls, and over 50 % died between the 5th and 6th week. Uninoculated thymectomized animals are still alive after 8 weeks.

As indicated by preliminary experiments, rats thymectomized at birth and inoculated when 2–3 weeks old with polyoma virus are still capable of developing polyoma tumours.

In a group of 28 rats, thymectomized at birth and inoculated subcutaneously 2–3 weeks

afterwards (10^8 $TCID_{50}$ virus). 7 up to now developed a polyoma tumour, while none in the sham-operated control group. All the tumours found till now were large cerebral hemangiomas. This tumour type and its particular localization are exceptionally encountered in our strain of rats, inoculated at birth with the polyoma virus. No other tumour localization was found till now. Five of the tumour-bearing rats had positive haemagglutination-inhibition antibody titres (1/512, 1/256), 2 had negative titres (1/32). As previously described, polyoma tumours in rats contain a new cellular antigen foreign to the rat tissues and therefore could be considered in a certain way, as a tissue homologous for the host own tissues. This fact could explain why rats, inoculated after the neonatal period with the virus, do not develop polyoma tumours, being immunologically competent to reject these foreign tumour antigens. However, rats thymectomized at birth will accept these foreign cellular antigen as they accept homografts and therefore may develop polyoma tumours when inoculated after the neonatal period.

Nucleotide Composition of RNA-Viruses. By C. Cocito, P. Laduron and P. De Somer (*Virus Department, Rega Institute, University of Louvain, Belgium*)

A micro method for electrophoretic analysis of nucleotides (Cocito & Laduron, in the Press) has been used to study the composition of RNA from animal viruses. Stocks of [^{32}P]-labelled myxoviruses were obtained by injecting [^{32}P]orthophosphate into the allantoic cavity of 10-day-old embryonated eggs which were infected with the virus 24 hr. later. From the allantoic fluid, collected after 2 days, the virus was purified by repeated cycles of adsorption at 0° on 2·5 % (v/v) chick erythrocyte suspensions followed by elution for 2½ hr. at 37° with 0·15 M-NaCl buffered at pH 7·4 with 0·05 M-Na phosphate. Viral nucleic acids were extracted with water-saturated phenol at 3°, precipitated with ethanol at −15°, dialysed at 3°, hydrolysed for 18 hr. at 37° with 0·3 M-KOH, neutralized with $HClO_4$ at 0 °, desalted and concentrated *in vacuo*. The resulting [^{32}P]-2′3′ribonucleotides were placed on strips of cellulose acetate, with suitable amounts of unlabelled nucleotides as markers for ultraviolet-absorption. Electrophoresis was carried out for 3 hr. at 300 volts, with a 0·01 M pH 3·15 Na citrate/citric acid buffer. The disposition of the ribonucleotides was the following: CMP, AMP, GMP, UMP, with a migration of the slowest CMP component of 2 cm./hr. Spots were eluted with 0·01 M-KOH at 37°, and radioactivity measured with a gas-flow counter. Nucleotide composition of RNA from two myxoviruses purified in this way was as follows: Newcastle disease virus, CMP = 24·6, AMP = 19·4, GMP = 24·1, UMP = 31·9; influenza virus, strain PR 8, CMP = 22·8, AMP = 26·1, GMP = 21·5, UMP = 29·6.

Another procedure used to purify viruses produced in *in vitro* cultures will be discussed. For example, the nucleotide composition of Coxsackie group B, grown in monolayers of HeLa cells, was CMP = 23·2, AMP = 24·5, GMP = 23·1, UMP = 29·2. The composition of the RNA from other myxo- arbor-, entero- and tumour-viruses grown either *in vitro* or *in vivo*, will be presented.

Induction and Repression of an Enzyme in *Pseudomonas aeruginosa*. By W. J. Brammar and P. H. Clarke (*Department of Biochemistry, University College London*)

A strain of *Pseudomonas aeruginosa* has been shown to produce an aliphatic acylamide amidohydrolase (3:5:1:4). This enzyme is induced by growing the organism in media containing the substrate amides (acetamide and propionamide) or one of a number of other amides which are inducers but not substrates (Kelly & Clarke (1962), *J. gen. Microbiol.* 27, 305).

The enzyme also acts as an acyl transferase, catalysing the transfer of the acyl radical of the amides to hydroxylamine to form the corresponding hydroxamic acids. Coordinate induction of transferase activity (acetamide as substrate), and hydrolytic activity (propionamide as substrate) was shown for a wide range of enzyme activities using both substrate and non-substrate inducers. There was no separation of these activities by purification procedures using $(NH_4)_2SO_4$ precipitation and column chromatography on DEAE

cellulose. Kelly & Kornberg ((1962) *Biochim. biophys. Acta*, **64**, 190) showed that the ratio of propionamide transferase to propionamide hydrolase activity was constant over a 35-fold purification.

Measurements of transferase activity, with acetamide as substrate, were used to follow enzyme synthesis by logarithmically growing cultures in succinate medium under conditions of gratuity. Acetamide, *N*-methyl acetamide and *N*-acetyl acetamide were effective inducers under these conditions. After a lag of about one generation, enzyme synthesis proceeded at a constant differential rate for some time. Enzyme induction by *N*-acetylacetamide was repressed by acetate (the product of enzyme action), by certain other metabolites, and by the substituted amides cyanoacetamide and thioacetamide, neither of which was a substrate or inhibitor of the enzyme. Enzyme induction by acetamide under these conditions was repressed by the same compounds, but at different concentrations.

The kinetics of enzyme induction in acetamide medium, when the enzyme is necessary for the initiation of growth, are more complex.

Cuticle Decomposition by Micro-organisms in the Phyllosphere. By JAKOBA RUINEN (*Laboratory for Microbiology, Wageningen, Netherlands*)

Yeasts and other micro-organisms are common inhabitants of the phyllosphere (Di Menna, M. E. (1959*a*), *N.Z. J. Agric. Res.* **2**, 394; (1959*b*), *J. gen. Microbiol.* **20**, 13; Ruinen, J. (1961), *Plant and Soil*, **15**, 81). The yeasts belong to a small number of species that are not the same as those of the soil yeasts occurring in the same locality. The phyllosphere yeasts in fact cannot survive in the soil (Di Menna, M. E. (1962), *J. gen. Microbiol.* **27**, 249).

In a survey of the phyllosphere of tropical vegetation, 200 yeast isolates have been obtained. All of these leaf yeasts are lipolytic and produce extracellular lipids (Deinema, M. H. (1961), *Meded. LandbHoogesch., Wageningen* **61**, 1; Ruinen, in the Press). Several of the isolates, when tested in the laboratory on cuticles of Aloe species and iris, have produced microscopic evidence of cutin decomposition within 24 hr. The presence of cutin-decomposing enzymes in other fungi has been demonstrated by Linskens, H. F. & Heinen, W. ((1961), *Naturwissenschaften*, **47**, 18; Heinen, W. (1960), *Acta bot. néerl.* **9**, 167; (1961), *Acta bot. néerl.* **10**, 171). It may be significant that the same activity is found in strains of Azotobacter, Beijerinckia and other bacteria from leaf surfaces.

In the natural habitat, where most of the nutrients must diffuse through the outer wall of the epidermis, it seems likely that the lipolytic activity of the micro-organisms facilitates the supply of nutrients necessary for the maintenance of a dense phyllosphere population.

The Fine Structure of the Cell Boundary and Photosynthetic Lamellae of *Anacystis montana* (Lightfoot) f. minor (Wille). Drouet and Daily. By P. ECHLIN (*Botany School, University of Cambridge*)

The cell boundary of blue-green algae has previously been described by Ris & Singh ((1961), *J. biophys. biochem. Cytol.* **9**, 63) and is composed of at least three layers, an outer membrane, an inner investment and a bipartite inner, or plasma membrane. Recent studies have shown that the outermost electron-dense layer is highly convoluted into a series of blebs or protrusions. The electron-lucid inner investment follows the contours of the protrusions while the bipartite plasma membrane remains unaltered. The significance of these protrusions in the outer membrane is not clearly understood, but it is thought that they may be connected either with the synthesis or attachment of the narrow mucilagenous sheath.

Studies have revealed the presence of a complex series of fine lamellae, approximately 150 Å thick, which appear to be concerned with the cellular photosynthetic mechanism. Earlier workers had found that the lamellae were confined to a rather narrow peripheral band in the cell. This present study indicates that the lamellae may in some instances fill a large part of the cell. The lamellae and associated granules and vesicles generally appear in one of three different forms: (*a*) tripartite, or in some instances, quadri-partite

structures, in which the lamellae are composed of two membranes and are not associated with a large number of granules; (*b*) tri-partite lamellae in which the interlamellar space is filled with large electron-lucid granules—possibly the products of photosynthesis, and (*c*) discontinuous lamellae that appear to be composed of a complex series of vesicles and granules. Work is in progress to elucidate the relationship of these ultra-structural changes to the physiologic state of the organism.

Influence of Root Secretions on the Symbiotic Association of Rhizobium and Leguminous Plants. By T. A. Lie (*Laboratory for Microbiology, Wageningen, Netherlands*)

The formation of root nodules in bean plants (*Phaseolus vulgaris* L.), grown in nutrient solution, is very poor under hot summer conditions. Satisfactory nodulation has been found, however, in the presence of root secretions of nodulated leguminous plants or an extract of root nodules.

The active substance is soluble in water and ethylether, but insoluble in chloroform. It is inactivated above 80°. No nitrate or aminonitrogen can be detected in the purified extract, but the presence of a pentose is likely. The ultraviolet spectrum of the extract exhibits a strong absorption at approximately 250–270 mμ. Evidence has been provided concerning a relationship between biological activity and absorption at about 260 mμ.

These results are in accordance with the 'preplanting' effect reported by Thornton, H. G. ((1929), *Proc. roy. Soc.* B, **104**, 408), and Nutman, P. S. (1953, 1957), *Ann. Bot., Lond.* **17**, 95; **21**, 322). Although the presence of nitrate in the tap water used by these authors invalidates to a certain extent the influence of 'preplanting' (Gibson, A. H. & Nutman, P. S. (1960), *Ann. Bot., Lond.* **24**, 420) the experiments with excised root nodules (Nutman, P. S. (1952), *Ann. Bot., Lond.* **16**, 80), still suggest a regulation of the existent root nodules on later infections. This is further supported by the observation that the infected root hairs are located in well separated zones originating from the primary infections (Nutman, P. S. (1962), *Proc. roy. Soc.* B, **156**, 122).

Some Aspects of the Leaf-Nodule Symbiosis of *Psychotria*. By Ysolina M. Centifanto and W. S. Silver (*Department of Bacteriology, University of Florida, Gainsville, Florida, U.S.A.*)

This investigation concerns the obligate, cyclic, leaf-nodule symbiosis in the tropical plant, *Psychotria bacteriophila*. In this symbiotic association plants naturally or experimentally freed of the bacterial endophyte are dwarfs. Although this system has been previously studied (von Faber, F. C. (1912), *Jb. wiss. Bot.* **51**, 285; (1915), *Jb. wiss. Bot.* **54**, 243), a definitive description of the organism and a biochemical basis of the symbiosis have not been made. We have repeatedly isolated the endophyte from germinating seeds on N-free media. When the leaves of bacteria-free plants were abraded with a carborundum-culture mixture nodulation was induced. The isolated endophyte was a motile, Gram-negative rod with a refractile granule at each end. On nitrogen-free media, colonies were small, smooth, colourless and quite mucoid. The pH tolerance for growth was wide (pH 5–9) and growth occurred from 15 to 40° but not at 50°. That the isolant fixes nitrogen was shown in several ways: (*a*) Extensive growth was obtained on N-free media within 4 days. but growth yield on peptone media was more rapid. Reduced O_2 tension increased the growth rate on N-free media. (*b*) Washed cells incorporated N_2^{15} from an N^{15}-enriched atmosphere. (*c*) The culture has been successfully carried for some 30 transfers on N-free agar. Atmospheric nitrogen was also fixed by nodulated plants for such plants have grown and shown no symptoms of N-deficiency when supplied with N-free nutrient for over 4 months. N_2 fixation is not the organism's sole function as a symbiont for dwarfism in nodule-free plants cannot be reversed by the addition of fixed nitrogen. Since dwarfs characteristically do not elongate at the stem internodes it would appear that a plant hormone is involved in the symbiosis. The nature of this substance has not yet been elucidated.

Symbiosis of Hydra with Algae. By L. MUSCATINE* and H. M. LENHOFF (*Howard Hughes Medical Institute, Miami, 36, Florida, U.S.A.*)

Green and albino (= algae-free) *Chlorohydra viridissima* were grown and maintained in the laboratory in an artificial medium. With daily feeding, maximum logarithmic growth (k = 0·54) occurred in 10^{-3}M-$CaCl_2$, 10^{-3}M-$NaHCO_3$, 10^{-4}M-KCl, 10^{-4}M-$MgCl_2$, 10^{-3}M Tris (hydroxymethyl) aminomethane buffer, pH 7·6, in de-ionized water. Calcium, sodium, and magnesium ions were required for growth of both green and albino individuals; potassium, though not required, enhanced growth.

When fed daily on *Artemia* nauplii, green and albino hydra grew at nearly identical logarithmic rates. But with limited food, growth of the green hydra always exceeded that of the albinos. On starvation, the green hydra survived for 2–3 weeks slowly diminishing in size, whereas the albinos disintegrated within 6–10 days. Only 15–20 % of the normal complement of algae were shown to be needed to prolong the survival of the hydra. These and radioisotope studies show that the algae were not essential to the host for removal of respiratory or excretory wastes, but rather for processes associated with efficient utilization of food substrates.

Both green and albino were able to retain 85–95 % of the ^{35}S-labelled food administered. However, after 5 days of starvation, green hydra contained a larger proportion of the ^{35}S in proteins, while albinos contained more of the ^{35}S in amino acids and small peptides. Furthermore, starving albinos lost ^{35}S-labelled material to the external medium at twice the rate of green individuals, indicating a higher turnover rate and shorter biological half-life of labelled tissues in *C. viridissima* devoid of algae. This loss of material could be diminished by grafting green tissues to albinos.

Green hydra incorporated $^{14}CO_2$ rapidly via algal photosynthesis. 10–12 % of the total carbon taken up was transferred to the animal tissues in 48 hr. About half of the transferred carbon was incorporated into host protein, while the remainder was found in small molecules.

It is hypothesized that augmentation of the host is mediated at the cellular level by trace amounts of substances produced by the algae.

A Bacterial Endosymbiote in an Osmotrophic Flagellate? By B. A. NEWTON† (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The fact that protozoa harbour organisms of other species has long been recognized and there is an extensive literature concerned with 'protozoon-bacterium complexes' (reviewed by Kirby, J. jr. (1941), *Protozoa in Biological Research*, p. 1009; edited by G. N. Calkins & F. M. Summers, Columbia University Press, N.Y.). However, as pointed out by Sonneborn, T. M. ((1959), *Advanc. Virus Res.* **6**, 229), it is difficult to establish criteria to distinguish between cytoplasmic genetic elements or particles of endogenous origin and symbiotic intracellular organisms or particles of exogenous origin: consequently, an unequivocal identification of intracellular structures in protozoa as bacteria has been achieved for relatively few cases.

This communication will describe a basophilic, rod-shaped structure (a 'bipolar-body') which occurs in the trypanosomid flagellate *Crithidia (Strigomonas) oncopelti*. Organisms contain one or two of these structures. Chemical analysis of isolated bipolar-bodies (Newton, B. A. & Horne, R. W. (1957), *Exp. Cell Res.* **13**, 563), showed that they are composed largely of ribonucleoprotein; no polysaccharide or metaphosphate was detected and cytochemical techniques failed to establish the presence of deoxyribonucleic acid. Isolated bipolar-bodies are sensitive to osmotic shock and electron-microscope studies revealed the presence of a thin boundary membrane; some rods were observed to have constrictions which strongly suggest a division process.

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C. oncopelti is atypical of the zoomastigophora in that it grows readily in a simple, chemically defined medium containing methionine as the sole amino acid (Newton, B. A. (1956), *Nature, Lond.* **177**, 279). Recently Gill, J. W. & Vogel, H. J. ((1962), *Biochim. biophys. Acta*, **56**, 200) reported that this flagellate synthesizes lysine from aspartate via α,ϵ-diaminopimelic acid (a pathway thought to be typical of bacteria and blue-green algae but absent in euglenids and higher fungi (Vogel, H. J. (1959), *Fed. Proc.* **18**, 345). The terminal enzyme in this pathway, diaminopimelic decarboxylase, was found to be localized in a cell fraction containing a high proportion of bipolar-bodies. These findings led the authors to state that '...the relative nutritional self-sufficiency attributed to this protozoon may only be apparent' and they conclude that the bipolar-bodies are bacteria-like organisms which furnish lysine and probably other metabolites to the protozoon, which in turn provides osmotic protection and perhaps other advantages for the endosymbiote. As further evidence in support of this conclusion they report (Gill, J. W. & Vogel, H. J. (1963), *J. Protozool.* (in the Press)), that the flagellate can be 'cured' of the endosymbiote by growth in the presence of penicillin. Results of experiments which bear on the truth of these conclusions will be presented.

SYMPOSIUM ON 'SYMBIOTIC ASSOCIATIONS'

This Symposium was held on Tuesday and Wednesday, 9 *and* 10 *April. The principal contributions have been published by the Cambridge University Press as the* 13*th Symposium of the Society. Their titles are as follows:*

Integrative and Disintegrative Factors in Symbiotic Associations. By R. Dubos and A. Kessler (*The Rockefeller Institute, New York*)

Bacteriophage Lysogeny. By W. Arber (*Laboratoire de Biophysique, Université de Genève, Switzerland*)

Experimental Studies of Lichen Physiology. By D. C. Smith (*University Department of Agriculture, Oxford*)

Factors Influencing the Balance of Mutual Advantage in Legume Symbiosis. By P. S. Nutman (*Soil Microbiology Department, Rothamsted Experimental Station, Harpenden*)

The Root Nodules of Non-Leguminous Angiosperms. By G. Bond (*Department of Botany, University of Glasgow*)

The Biochemistry of Nitrogen Fixation. By D. J. D. Nicholas (*Research Station, Long Ashton, Bristol*)

Some Effects of Forest Tree Roots on Mycorrhizal Basidiomycetes. By J. B. E. Melin (*Institute of Physiological Botany, Uppsala, Sweden*)

Defence Reactions in Orchid Bulbs. By J. Nüesch (*Ciba Ltd., Basle*)

Vesicular-Arbuscular Mycorrhiza: an Extreme Form of Fungal Adaptation. By B. Mosse (*Soil Microbiology Department, Rothamsted Experimental Station, Harpenden*)

Algae and Invertebrates in Symbiosis. By M. R. Droop (*The Marine Station, Millport, Scotland*)

Symbiosis and Aposymbiosis in Arthropods. By M. A. Brooks (*Department of Entomology and Economic Zoology, University of Minnesota, St Paul, Minnesota*)

Ambrosia Beetles and their Fungi, with Particular Reference to *Platypus cylindrus* Fab. By J. M. BAKER (*Forest Products Research Laboratory, Princes Risborough*)

Symbiotic Associations: The Rumen Bacteria. By R. E. HUNGATE (*University of California, Davis, California*)

The Growth and Metabolism of Rumen Ciliate Protozoa. By G. S. COLEMAN (*Biochemistry Department, Institute of Animal Physiology, Babraham, Cambridge*)

Studies on Bacterial Associations in Germ-Free Animals and Animals with Defined Floras. By M. LEV (*National Institute for Research in Dairying, Shinfield*)