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THE JOURNAL OF GENERAL MICROBIOLOGY

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

THE PREPARATION OF PAPERS

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

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

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

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

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

A paper should be submitted in double-spaced typing (top copy) with a 1½ in. left-hand margin, and on paper suitable for ink corrections. The paper should be written

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.

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

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

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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.

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

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

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

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

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

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

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

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

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

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

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

BACTERIA. Author's 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.

JULES BORDET

(*Facing p.* 1)

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Jules Bordet 1870–1961

With the death of Jules Bordet at Brussels on 6 April 1961 there disappeared the last of the Pasteurians of the great epoch. Jules Bordet was born at Soignies, a small town about 40 km. from Brussels, on 13 June 1870. The Bordet family, originally from Rolampont on the plateau of Langres (France) settled on present-day Belgian territory when an ancestor came to live at Liège at the end of the eighteenth century; there the father of Jules Bordet was born in 1834. Appointed a school teacher at Soignies the father married and had two sons, Charles (b. 1868) and Jules (b. 1870). When the father was appointed in 1874 to the École Moyenne of Schaerbeek, a district of Brussels, the family moved to the capital.

Jules Bordet had his secondary education at the Athénée Royal of Brussels. He showed an interest in chemistry and worked with great enthusiasm in a little laboratory which he constructed in his home. At 16 he entered the University of Brussels where he obtained, in 6 years instead of the usual 7, his Diploma of Doctor of Medicine, in 1892. The same year he published in the *Annales de l'Institut Pasteur* a memoir entitled 'Adaptation des virus aux organismes vaccinés' (Bordet, 1892). In this he established that serial passage of *Vibrio metchnikovi* through a vaccinated animal led to the selection of specially virulent microbes, little susceptible to phagocytosis. This memoir was the result of a lively interest in experimental research which had led the young student, during his medical studies, to frequent the laboratory of Leo Errera of the Botanical Institute of the University. There he experimented on the chemotactic sensitivity of the gametes of certain algae. The work of Massart and of his brother Charles Bordet on the leucocytic chemotaxis (Massart & Bordet, 1890) undoubtedly did much to direct Jules Bordet towards microbiology and the work which was the subject of the 1892 paper. This led to his obtaining a travel bursary from the Belgian Government, with which he was able, after serving as a doctor at the hospital at Middelkerke, to go to the Institut Pasteur in Paris. He was in Paris from 1894 until 1901. Bordet entered Metchnikoff's laboratory, where great scientific activity by a group of brilliant workers was in progress. He became friendly in particular with Cantacuzène, Auguste Marie, Salimbeni and Besredka. His strong personality, his activity, his infectious gaiety and ardour for work imposed themselves on this society of enthusiastic young men moved by the desire for knowledge. The discovery in 1895 of the roles of alexine and antibody in the Pfeiffer phenomenon made him a star. There then followed a series of papers which in a few years laid the foundations for the understanding of humoral immunity and serological specificity.

In 1897 Bordet's residence in Paris was interrupted by a mission to the Transvaal to study rinderpest, which he undertook on behalf of the Institut Pasteur; there he met a German mission led by Robert Koch. Bordet devised a method for the control of rinderpest which consisted in injecting healthy animals with a little convalescent serum from other animals and then putting the injected animals in contact with infected animals in order to confer immunity by a mild attack of the disease.

When he returned to Paris Bordet again began his research work and new dis-

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coveries were made in rapid succession: haemolytic sera (1898) and precipitating sera (1899), which helped to establish on immunological grounds the reality of zoological species; complement fixation (done for the first time on 16 March 1900 as shown by his experimental notebook); and in 1901 with his brother-in-law O. Gengou the application of this reaction to the demonstration of 'sensibilisatrices spécifiques' in various infections.

Bordet's private life had meanwhile undergone a profound and happy change in 1899 when he married the wife whose love, intelligence and goodness never ceased to sustain him until he died, thus bringing to an end an admirable union of two people who had shared an exceptionally happy life. In 1900 a daughter was born; later followed a second daughter and then a son.

In 1901 Bordet returned to Brussels. In the preceding year the province of Brabant, in which Brussels is situated, had decided to create an anti-rabies and bacteriological institute and offered its directorship to Bordet who, in spite of pressing invitations from the Institut Pasteur in Paris, decided to return to his own country to start the new institute. This was provisionally installed in March 1901 in the buildings of the Institut de Physiologie Solvay de l'Université Libre in Brussels. The creation, organization and management of the new laboratory demanded much labour from Bordet. It was a flattering distinction and a valuable encouragement when, in 1903, Madame Pasteur gave permission for the Institut du Brabant to take the name of Institut Pasteur de Bruxelles, which it jealously preserves. In 1905 the Institut moved to its present building, which had been built for it and which it still occupies. Henceforth the burden of administration and of teaching took more and more of Bordet's energy, though it did not hinder his research work. Papers continued to appear: studies of the antigen–antibody reaction; the discovery of the whooping cough bacillus (Bordet & Gengou, 1906 *a*, *b*) a study of blood coagulation; the discovery and cultivation of the organisms of avian diphtheria (1907); a description of the agent of bovine pleuropneumonia; the discovery of conglutination (Bordet & Streng, 1909) and of co-agglutination (Bordet & Gengou, 1911); researches on anaphylatoxins (1913*b*).

In 1907 the Université Libre of Brussels appointed Bordet to the Chair of Bacteriology in the Faculty of Medicine. Until 1935 he taught and lectured, giving an abundance of experimental facts, expounded with precision and simplicity in a logical and methodical sequence of extreme clarity. His lectures were followed avidly by the medical students, won by the infectious enthusiasm of this exceptionally gifted teacher.

The First World War made scientific research difficult in an occupied country. During these sombre years Bordet undertook the editing of his *Traité de l'immunité dans les maladies infectieuses* which appeared in 1919. Bordet, isolated by the hostilities, received great moral support when in 1916 the Royal Society elected him a foreign member. With the return of peace international scientific activity began again, but new tasks claimed Bordet. Belgium had suffered greatly during the war; the Université Libre of Brussels in 1920 sent to the U.S.A. a delegation to seek the aid of the Rockefeller Foundation to build new institutes for the Faculty of Medicine, and Bordet was nominated to this mission. During this trip he learnt that the Nobel prize for 1919 had been awarded to him for his work on immunity. When he returned to Belgium he was welcomed by the Royal Academy of Medicine

at a special meeting to which many of his foreign colleagues came. In response to the speeches about himself Bordet made the following charming comment: 'It is true that Nature from time to time sheds a little of her mystery; sometimes one succeeds in picking up some of the petals which she strews in the wind'. The Nobel Prize was the culmination of many honours conferred on Bordet; his international reputation was further enhanced. In 1922 he spoke at Strasbourg, in the presence of the President of the Republic of France, for foreign scientists at the commemoration of the centenary of the birth of Pasteur. In 1930 he was President of the First International Congress of Microbiology, held in Paris, and in 1946 in Paris he gave, in the name of his foreign colleagues, an admirable discourse at the meeting in the Sorbonne to commemorate the 50th anniversary of the death of Pasteur. In 1933 after the nearly simultaneous deaths of Roux and Calmette, Bordet was nominated President of the Scientific Council of the Institut Pasteur in Paris. There each year he lectured on immunology, drawing conclusions from new discoveries with a flair for the essential and a capacity for interpretation which made the lectures a periodical review of progress in immunology.

The accumulation of more honours and duties which came to him in the scientific and medical life of his country did not prevent Bordet from continuing to devote himself to his true mission and dearest occupation, experimental research. Between the two World Wars most of his activity was concerned with bacteriophage, then a new discovery. With various colleagues he established the antigenic power of phage and the hereditary character of lysogeny; he noted the role of calcium and microbial variation in susceptibility to bacteriophage.

In 1940, at the age of 70, Bordet gave up the directorship of the Institut Pasteur of Brussels but, in spite of the difficulties of a second enemy occupation of Belgium, he carried on work with his son, who succeeded him as Director of the Institut. Unfortunately Bordet's sight now began to fail, putting an end to his laboratory work. He maintained, however, a very active membership of numerous committees. In 1950 there was a celebration in the great hall of the Université Libre of Brussels on the occasion of his 80th birthday; numerous public authorities, foreign scientists (whose spokesman was Sir Alexander Fleming), Academies, Universities, friends and pupils, united to pay him a moving tribute. His speech of thanks constituted a fervent profession of faith in the value and future of science. In retirement he lived with his wife in an appartment near the University and maintained his interest in everything happening in the world, especially in the world of science. His greatest pleasure was the frequent visits he made to the Institut, to which he remained attached by the strongest bonds. His health, which had always been exceptionally robust, only failed in the last months of his life; he died peacefully on 6 April 1961.

The scientific work of Jules Bordet

The scientific work of Jules Bordet is remarkable both in extent and depth. One is astonished by the number of his fundamental discoveries in immunology and by the contributions of the first order that he made in bacteriology, about blood coagulation and in the study of bacteriophage. On his arrival in Metchnikoff's laboratory an immunological problem first attracted Bordet. Pfeiffer and Issaeff had just noted (Pfeiffer, 1894; Pfeiffer & Issaeff, 1894) that cholera vibrios, when

injected into the peritoneum of a guinea pig immunized against this microbe, changed into spherical non-motile granules which soon died. This bacteriolytic effect did not occur in an untreated guinea pig, however, but did occur in the peritoneum of untreated guinea pigs receiving cholera antiserum along with the vibrios. Since antiserum had no bacteriolytic activity *in vitro*, Pfeiffer imagined that the endothelial cells of the peritoneum were necessary for the bacteriolysis. On the other hand, Metchnikoff, observing the granular transformation of vibrios *in vitro*, in peritoneal exudate of an immunized guinea pig, attributed the bacteriolytic power to leucocytes present in the exudate (Metchnikoff, 1895). Bordet, however, noticed that the cell-free antiserum was bacteriolytic *in vitro* provided it was fresh (Bordet, 1895). After heating to 55° or storage the antiserum lost its bacteriolytic power, though it still contained agglutinins for the vibrio. Bordet then discovered that a little fresh serum from a non-immunized animal would restore bacteriolytic power to a heated or aged antiserum. He drew the conclusion, 'the only one however which it was possible to deduce' he said later, that the bacteriolysis was due to the combined action of the two substances: heat-resistant antibody found only in the serum of the immunized animal, and heat-labile substance present in the sera of both immunized and non-immunized animals. He realized that the serum of animals immunized against *Vibrio cholerae* agglutinated and lysed *V. cholerae* only, just as sera of animals immunized with *Vibrio metchnikovi* agglutinated and lysed only *V. metchnikovi*; the antibodies were specific. In contrast, fresh serum from a non-immunized animal restored to heated antisera lost bacteriolytic power. Moreover, it was not necessary even that the heated antiserum and the fresh normal serum should come from the same animal species. The substance in the serum which collaborated with the specific antibody was therefore unique. Bordet recognized that this substance was like the alexine of Buchner (1889*a–c*), that was a principle responsible for more or less marked bactericidal or haemolytic activity discovered in normal sera by that author.

Normal sera, however, can agglutinate certain micro-organisms and the red blood cells of other species of animal. Since injection of microbes considerably enhanced the agglutinating power of the serum for the microbe, Bordet asked himself whether the injection of rabbit red cells into a guinea pig might not similarly enhance the power of the guinea pig serum to agglutinate these foreign red cells. The results of the experiments surpassed his expectations: not only did the serum of the immunized guinea-pig agglutinate rabbit red cells but in a few minutes lysed them completely (Bordet, 1898). The haemolytic serum thus discovered was found to act exactly like the bacteriolytic serum. The haemolytic power disappeared on heating to 55° and could be restored by adding alexine. The combined action of non-specific alexine and specific antibodies was thus revealed as a mechanism involved in the reaction of the living animal against the introduction into its body of innocuous foreign material, such as the red cells of another animal species. The elaboration of specific antibodies against microbes was only one aspect of the same general capacity of the living organism to defend itself against penetration by foreign elements.

In 1895 Bordet had established the first example of an *in vitro* serodiagnostic method by showing that a trace of heated cholera antiserum specifically agglutinated a suspension of cholera vibrios and not a suspension of vibrios of a different species (Bordet, 1895). Thus, just as the specificity of sera which agglutinated different

vibrios permitted the certain distinction of cholera vibrios from other vibrios, so the remarkable specificity of haemolytic sera permitted animal species to be distinguished by components of their cells. Bordet showed in 1900 that it was the stroma of red cells which carried the antigenic specificity and with Renaux (1926) that zoological specificity resided in the lipids of the stroma. But since 1899, the work of Bordet and of Tschistovitch on specific precipitins against animal proteins had shown that the humoral constituents of the organisms were equally important in zoological specificity. Bordet discovered that injection of cow's milk into a rabbit gave a serum which precipitated the casein of that milk (Bordet, 1899*a*). A little later he (1899*a*) and Tschistovitch (1899) simultaneously observed that the sera of animals immunized with the serum of a foreign species specifically precipitated the proteins of this serum. The use of these precipitating sera in medicolegal diagnostic tests of blood stains is well known.

The combined action of antibody and alexine which Bordet had proved necessary for bacteriolysis and for haemolysis by antisera was elucidated when in 1900 he demonstrated for the first time the phenomenon of alexine (complement) fixation. He had shown that after contact with a sufficient dose of cholera vibrios the specific antiserum lost the power of making new vibrios susceptible to lysis by alexine. The antibody therefore, in fixing itself on the vibrios, 'sensitized' the micro-organism to the action of the alexine, hence the name 'sensibilisatrice' (sensitizer) which Bordet gave it. In a crucial experiment he showed that alexine itself also fixed on the micro-organisms but only when these had been sensitized by specific antibody.

Ehrlich & Morgenroth (1899) and Bordet (1899*b*) noted independently that red cells would absorb antibody from heated antiserum and after centrifugation were haemolysed by alexine. Bordet (1899*b*) also noted that the antibody was fixed so firmly to the red cells that even after several washings the red cells retained the ability to be lysed by alexine. In 1900 Bordet showed that the alexine was firmly fixed to red cells sensitized with specific antibody and that it was possible in this way to remove all alexine from fresh serum.

The role of the sensitizer was to confer on the antigen (vibrio or red cell) the ability to fix alexine. Bordet confirmed the non-specific character of alexine, it was the same alexine which affected red cells and transformed cholera vibrios into granules (Bordet, 1900, 1901). From 1901 on, Bordet and Gengou applied the reaction of complement fixation to the serodiagnosis of numerous infections (e.g. typhoid fever, anthrax, swine erysipelas), by which the presence of the specific sensitizer in the sera of the sick animal could be revealed (Bordet & Gengou, 1901*b*). The later application of this method by Wassermann to the diagnosis of syphilis and more recent applications amply demonstrate the fruitfulness of Bordet's discovery.

In the years which followed Bordet worked continuously in this fertile field of humoral immunity where so many discoveries were to be made. In 1899 he recognized in the sera of non-immunized animals antibodies analogous to those of immune sera, notably in that they were sharply specific (Bordet, 1899*a*). In 1899 and 1904 he found that the antibodies and alexine in sera were themselves antigenic when injected into animals of different species; and thus obtained anti-alexines, anti-agglutinins, and anti-sensitizer. The specificity of these anti-antibodies depended

on the zoological source of the antibody: the antiserum of a guinea pig immunized with rabbit serum neutralized indifferently all the antibodies obtained in the rabbit by immunization with various antigens (Bordet, 1904).

In 1906 Bordet & Gay explained a phenomenon described in 1902 by Ehrlich & Sachs, who had observed that guinea pig red cells were haemolysed by a mixture of normal bovine serum heated to 55° + fresh normal horse serum, although neither serum alone was active. Ehrlich & Sachs (1902) concluded that the normal bovine serum contained a substance that sensitized the red cells to action of the alexine in the normal horse serum. Bordet & Gay (1906) recognized a phenomenon which had escaped the other two workers, namely, the marked agglutination of the guinea-pig red cells produced in the mixture of the two sera. They suspected that the explanation of Ehrlich & Sachs was incorrect and that a new phenomenon was being observed. By ingenious experiments Bordet & Gay established that the bovine serum contained colloidal materials which adsorbed to red cells already loaded with sensitizer and alexine, and as a result both agglutinated and lysed them. In the experiments of Ehrlich & Sachs the fresh horse serum supplied both sensitizer and alexine but, as is known, horse alexine does not lyse even strongly sensitized guinea-pig red cells. The heated bovine serum supplied the additional colloids which, on fixation to the red cells charged with sensitizer + alexine, agglutinated them and promoted their haemolysis (Bordet & Gay, 1906). This phenomenon of conglutination as Bordet named it (Bordet & Streng, 1909) occurs also with microbes (Streng, 1909), and constitutes a very sensitive test for the fixation of alexine.

The strong absorptive capacity of the antigen + antibody complex is evident in another phenomenon described by Bordet & Gengou (1911) under the name of co-agglutination. When rabbit serum was added to a mixture of defibrinated normal guinea-pig blood and the serum of guinea pig immunized against rabbit serum, there was a marked agglutination of the red cells. Analysis showed that the antigen + antibody complex formed by the union of rabbit protein and the corresponding guinea pig antibody led to the flocculation of the red cells—the first recorded example of an indirect haemagglutination reaction (Bordet & Gengou, 1911)

During these years of intense activity in the field of humoral immunity Bordet was preoccupied with the mechanism of the union of antigen with antibody and maintained a long controversy with Ehrlich on this subject. To Ehrlich's theory of the union of toxin and antitoxin in fixed proportions, based on the hypothesis of a strong affinity between the two substances, and a similarity of their combination to that of a strong monovalent acid with a strong base, Bordet proposed his theory of the union of antigen and antibody in variable proportions, as in adsorption. In his own words 'les réactions anticorps–antigène consistent en accolements fort analogues aux phénomènes d'adsorption dûs à l'affinité intermoléculaire, lesquels offrent ce caractère important que les substances réagissantes s'unissent en proportions très variables. De ce point de vue, la réaction de l'anticorps sur l'antigène peut se comparer à la condensation d'une couleur d'aniline sur le papier-filtre...'. Bordet's theory explains how under-neutralized toxin no longer kills the animal which receives it, although still producing certain toxic phenomena. According to the theory of union in variable proportions these mixtures contain not an excess of pure toxin along with completely neutralized toxin, as would be required by the

theory of union in fixed proportions, but only of partially neutralized toxin, that is to say, toxin which has absorbed uniformly a quantity of antitoxin too small to neutralize the toxicity completely. The later work of Heidelberger and his colleagues confirmed by quantitative chemical analysis of specific precipitates that antigen and antibody indeed combine in variable proportions.

In 1913 Bordet's interests turned to the problem of anaphylaxis and particularly to the question of anaphylatoxins. Various authors, notably Friedberger, had produced shock in the guinea-pig very like anaphylactic shock by the intravenous injection of a liquid obtained by adding to fresh guinea pig serum, antigen + antibody complexes such as sensitized red cells or specific precipitates; heated guinea-pig serum was inactive in these conditions. Friedberger thought that alexine, on absorption to the antigen + antibody complex, decomposed the antigen to produce a poison, anaphylatoxin. Bordet discovered that fresh guinea pig serum could be activated in this way by a little viscous suspension of agar. Intravenous injection of serum thus treated in fact induced anaphylactoid shock (Bordet, 1913*a*, *b*). The agar seemed therefore to behave like antigen + antibody complexes, showing like them, as Bordet (1924) thought, absorptive properties with respect to serum constituents and alexine.

The outstanding contribution of Bordet to bacteriology was the discovery of the organism of whooping-cough, first seen by Bordet in 1900 in sputum obtained from one of his daughters, who had the disease. This small Gram-negative coccobacillus was unable to grow on the usual media. Bordet & Gengou failed to find the organism again until 1906 when Bordet collected sputum from his son, now stricken with whooping-cough, during the early stages of the disease. In this specimen he found the coccobacillus and succeeded in cultivating it on the Bordet–Gengou medium which, with only minor modifications, is still used (Bordet & Gengou, 1906*a*, *b*). Bordet & Gengou recognized that the bacillus was very common in the respiratory passages in the early stages of the disease but later it became scarcer. They found that 2–3 weeks after onset the sera of whooping-cough patients contained a specific antibody to the whooping-cough bacillus. The bacillus was very toxic, containing an endotoxin which even in very small doses produced widespread ecchymosis, pleural effusion, dyspnoea and tissue necrosis at the site of inoculation. This endotoxin they considered was responsible for the irritation of the respiratory mucous membrane which initiates the fits of coughing (Bordet & Gengou, 1907, 1909). Bordet & Gengou also prepared a phenolized vaccine which was used successfully as a prophylactic, notably in Denmark by Madsen. In 1910 Bordet & Sleeswijk recognized in the whooping-cough bacillus the first example of antigenic variability in a microbial species. For initial isolation from an infected person, the organism required blood for its growth; it could be 'trained', however, to grow in nutrient agar, but the organism trained to grow on nutrient agar differed antigenically from the original isolate maintained in blood agar. The strain maintained in blood agar contained an antigen easily agglutinable by the serum of a rabbit immunized with this strain, but this antigen was lacking in the strain adapted to grow on nutrient agar alone. To Bordet also (1909, 1910) is due the earliest description of the morphology of the organism of bovine pleuropneumonia, and in collaboration with Fally that of the organism of avian diphtheria (Bordet, 1907; Bordet & Fally, 1910).

Between 1901 and 1920 Bordet devoted much activity to another question which

greatly interested him, that of blood coagulation. The numerous papers which he published, alone or with his colleagues Gengou and Delange, contain a wealth of facts (Bordet, 1920). Bordet and Gengou first showed that not only whole blood but also the plasma freed from cells coagulated on contact with wettable foreign bodies; and that covering the internal surface of glass tubes with paraffin wax kept the plasma liquid. The coagulation of plasma on contact with glass is due to the ultimate formation of thrombin which requires the presence of calcium, and the anticoagulant action of sodium oxalate is due to the binding of calcium as oxalate. Once formed, thrombin added to plasma in the presence of calcium not only brought about coagulation but considerably accelerated the formation of new thrombin. The latter possesses, according to Bordet & Gengou (1901*a*, 1903*a*, *b*, 1904*a*, *b*) ability to stimulate its own production. Bordet & Delange (1912*a*) showed that the thrombin activity appears to be the result of the combined action of two substances: the first, which they named serozyme, was furnished by the liquid part of blood; the second, named cytozyme, came from the cells of the blood, notably from platelets, and was also present in tissues. Cytozyme, which was soluble in ether, ethanol and chloroform but was precipitable by acetone, behaved like a phospholipid (Bordet & Delange, 1912*b*, 1913). Serozyme, probably of a protein nature, did not exist as such in circulating blood or in oxalated plasma but in the state of pro-serozyme, which was incapable of uniting with cytozyme. It was contact with the wettable walls of a test-tube which, in the presence of calcium, brought about the transformation of pro-serozyme to serozyme, a reaction accelerated by cytozyme. The latter had the property of uniting with serozyme to form thrombin which coagulated fibrinogen. The latter played in coagulation a passive role since thrombin could be produced in its absence, as Bordet (1919) showed by depriving oxalated plasma of its fibrinogen by saturation with sodium chloride and showing that on contact with a foreign body serozyme appeared in this plasma, and that this serozyme was capable of uniting with cytozyme, thus producing thrombin.

From 1920 on Bordet took a lively interest in bacteriophage which Twort and d'Herelle had just discovered. With Jaumain, Bordet noted that lysis of bacteria by bacteriophage required not only that the bacteria should be living but that nutrients should be available; lysis was not produced in physiological saline (Bordet & Ciuca, 1921*c*). In certain bacterial species sensitivity to phage was dependent on the antigenic make-up, the S form being very sensitive and the R form resistant to the same phage (Bordet, 1922). He discovered that some phages lysed in the presence of oxalate, whereas others were inactive under these conditions (Bordet, 1926), and that these latter phages required considerable quantities of calcium for lysis, different for different phages (Bordet & Bordet, 1941). Bordet & Ciuca (1921*a*) discovered that bacteriophage was antigenic. Bordet was particularly concerned with the problem of lysogeny. In particular he showed the essential difference between lysogenic bacteria and bacteria contaminated with phage which could develop after lysis of a sensitive strain of bacterium. Contaminated bacteria were easily freed from phage by isolation procedures, but true lysogenic bacteria preserved their lysogeny indefinitely (Bordet & Ciuca, 1921*b*; Bordet, 1925). Bordet also noted that certain phages made some bacterial strains definitely lysogenic; this is, lysogenization (Bordet, 1925). It was in Bordet's laboratory that McKinley (1925) noted that the lysogenic power persisted in lysogenized bacteria in the

presence of phage antiserum; the lysogenic character was therefore perpetuated in the absence of free phage. Moreover, J. Bordet & P. Bordet (1946) recognized that if the addition of sodium oxalate to the culture medium prevented the propagation of the phage from a lysogenized bacterium on a sensitive bacterium, it did not, however, prevent the liberation of this phage by the lysogenized bacterium. Bordet saw clearly that it was the lysogenic power which was transmitted by the lysogenized bacterium to its descendants and not the phage itself. Bordet & Renaux (1928) explained this as follows: '...in the case of the Lisbon organism each individual microbe washed completely free from culture medium and carefully separated from its congeners kept within itself the ability to cause lysis of the Shiga bacillus; that is to say, could furnish descendants having this lysogenic power and which moreover developed quite normally without showing lysis'.

CONCLUSION

The work of Jules Bordet shows clearly the essential character of his scientific thought. Basically there was a wide and profound curiosity which drove him to research through a need not only to know, but to understand. This curiosity, fed by wide learning, was fruitful because it was never satisfied by a superficial answer to a question, but demanded detailed understanding of the data and a solution which was complete, logical and clearly demonstrated. His mind presented to him in unambiguous terms the problem to be solved and separated the contingent factors from the essential and really basic ones. Without apparent effort, by a kind of instinct, the intellectual interrogation became concrete in experimental form. The experimental scheme was always simple and precise, however profound and subtle; Bordet shaped the experiments in the form of an unavoidable dilemma, chosen with such logic that a clear answer must result. It is perhaps this mastery in the design of experiment which most deserves admiration.

Bordet was a very exact observer; as his imagination was lively in the selection of subjects for experiment, leading to daring associations of ideas, so his mind was quick, methodical and prudent in the observation of fact. He always insisted on the capital importance of proper experimental controls designed to leave no room for doubt, never risking a conclusion that was susceptible of ambiguous interpretation because a different possibility might have been neglected. He considered that when an experimental fact had been correctly observed under adequate conditions, and when all possibilities of error had been removed, thanks to a judicious use of controls, it remained for the research worker to play a rigorous game against himself in which he had to try to refute his own discovery, and to admit it at last, only when it could no longer be denied. A striking example of this rigour and prudence is given by the following story, which concerns the spirochaete of syphilis described by Schaudinn in 1905. In October/November 1902, Bordet & Gengou examined a syphilitic chancre and discovered a spirillum in the specimen. Bordet wrote about this discovery in a letter (28 April 1905) addressed to Metchnikoff which is now preserved in the archives of the Academy of Sciences of Moscow. In an article entitled 'A propos de la découverte du tréponème pâle' which appeared in the *Revue medicale de Bruxelles* (1961, **17**, 333) S. Zylbersac published the text of this letter of which he had obtained a photograph thanks to the Secretary of the Academy of

Sciences, Professor Fedoseef. It is from the text published in this article that we extract the passages cited here.

In particular Bordet said:

'In the deeper layers of a chancre... we have found (having studied the preparation for a long time) a spirillum hardly more coloured than the background, of such extraordinary fineness that if one were not warned, one could examine preparations for a long time without discovering it.' Further on: 'Naturally we felt very excited after having seen this, but in spite of very close examination our later work has been negative...'. 'We have however discovered spirilla which appear to be identical in a sore of the mucous membrane of the throat, but even a non-syphilitic throat contains spirilla often so similar that we have not dared to attribute any value to our results.' Because of this unreproducibility of results and inability to be certain Bordet & Gengou refrained from publishing their observations. As said in the letter to Metchnikoff 'We have been discouraged and we have not concluded that the spirillum has anything to do with syphilis, for it is certain that one can only see it when it is abundant, but we have not judged either that our single positive result ought to be taken too seriously.'

Bordet showed the same prudence and intellectual honesty in the interpretation of experimental results, never exceeding what the facts showed and hazarding an explanation only as a working hypothesis for future experiments. But if he did not let the facts say too much, he drew from them, however, everything that a penetrating and ranging mind could deduce. The sure intuition with which he perceived the really new and fruitful idea in the observed facts was only equalled by the ease with which he passed from the experimental fact to its significance and the law which it illustrated.

Exceptional in his scientific gifts, Jules Bordet was attractive also by other aspects of his rich personality. He was a writer of high order, using the French language with a rare elegance and precision. His *Traité de l'immunité dans les maladies infectieuses* is not only an assemblage of the fundamental bases of immunology but also a classic of scientific writing. The limpidity and elegance of his style, his ability to express in common words the most difficult scientific problems and the colourfulness of his comparisons led to his often being asked to write general reviews or articles for high-level popularization; these are models of their kind.

In his public discourses the elevation and vigour of his thinking would have sufficed to capture his audience, but his remarkable oratorical talent contributed yet more to the dissemination of his thoughts. His teaching at the Faculty of Medicine of the University of Brussels will never be forgotten by anyone who had the privilege of attending his clear vivid lectures, delivered with such infectious enthusiasm.

He trained his students by example, by daily contact in the laboratory, by conversations without formality during which, by his advice or criticism, he indicated a method of work and a rigorous scientific discipline without crushing the personalities of his colleagues. He left them in complete liberty, not imposing upon them any *esprit d'école* in the strict sense of the term, but generously according them the most precious gifts which he could make: sincere appreciation of their value, enlightened advice about their work, and severe but constructive criticism of their efforts.

The man himself was as attractive as the research worker and teacher. His equanimity, his spontaneity, the simplicity and charm of his welcome made human contacts extremely easy. He mixed with this a sincere interest in his fellow men; his relations with the staff of the Institut Pasteur of Brussels, from his immediate colleagues to the most junior of his technicians, were marked by a friendliness and reciprocal confidence which developed in an almost family way. But this interest in the person extended to an interest in all mankind. Jules Bordet had very high ideals about the mission of the research worker, more especially of biologists. He never lost sight of the good that men can draw from the discoveries of learned men and the possibilities which are offered to the latter to play a role in the struggle against misery, suffering and disease. This is what he expressed at Paris in 1930 in his address as President of the First International Congress of Microbiology when he said:

'Assuredly every scientific discovery gives lively satisfaction about the difficulties overcome, but no joy can compare with that which the bacteriologist experiences when he thinks that profound unhappiness or cruel suffering will be mitigated, that much mourning will be avoided, that a country where innumerable lives were sacrificed will soon show itself to be hospitable; in a word that inestimable benefits will spread through the world because one day someone in a laboratory did an experiment which penetrated some mystery of etiology or mode of transmission of some noxious microbe, revealed a diagnostic test or suggested a more successful prophylactic or therapeutic measure.'

Bordet did not see in science only a tool which men could use in order to understand nature, to dominate it and to use it for his own purposes; he placed science at the centre of philosophy and ethics. Interested in all the problems with which man is faced, Bordet was much preoccupied with the philosophical, moral and social roles of science. Convinced of the excellence of scientific method, he earnestly wished that men should use it to solve the moral, political or social problems which face them. He published in 1945 a little work entitled *Brèves considérations sur le mode de gouvernement, la liberté et l'éducation morale.* I extract from it two passages characteristic of the high conception which Bordet had of science and which may serve as the conclusion to this article.

'It is indispensable that agreement should be reached about the basic precepts of a universal ethic which should be acceptable by all men of good will, whatever their philosophical or religious convictions. One thinks in this context of science, for it is universal.'

'What has given birth to science and sustains it in its eternal task is a pure idealism, this burning and disinterested desire to know, the infinitely precious privilege which human nature possesses, and which explains and justifies the primacy of our species. But this is integrated with science, which sets the example and is its living witness. Science can embrace in its religion of truth all the aspirations of men of good will, it can unite them in its wish to serve civilization. Scientific culture, provided that it does not limit itself to the knowledge of techniques and that it reaches towards general ideas, develops in the mind qualities of equilibrium, comprehension and impartiality, which make it understandable how useful and wise it is to unite science and ethics so that they may inspire each other.'

J. BEUMER

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A SYMPOSIUM ON METABOLIC ACTIVITY AND BACTERIAL STRUCTURE

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CONTENTS

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Cell-Wall Structure and Biosynthesis

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The external structures responsible for the rigidity and integrity of bacterial cells are generally referred to as the 'cell walls' and apart from their obvious mechanical functions very little direct work has been done on the biochemical activities associated with these structures. It is generally agreed that isolated cell-wall preparations of Gram-positive bacteria are comparatively devoid of enzyme systems localized in other structures and fractions derived from disintegrated cells. With the cell walls of Gram-negative bacteria the situation is less certain as there have been several reports of enzymic activities intimately associated with 'envelope' preparations of these organisms (Marr, 1960; Hunt, Rodgers & Hughes, 1959; Salton, 1961*a*). Although the enzymic functions localized in cell walls have not been investigated extensively, walls have attracted interest in a broader biochemical sense. This report will thus be confined to some aspects of the chemistry and biosynthesis of these structures and it is not intended to be a complete review of this rapidly expanding topic (for recent reviews see Work (1961) and Salton (1961*b*)).

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Structure of cell walls of Gram-positive bacteria

The general chemical composition of the cell walls of Gram-positive bacteria has been the subject of numerous investigations and the broad features of amino acid, amino sugar and monosaccharide constitution are now well known (Cummins & Harris, 1956, 1958; Work, 1957, 1961; Salton, 1953, 1961*b*). However, the arrangement of the simple constituents identified by paper chromatography of the hydrolysed walls has remained uncertain until recent years. Thus the variety of polymers in the walls of Gram-positive bacteria can only now be defined a little more clearly.

The following classes of polymeric substances have been isolated from walls and partially or fully characterized: (1) mucopeptides or peptidopolysaccharides; (2) oligosaccharides, polysaccharides; (3) teichoic acids; (4) teichuronic acid.

It is apparent that the walls of some Gram-positive bacteria may be made up entirely of mucopeptides (1), while others contain in addition one or more of the other three classes of polymers. Mucopeptides composed of three or four principal amino acids, together with glucosamine and muramic acid, have been identified in all bacterial cell walls. Additional cell-wall components such as the teichoic acids (Armstrong *et al.* 1959) and polysaccharides are less widely distributed and the teichuronic acid polymer has so far only been reported in walls of *Bacillus subtilis* (Janczura, Perkins & Rogers, 1961). Of all the four classes of substances isolated from walls, only the chemical structure of the teichoic acid has been fully established by the beautiful work of Baddiley and his colleagues (Armstrong, Baddiley & Buchanan, 1961).

The term 'mucopeptide' was originally proposed by Mandelstam & Rogers (1959) to describe the amino sugar–amino acid-containing complexes of the walls now recognized as the structural 'backbone' common to all cell walls of Gram-positive bacteria. Whether the 'mucopeptide' of a given wall is made up of one type of 'polymer' is certainly not known at present and as shown by Salton (1956) a complex mixture of 'fragments' is obtained when a mucopeptide wall is digested by the enzyme, lysozyme. There is, however, little doubt now that the mucopeptide forms the rigid backbone component composed of covalently bonded amino acids and amino sugars. That the other cell-wall compounds are attached to the mucopeptide by weaker linkages now seems likely from the extractibility of the teichoic acids (Archibald, Armstrong, Baddiley & Hay, 1961) and the teichuronic acid of *Bacillus subtilis* (Janczura *et al.* 1961) with trichloroacetic acid (TCA) in the cold and the removal of oligosaccharide and polysaccharide residues with both picric acid (Holdsworth, 1952) and formamide (Krause & McCarty, 1961). In all cases the other wall 'polymers' have been obtained in solution, leaving behind insoluble mucopeptide residues, still possessing the structural rigidity and appearance of the native cell wall as seen in the electron microscope (Archibald, Armstrong, Baddiley & Hay, 1961; Krause & McCarty, 1961).

With the isolation and characterization of muramic acid (Strange & Dark, 1956; Strange & Kent, 1959) and its identification in the Park nucleotides (Park & Strominger, 1957) the key role of this amino sugar in the structure of the wall and spore mucopeptides became apparent. Thus the broad outline of mucopeptide structure has been established from the studies of products isolated from walls and mucopeptides digested with lysozyme and streptomyces amidase (Salton, 1956,

1957; Brumfitt, Wardlaw & Park, 1958; Ghuysen & Salton, 1960; Ghuysen, 1960; Salton & Ghuysen, 1960; Ghuysen, 1961; Primosigh, Pelzer, Maass & Weidel, 1961).

The mucopeptide is envisaged as possessing an acetyl amino sugar 'backbone' containing alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues with peptides linked through the COOH group of muramic acid. However, it is obvious that not all of the *N*-acetylmuramic acid residues carry peptide substituents since *di-* and *tetra*-saccharides have been isolated in lysozyme digests of walls (Perkins, 1960; Salton & Ghuysen, 1960). These observations suggest that *N*-acetylglucosamine and *N*-acetylmuramic acid are in the form of amino sugar repeating units. An examination of the products of partial acid hydrolysis of the walls of *Micrococcus lysodeikticus* with concentrated H_3PO_4 for 12 hr. at 37° also

Table 1. *Products identified in lysozyme and* H_3PO_4 *digests of* Micrococcus lysodeikticus *walls*

Lysozyme	H_3PO_4, 12 hr.; 37°
Disaccharide*	Free AG† and AMA† Disaccharide*
Tetrasaccharide*	Trisaccharide?* Tetrasaccharide* Oligosaccharides*
Mucopeptides	Mucopeptides

* Compounds composed of both AG and AMA. † AG, *N*-acetylglucosamine; AMA, *N*-acetylmuramic acid.

revealed the presence of acetyl amino sugar compounds composed of *N*-acetylglucosamine and *N*-acetylmuramic acid and the nature of some of these products is compared with those obtained by lysozyme digestion in Table 1.

Confirmation that some of the muramic acid residues in the wall mucopeptide possess free carboxyl groups has been obtained by esterifying the walls of *Micrococcus lysodeikticus* with methanol–HCl and reducing the esterified walls with $LiBH_4$ in tetrahydrofuran. Upon acid hydrolysis (4N-HCl, 4 hr. 100°) of the walls subjected to this procedure much of the cell-wall muramic acid has been converted to a neutral amino sugar compound and the sequence of the reactions is shown in Fig. 1 (Salton, 1962).

The precise nature of the repeating unit in mucopeptides is not known. Several low-molecular-weight mucopeptides have been isolated from lysozyme-digested walls of *Micrococcus lysodeikticus* (Ghuysen & Salton, 1960) and from *Escherichia coli* mucopeptide (Primosigh, Pelzer, Maass & Weidel, 1961). Whether these simple mucopeptides form the repeating units of 10,000–20,000-molecular-weight compounds obtained from wall digests (Salton, 1956) cannot be said at present.

The problem of determining the subunit size of wall mucopeptide by conventional *N*-terminal and *C*-terminal amino acid analysis (Salton, 1961*c*) presents some difficulties, especially when the wall structure contains teichoic acids possessing ester-linked alanine. Free amino groups and *C*-terminal residues can be readily identified by reacting walls with fluoro-dinitrobenzene and anhydrous hydrazine respectively (Salton, 1961*c*). Surprisingly few free amino and *C*-terminal groups are found in some bacterial cell walls, suggesting either a high degree of cross-linking or protection of the reactive groups with substituents.

Where a variety of '*N*-terminal' and '*C*-terminal' groups have been detected in the wall of a given species, it is difficult to interpret the information in terms of subunit size. This has frequently been found to be the case with a wide variety of bacterial walls (Salton, 1961*c*). However, the data for walls of *Micrococcus lysodeikticus* and some of the lysozyme digest products appear to be consistent and have given a subunit size of around 2000, as shown in Table 2. A tentative model of a mucopeptide structure compatible with the data available at present is illustrated in Fig. 2.

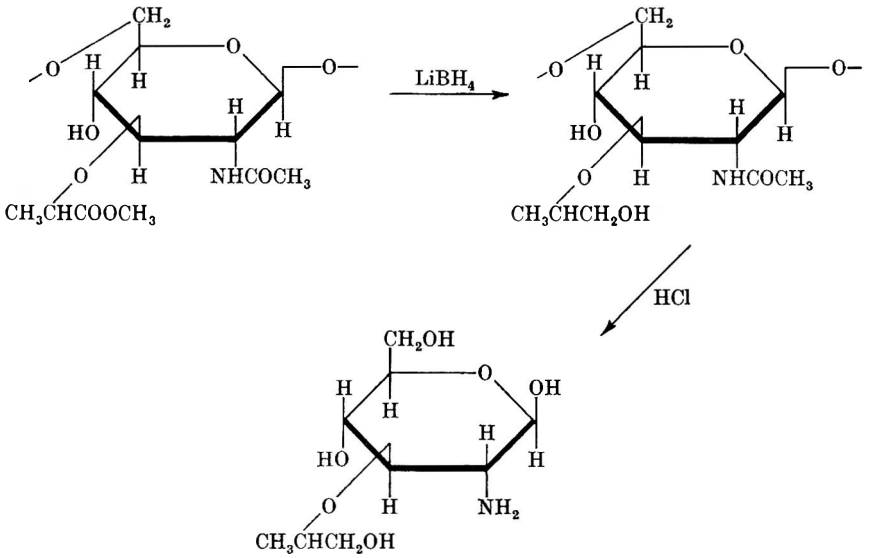

Fig. 1. The sequence of reactions for detecting muramic acid residues (in walls) possessing free carboxyl groups involves esterification with methanol–HCl followed by reduction with lithium borohydride giving the modified 'muramic acid' compound on acid hydrolysis.

Table 2. *Subunit size of walls and soluble products from* Micrococcus lysodeikticus

	Wall	Lysozyme NDF	Mucopeptides
Molecular weight	—	10,000–20,000	1200 2400
Subunit from			
ϵ-NH_2 lysine	2500	2500	—
C-terminal glycine	2000	2000	—

Many structural problems remain to be solved and it is not known at present how subunits of the type proposed in Fig. 2 would be joined together to give the higher-molecular-weight (10,000–20,000) components possessing peptide to disaccharide (*N*-acetylmuramic acid–*N*-acetylglucosamine) ratios greater than 1.

Structure of walls of Gram-negative bacteria

Isolated walls of Gram-negative bacteria are both physically and chemically more complex than those of most Gram-positive organisms (Salton, 1961*b*). The protein, lipid, polysaccharide complexes form part of the wall and in addition a

rigid mucopeptide layer has been clearly demonstrated in the elegant studies of Weidel, Frank & Martin (1960). Thus there is now abundant evidence that walls of Gram-negative bacteria possess a mucopeptide of similar composition to that found in Gram-positive bacteria and it is the mucopeptide component which represents the 'basal structure' containing muramic acid and diaminopimelic acid and D-glutamic acid as the most characteristic substances (Work, 1957).

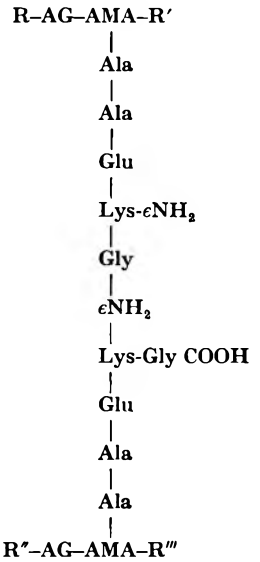

Fig. 2. A tentative model of a mucopeptide subunit based on data obtained from *Micrococcus lysodeikticus* walls.

Biosynthesis of cell walls

Interest in the problem of cell-wall biosynthesis was stimulated by the discovery that penicillin action on bacteria involved an inhibition of mucopeptide formation and consequent accumulation of nucleotide precursors (Park & Strominger, 1957; Park, 1952; Mandelstam & Rogers, 1959). Various aspects of the biosynthesis of walls have been dealt with in reviews by Strominger (1960), Work (1961) and Salton (1961*b*) and the present discussion briefly covers only certain facets of the problem.

Amino acids

The simplest level at which wall synthesis has been studied is at the level of biosynthesis of some of the most characteristic building blocks (amino acids and amino sugars) of the wall mucopeptide. Thus the origins of the D-isomers of the amino acids alanine, aspartic acid and glutamic acid as well as the formation of α,ϵ-diaminopimelic acid (DAP) in its various isomeric forms (LL, DD and *meso*) are relevant to the problem of the biosynthesis of walls. Pathways for the biosynthesis of the latter amino acid (DAP) have been discussed in detail elsewhere (Rhuland, 1960; Work, 1961).

Alanine racemase has been known for some time in bacteria (Wood & Gunsalus, 1951) and the presence of such an enzyme could fulfil the function of providing

D-alanine for cell-wall precursors. The origin of D-glutamic acid in bacteria has been investigated in relation to the formation of the γ-D-glutamyl capsular polypeptides (Thorne, 1956). No glutamic racemase could be detected in the organisms capable of producing D-glutamyl peptides, but they did, however, contain enzymes effecting an indirect conversion to D-glutamic acid by a transamination reaction between D-alanine and α-ketoglutamic acid (Thorne, 1956). Whether cell-wall D-glutamic acid arises by a similar pathway cannot be said at present. That the D-glutamate may also originate from a specific racemase in some organisms has now been demonstrated with an enzyme purified from extracts of *Lactobacillus fermenti* by Tanaka, Kato & Kinoshita (1961). The *meso* isomer of diaminopimelic acid occurs more widely in cell walls than does the LL isomer (Salton, 1961*b*; Work, 1961) and a racemase which interconverts the LL or *meso* isomer to an equilibrium mixture of the two has been described by Antia, Hoare & Work (1957). The origin of the D-isomer of aspartic acid in bacterial cell walls has not, so far as the author is aware, been investigated.

Another enzymic reaction which may have a bearing on the biosynthesis of cell-wall compounds includes the amino acid-activating system for D-alanine detected by Baddiley & Neuhaus (1959) in *Lactobacillus arabinosus*.

Amino sugars

The two amino sugars *N*-acetylglucosamine and *N*-acetylmuramic acid occur in all of the bacterial cell walls so far studied. The biosynthesis of amino sugars has been discussed in some detail in the excellent review by Roseman (1959). The pathways for the formation of *N*-acetylglucosamine and *N*-acetylgalactosamine have been studied with bacterial enzymes by Roseman and his colleagues (Roseman, 1959). It is thus likely that cell-wall acetylglucosamine originates from some of these described enzymic reactions.

The biosynthesis of muramic acid (the 3-O-lactyl ether of D-glucosamine) has not been fully investigated. Strominger (1960) reported the following enzymic reaction of uridine diphosphonucleotides (UDP) in extracts of *Staphylococcus aureus*:

UDP-acetylglucosamine + phosphoenolpyruvate → UDP-acetylglucosamine – pyruvate + Pi,

UDP-acetylglucosamine – pyruvate → UDP-acetylglucosamine – lactic acid.

Richmond & Perkins (1960) also investigated the origin of the lactic acid residue of muramic acid and their results were consistent with the idea that phosphoenolpyruvate was the immediate precursor.

Whether muramic acid is synthesized at the nucleotide level has not been conclusively established. Zilliken (personal communication) has suggested the biosynthesis of muramic acid at a *di-* or *tri*-saccharide level by the addition of an unknown 3-C fragment to form the O-lactyl side chain.

The possibility of a more direct route of biosynthesis has been explored in the author's laboratory using extracts of *Micrococcus lysodeikticus* (Salton, unpublished observations). The investigations of Roseman (1959) have suggested that the amino sugar phosphates are probably the most important intermediates for the formation of *N*-acetyl amino sugars. To test the possibility that muramic acid synthesis may occur via glucosamine-6-phosphate (GM-6 P) crude extracts of *M. lysodeikticus*

were incubated with GM-6 P and phosphoenolpyruvate in M/15 phosphate buffer at pH 7·0. A compound which corresponded to muramic acid-6-phosphate (prepared by incubating Boehringer crystalline yeast hexokinase, ATP, Mg^{2+} and muramic acid and separated by paper chromatography) was detected in the reaction mixture. Thus these results tentatively suggest that muramic acid may originate from the following reaction:

Glucosamine-6-phosphate + phosphoenolpyruvate→muramic acid-6-phosphate + Pi.

Confirmation of this pathway must await further purification of the enzyme system present in the crude extracts. Which of the three possible pathways suggested for biosynthesis of muramic acid will be the principal one involved in wall formation remains to be established. It is perhaps relevant to this problem that muramic acid-6-phosphate has already been isolated from bacteria by Ågren & de Verdier (1958) as well as a compound believed to be UDP-muramic acid-6-phosphate.

Cell-wall intermediates and biosynthetic pathways

The original observations of Park (1952) on the structure of the nucleotides accumulating in penicillin-treated *Staphylococcus aureus* gave the first indications of the types of compounds acting as cell-wall precursors (Park & Strominger, 1957). Compounds of identical structure have also been obtained by Rogers & Perkins (1960) when 5-fluorouracil was used as an inhibitor of mucopeptide biosynthesis. Thus the use of substances inhibiting wall formation has greatly facilitated the detection and isolation of cell-wall nucleotide precursors (Strominger, 1960).

The number of nucleotides isolated from both untreated and penicillin- or fluorouracil-inhibited bacteria has increased rapidly during the past few years, and some of the earlier data have been summarized by Strominger (1960) and Salton (1961*b*). Uridine nucleotides containing muramic acid and amino acids including diaminopimelic acid have also been isolated from *Escherichia coli* by Strominger (1960) and Comb, Chin & Roseman (1961). Until recently only uridine nucleotides containing muramic acid had been found in bacteria, but the detection in extracts from *Aerobacter cloacae* of adenyl nucleotides containing muramic acid and peptides is of considerable interest (Cooksey, Anwar, Roy & Watson, 1961). The formation of thymidine diphosphorhamnose by extracts of *Streptococcus faecalis* which contains considerable quantities of rhamnose in the wall has now been established by Pazur & Shuey (1960) and it seems likely that this nucleotide may also be a cell-wall precursor compound.

Transfer of the amino sugar–peptide residues as well as polyol and sugar moieties of the various nucleotides to mucopeptide and/or wall has not been conclusively demonstrated. However, the nature of the nucleotides accumulating in the presence of various antibiotics makes it extremely likely that some of the suggested pathways for wall biosynthesis (Strominger, 1960; Salton, 1961*b*) may not be far from reality.

The site of cell-wall biosynthesis within the bacterial cell has not been established, but studies of the anatomy of thin sections of cells support the view that wall formation may occur in a limited area rather than over the entire surface of the protoplast. It seems inevitable that the membranes or membranous organelles are concerned in some way in wall synthesis. The hypothesis that the intermediates

are formed at intracellular levels and that the transferring and/or 'polymerizing' mechanisms are located in the membrane in intimate contact with the cell-wall acceptor seems an attractive basis for further experimental exploration.

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Metabolism, Transport, and Morphogenesis: Which Drives Which?

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In the course of the last forty or fifty years, the profound insight that biochemists have obtained into the biological processes known collectively as energy metabolism has led to the belief amongst many biochemists and cell biologists that the processes of transport, growth, and morphogenesis are 'driven' by the 'energy released through metabolism'. This popular belief has, to some extent, substituted the imaginary demon of 'metabolic energy' for the *vis vitae*. In the case of 'active transport' across membranes, for example, many of us tend to visualize a kind of grabbing or catapult system that is triggered off by contact with its substrate so that it traps the substrate and, with the aid of 'metabolic energy', forces the substrate through the membrane, where it releases it and passes back again in readiness to grab another. With morphogenesis it is somewhat harder to portray how we may imagine the process to be 'driven' by metabolic energy, but I am sure that our mental images often tend to include miniature conveyor belts, substrate-specific mechanical shovels, and all kinds of engineering devices. As Waddington (1959) has pointed out in an excellent little cameo on physico-chemical aspects of morphogenesis, 'It is perhaps remarkable that biology, which was for so long a morphological science, has made so little attempt, now that it has passed into the experimental phase, at a causal analysis of the mechanisms that give rise to the shapes which have been so extensively studied'. Waddington was mainly thinking of multicellular organisms, but although the microbiologist has the great advantage of studying morphogenesis in individual cells or in quite small aggregates, Waddington's conclusion about our ignorance of the morphogenetic mechanisms is nevertheless largely true.

Of course, the fundamental advances in the knowledge of the molecular mechanisms of transport, growth and morphogenesis have had to await the development of their foundations in classical biochemistry. But I agree with Waddington and with other biologists who are suggesting, in effect, that it would now be helpful if the biochemically minded were more often to interrupt their preoccupation with the chemical foundations, to straighten their backs, as it were, and to look carefully at the master plans of our science—that is, at whole living organisms.

When we consider whole adapting and developing bacteria and give our thoughts some licence within the context of the present symposium, we may realize that the processes of growth and morphogenesis represent the movements of chemical groups (atomic nuclei and electrons) at certain rates and in certain definite directions in space, from nutrients initially present in the growth medium to polymers or metabolic intermediates (including inorganic ions and water) within the organism. Thus, growth and morphogenesis represent transport processes which, in common with the more popular membrane transport, must be described by vector quantities, having both magnitude and direction in space. They can be represented by arrows,

the length of which will denote their magnitude. On the other hand, orthodox chemical reactions are scalar quantities, having only magnitude and not direction in space. They can be represented by dots of different size, or by numbers. It is a matter of experience—and of theory (see Curie, 1908)—that scalar or directionless quantities cannot determine the direction of vector quantities. And so, the spatially directionless processes of metabolism (as usually understood) cannot force the movement of any chemical against the natural direction of diffusion (see Jardetsky & Snell, 1960; Kedem, 1961). This is equivalent to saying that if a substance appears to be moving against the natural diffusion gradient in a resting or growing organism (that is, if it seems to be 'actively' transported) the substance in question cannot be doing so, but must either be moving as some unidentified derivative, or must be carried by a bulk flow (Mitchell, 1959*b*, 1961*a*, *b*). Pausing for a moment, you will see that the simple argument that we have just used implies that the cause of the process usually described as 'active transport' must be nothing more than the thermal or Brownian motion that gives rise to down-hill diffusion of molecular complexes, molecules, or stable ions, or the pressure gradient that gives rise to the down-hill movement of a mass of fluid (or of solid fibrils or particles), and which must be due, in the first place, to a diffusion or escaping process.

Apart from its intrinsic interest, this conclusion is very valuable to the experimentalist, for it may impel him not to rest content with the sort of 'active transport' model that is based upon macroscopic mechanical analogies, but to ask what the unidentified derivative of the apparently 'actively transported' substance may be, or whether the transport may be due to a bulk flow. One may object that this view of 'active transport' as a diffusion or down-hill escape, robs the idea of its glamour and mystery; and, for example, we would have to discard the romantic suggestion of Cohen & Monod (1957) that membrane transport is controlled by demons like those imagined (but not isolated and purified!) by Clerk Maxwell. I think, however, that we have to accept this possible loss of romance as one of the normal consequences of scientific thought and enlightenment; and there are indeed signs that the Parisian permease school are beginning to recognize this necessity (see Kepes, 1960). So far our argument has suggested that we can represent the process of membrane transport and the vectorial processes involved in the activities of maintenance and growth by processes of diffusion alternating with chemical transformation. The chemical transformation (promoted by an enzyme) gives rise to the disappearance of a given component and the appearance of another at a certain site so that diffusion of the appropriate components towards and away from the site of chemical change will occur. This description, as you see, seems to distinguish sharply between chemical transformation and diffusion.

During the last few years it has become apparent that certain types of catalysed chemical process (involving the exchange of covalent bonds) are not strictly scalar. These are what I have called group-translocation reactions, because the chemical process of group transfer is accompanied by the effective diffusion of chemical groups across an anisotropic catalytic system (Mitchell, 1957, 1959*a*, *b*, 1961*a*, *b*, *c*; Mitchell & Moyle, 1958*a*, *b*).

Figure 1 illustrates a working model of a hydroxyl-translocating ATPase as an example of a catalyst of group translocation. This model helps one to realize that although we are accustomed to thinking of the diffusion and the chemical change

of molecules in rather different terms, the processes involved in diffusion and chemical change are, in fact, very similar. The *diffusion* of a molecule or ion or molecular complex in a biological system describes the movement of the particle by the thermally activated breaking and making of the *secondary* bonds that tend to prevent the displacement of the particle relative to the neighbouring atoms. The *chemical*

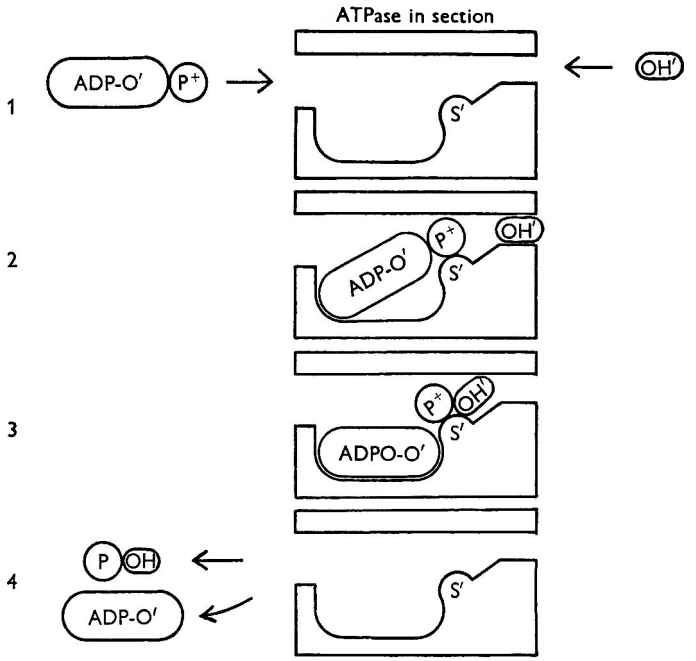

Fig. 1. Diagram of working model of anisotropic ATPase, translocating hydroxyl ion. Reaction shown in stages 1 to 4. The channel leading to the active centre from the left allows passage of adenosine triphosphate (ADP–O'–P+), phosphate (POH) and H^+, but not of OH'. The right-hand channel allows passage of OH' only. The phosphorylium group (represented as P^+—equivalent to monomeric metaphosphate) forms an intermediate with a negatively charged atom (S') in the enzyme.

transformation of a molecule or ion or molecular complex describes the movement of one of its constituent chemical groups by the thermally activated breaking and making, not only of *secondary* bonds, but also the *primary* bond that tends to prevent the escape of the group from its partner (or donor group) and its transfer to an acceptor group.

If, then, we think of chemical transformation as being due to the diffusion of chemical groups (and/or electrons) from group donors to group acceptors, guided or catalysed at certain points by the presence of group-transferring and group-translocating enzymes, it is evident that we will have to regard the gradient of escaping tendency, or the gradient of 'group potential'—as Lipmann (1941, 1960) called it—not only as the determinant of the direction of chemical reaction in the orthodox scalar sense, but also in the vector sense, meaning the direction of group diffusion or transport.

We have arrived now at a more homogeneous view of the processes of diffusion

and chemical reaction in which the first refers to the down-hill escape of molecular complexes, molecules or stable ions, and the second refers to the down-hill escape of chemical groups, electrons, or radicals. As recognized by Henry Eyring and others (see Glasstone, Laidler & Eyring, 1941), the processes of diffusional and chemical change are alike in being activated diffusion processes. Further, in condensed systems such as we mainly find in biology, these thermodynamic processes proceed by a partially reversible random bouncing of the particles from less probable configurations to more probable configurations in space. The activity of enzymes and catalytic carriers in such systems is due to their capacity for increasing the probability of certain transitional spatial configurations. This is the justification for thinking of metabolism as a spatially organized process of translocation catalysis (Mitchell, 1959*a*, *b*, 1961*a*, *b*, *c*).

It is now necessary to return to our argument about the impossibility of driving substances against the natural diffusion or escaping tendency by chemical reactions, for in the case of the group-translocation type of reaction, the chemical process as we have now defined it contains a spatially directed or vectorial element. In the case of the model of the ATPase that translocates hydroxyl ions, for example, we can see that the movement of hydroxyl across the molecule is dependent upon the electrochemical potential gradient of hydroxyl ion across the system—bearing in mind, of course, that the effective hydroxyl ion activity on the left is not that of the free hydroxyl ion, but is the activity of the OH′ group in inorganic phosphate.

Now, it is clearly possible that the vectorial transfer of hydroxyl ions through the anisotropic ATPase might influence the movement of other molecules or ions (say Na^+ or K^+) along with it by a momentum transfer process. But it is sufficient to note for the purposes of the present argument that even if this were the case, the direction and velocity of the group translocation reaction would still depend upon the diffusion gradient of the effective hydroxyl ion–alkali metal ion 'complex' across the catalytic system as a whole. The particles involved in translocation cannot as a group go against the direction of the natural escaping tendency.

To summarize, it seems that in the metabolic sequence (that we described earlier as diffusion alternating with chemical transformation) no step can properly be said to drive the processes of transport or metabolism forward, for every step in reality represents a tendency for molecules or chemical groups to escape from one place and pass to another, *spontaneously*.

THE CYTOSKELETON

One may well ask how the processes of vectorial metabolism that I am systematically formulating in this paper come to be organized in space, in the macroscopic sense. We will now go on to consider this question very briefly.

When the enzymes, catalytic carriers, and other regulators of diffusion in a system catalysing a metabolic process are distributed homogeneously or randomly, there will, of course, be no macroscopic spatial consequence of the movements of the substrates relative to the catalysts; for such movements, being directed at random, will have no vector component. This is metabolism as we often see it in the test tube. On the other hand, if the microscopically anisotropic catalysts are organized

in space upon or within a macroscopically anisotropic membrane or other structure, the corresponding spatially organized diffusion of the substrate molecules and chemical groups in the system represents the fundamental vectorial component of the metabolic process underlying 'active' transport and developmental processes generally.

The anisotropic array of catalysts required to produce the spatial and metabolic organization of the organism must, of course, be supported upon or within a specifically locating framework (Mitchell, 1959*b*, 1961*a*, *b*). Thus we have formulated, in explicit biochemical terms, the requirement for the cytoskeleton envisaged long ago, for example by Peters and by Needham (see Peters, 1939). In bacteria we are lucky in being able to recognize the cell-wall and the plasma membrane as the main cytoskeletal components—the backbone, as it were, of the whole spatial metabolic organization (see Mitchell & Moyle, 1956*a*).

I hope it will now be possible to see more clearly the practical aim of this rather abstract exposition. My aim has been to draw your attention to the spatial asymmetry of the individual catalysts and the spatial asymmetry of the organized polymolecular systems in which they reside. For this spatial asymmetry can properly be regarded as a primary cause (or specification) of the vectorial diffusion processes of organized metabolism and growth.

It seems rather unlikely that during the growth of micro-organisms many of the enzymes and catalytic carriers of the cell envelopes and cytoplasm could be synthesized at their sites of activity, for the necessary synthetic machinery could hardly be fitted into the available space; and indeed, there is evidence, which will be discussed in this symposium, that the proteins (or at least their subunits) are made in the microsomal particles. We must therefore consider the possibility that the very catalytic proteins that are to cause and control the translocation of specific substrates across the plasma membrane, or the translocation and incorporation of specific chemical groups into cell-wall polymers, and so on, must themselves possess the specificities that will allow them to diffuse to and become specifically bonded into their sites of activity in the polymolecular fabric of the cell envelopes and cytoplasm during growth and adaptation (Mitchell, 1959*b*, *c*, 1961*b*). Thus, we are given a new reason for investigating the idea, originally suggested by Green (1957), that enzymes and catalytic carriers have a dual specificity—one being the substrate specificity, and the other being a locational specificity dependent on the shape and properties of the surface of the protein. According to this conception the locational bonds would represent the articulations between the 'small bones' of the cytoskeleton.

It is against this kind of background that the studies of the distribution of enzyme activities between the plasma membrane and cytoplasm fractions of microorganisms are of real biological interest. I do not propose to dwell upon distribution studies; Dr Dawson will be dealing with them in some detail. But I think it would be appropriate to mention just a few germane facts. Some time ago, Keilin & King (1960) showed that after the succinic dehydrogenase protein of the cytochrome complex of the mitochondrial membrane fraction has been displaced (or 'solubilized') by changing the physical conditions of the system, it will, under normal physiological conditions, pass back into fresh succinic dehydrogenase-poor cytochrome complex, becoming 'insoluble' and functional again as part of the succinic oxidase system.

Dr Moyle and I have succeeded in showing a similar reversibility of the binding of α-ketoglutarate dehydrogenase in the membrane complex of *Micrococcus lysodeikticus* (see Mitchell, 1961*b*). Very recently, Green and his collaborators (Green, Tisdale, Criddle & Bock, 1961) have isolated a protein of low water solubility from mitochrondria which may represent an important structural element in the membrane, and they have obtained some evidence that this protein forms complexes with certain of the enzymes of the electron and hydrogen transport system. Miss Thomson and I have found that about 20 % of the total nitrogen of the plasma-membrane material of *M. lysodeikticus* is soluble in chloroform + methanol (2 + 1, v/v), and that practically all this nitrogen is accounted for by a protein of low water solubility, the amino acid analysis of which shows an unusually high proportion of non-polar amino acids. The surface-active properties of this protein suggest that it is an important structural element of the plasma membrane. The studies of protein synthesis that Dr McQuillen, Dr Dagley, and Dr Hunter are to describe at this symposium and the ideas on cell-wall synthesis that Dr Salton has already discussed presumably require spatially anisotropic molecular complexes in microsome and plasma membrane, and the experimental work on the reversible dissociation of these supramolecular complexes is beginning to support a jig-saw puzzle view of much of the cytoskeleton, many of the bits of the jig-saw staying together mainly because they fit. One would say, in more strictly chemical terminology, that the molecules of the cytoplasmic and membrane complexes are probably mostly linked together by residual valencies and by hydrophilic bonding, as, for example, in the case of the four subunits of haemoglobin (see Kendrew, 1961).

Now I would like to return to the main theme of my paper and describe very briefly some experiments that we have recently done in Edinburgh in an effort to demonstrate the anisotropy of certain enzyme systems in the plasma membrane of bacteria.

VECTORIAL METABOLISM

Many of the current ideas about the molecular mechanism of metabolically driven ion-transport begin with the assumption that the components of the metabolic electron and hydrogen transport system are arranged in a spatial sequence across the membrane through which transport occurs. Lundegardh (1945) was the originator of this idea, which we can represent as shown in Fig. 2. In terms of enzymology, this might imply that cytochrome oxidase (or other terminal catalyst for donating electrons to oxygen) would be situated on the right, and that DPNH dehydrogenase and succinic dehydrogenase, for example, would be at the left-hand side; so that the passage of electrons through the membrane (perhaps via the cytochrome Fe^{2+}/Fe^{3+} system) would form OH' ions on the right and H^+ ions on the left in equal numbers.

Since biochemists have often considered some such spatial arrangement of the cytochrome system and associated enzymes across mitochondrial and other membranes, and have assumed that it might be of physiological importance (see Robertson, 1960), I thought it would be useful to attempt to test the possible asymmetry of the oxidation-reduction processes across the plasma membrane of *Micrococcus lysodeikticus* by a suitable combination of metabolic and transport studies. But, before I talk about this work, it will be necessary to give you some background facts.

Some years ago, Dr Moyle and I found that the bulk of the cytochrome system of *Micrococcus lysodeikticus* is present in the plasma membrane of the cells. We also found that the plasma membrane contains several oxidative enzymes, including succinic oxidase. At pH 7, the plasma membrane of *M. lysodeikticus* is impermeable to hydrophilic solutes such as ATP, DPN, glucose, succinate, malonate, and ferricyanide (Mitchell & Moyle, 1956*b*, and unpublished observations). On the other hand, it is freely permeable to lipid-soluble solutes such as ethanol. Recently I have confirmed the observation of Gilbey & Few (1958) that H^+ ions pass only slowly through the plasma membrane of *M. lysodeikticus*, and have made the surprisingly elementary discovery that uncouplers of oxidative phosphorylation and assimilation, such as 2:4-dinitrophenol, salicylate, dicoumarol, and azide, catalyse the equilibration of H^+ ions in proportion to their uncoupling activity (Mitchell, 1961*d*).

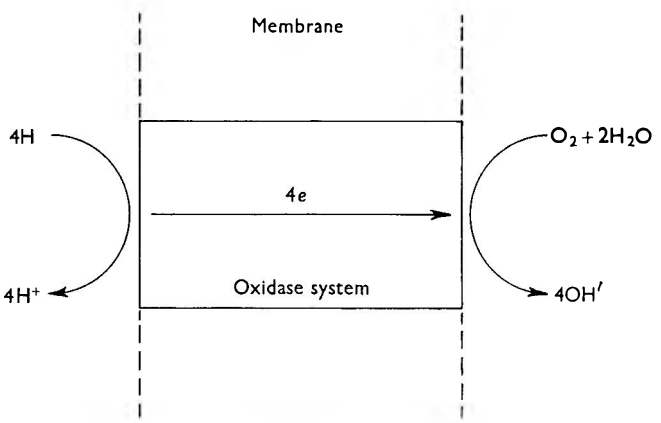

Fig. 2. Diagram of anisotropic oxido-reduction system after Lundegardh (1945).

With these facts as a background, let us focus attention upon the succinic oxidase system in the membrane of *Micrococcus lysodeikticus*. The intact cells oxidize externally added succinate rapidly, using molecular oxygen—but this oxidation is not depressed by the presence of malonate outside the cells. (Malonate, of course, is the classical competitive inhibitor of succinic dehydrogenase.) If, however, the pH of the cell suspension is briefly depressed to 5 so as to let in externally added malonate without otherwise damaging the cells, the oxidation of succinate is almost completely abolished. These observations demonstrate that the succinate and malonate accepting region of the succinic oxidase system of this organism is situated at the inner side of the plasma membrane as shown in Fig. 3. I should, perhaps, remind you that the succinate cannot pass spontaneously through the membrane as such, but goes via a highly specific catalytic system, probably as succinyl, which we will have to take for granted for the purpose of the present argument.

The second fundamental question, concerning the possible asymmetry of the succinic oxidase system that one would naturally ask is, where, in relation to the plasma membrane, is the cytochrome oxidase? A few years ago, Chance (1953*a*, *b*) described a carbon monoxide spectrum of the cytochrome oxidase of this organism, and so we carried out experiments designed to locate this terminal oxidase. To our

surprise we discovered, in the course of these studies, that the respiration of *Micrococcus lysodeikticus* is not depressed by 10^{-4} M-cyanide or 10^{-2} M-azide—indeed, it is stimulated by 30–50 %—and so we were forced to the conclusion that this highly aerobic organism does not contain a cytochrome oxidase of normal cyanide or azide sensitivity. This being the case, we had to abandon our plan to locate the

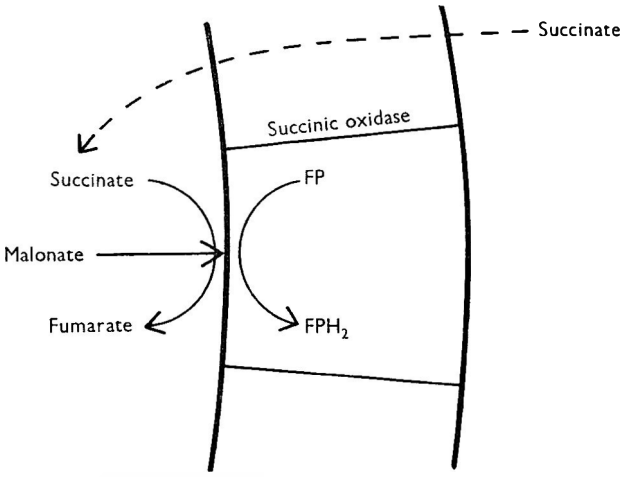

Fig. 3. Arrangement of succinic dehydrogenase flavoprotein (FP) relative to the plasma membrane of *Micrococcus lysodeikticus*. Malonate can inhibit only from inside.

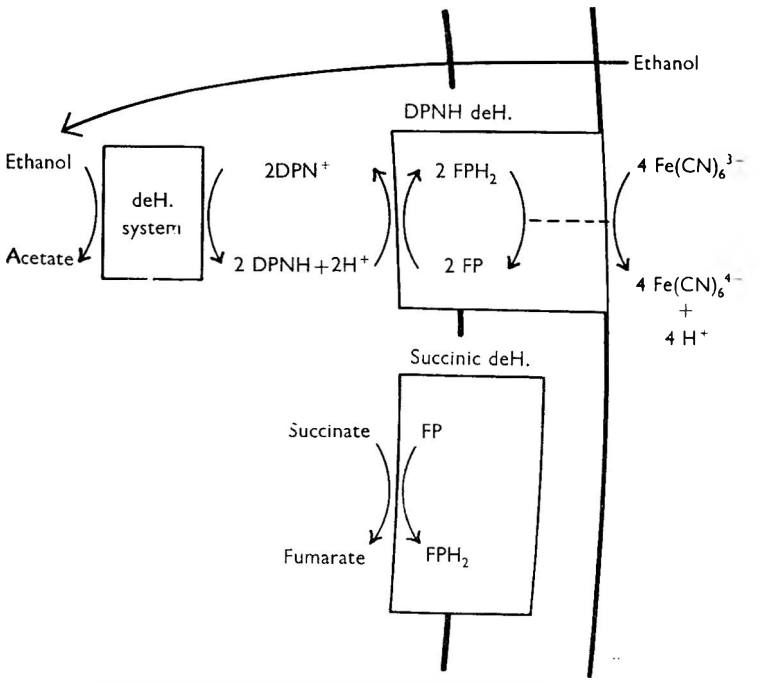

Fig. 4. Suggested arrangement of dehydrogenase systems in relation to reduction of external ferricyanide, forming hydrogen ions *outside* the plasma membrane of *Micrococcus lysodeikticus*. The dotted line may represent a quinol/quinone system.

oxidase with non-penetrating inhibitors. Instead, we decided to search for an outlet of electrons on the external surface of the plasma membrane by using the non-penetrating ferricyanide ion as electron acceptor in place of oxygen, and to look for a possible inhibitory action of ferricyanide on oxygen uptake. We observed that, although external ferricyanide could not substitute for or inhibit molecular oxygen uptake in the oxidation of succinate by suspensions of intact cells, it could accept electrons arising from the oxidation of ethyl alcohol to acetic acid via a DPN-linked dehydrogenase system. Further, alcohol oxidation was carried out at

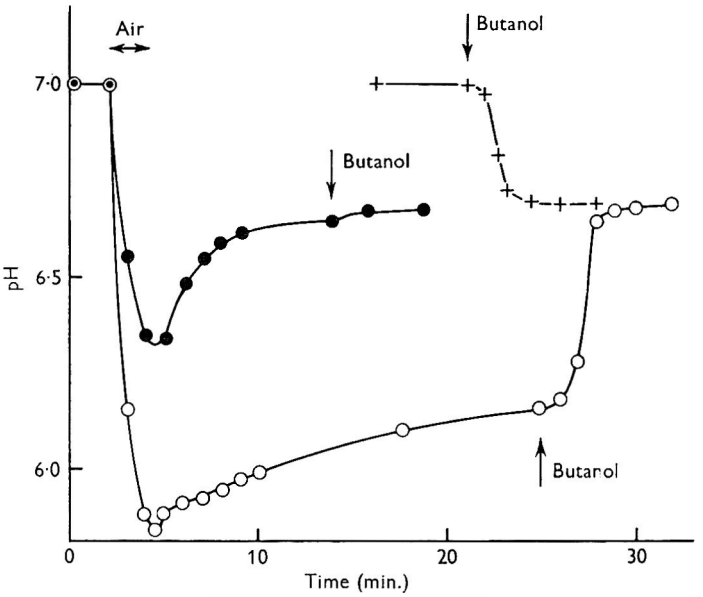

Fig. 5. Change of pH of suspension medium (0·1 M-KCl) by suspensions of *Micrococcus lysodeikticus* (10 mg. dry wt./ml.). The washed suspensions (which exhibit vigorous endogenous respiration) are initially adjusted to pH 7 anaerobically, bubbling with pure N_2. Lower curve (—○—○—), suspensions aerated for 2 min. and then immediately made anaerobic again. Butanol added (5 %, v/v) at arrow. Similar experiment in presence of 10^{-3} M-2:4-dinitrophenol (—●—●—). Experiment omitting aeration, and without addition of dinitrophenol (— + — + —).

the same very rapid rate by ferricyanide as by molecular oxygen; and the hydrogen ions produced in the course of the reduction of ferricyanide were found by titration in unbuffered incubation media to be liberated exclusively outside the membrane of the cells. Since externally added DPN could be shown not to participate in the DPN-linked dehydrogenase systems of intact cells, and since the normal DPN-linked reactions ceased if the cell membrane were damaged so as to allow the endogenous DPN to escape, we concluded that, using ferricyanide as terminal electron acceptor, the oxido-reduction systems are probably arranged in some such way as illustrated in Fig. 4.

We were particularly impressed by the fact that, using ferricyanide as terminal electron acceptor, the hydrogen ions produced by the oxidation of alcohol were all released outside the membrane. This led us to inquire whether a similar production of external acid occurred when oxygen was used as terminal electron acceptor; for

it would be expected that, since the reduction of oxygen can be equivalent to the production of hydroxyl ions, the hydrogen ions would be neutralized by hydroxyls if the site of the oxygen-terminal oxidase were outside the membrane, while they would not be neutralized if the oxygen-terminal oxidase were inside the membrane. We assumed, of course, that the oxygen, unlike the ferricyanide, can pass readily through the membrane. To try to locate the production of OH' ions from the molecular oxygen, we measured the effect of a brief period of aeration on the external pH of

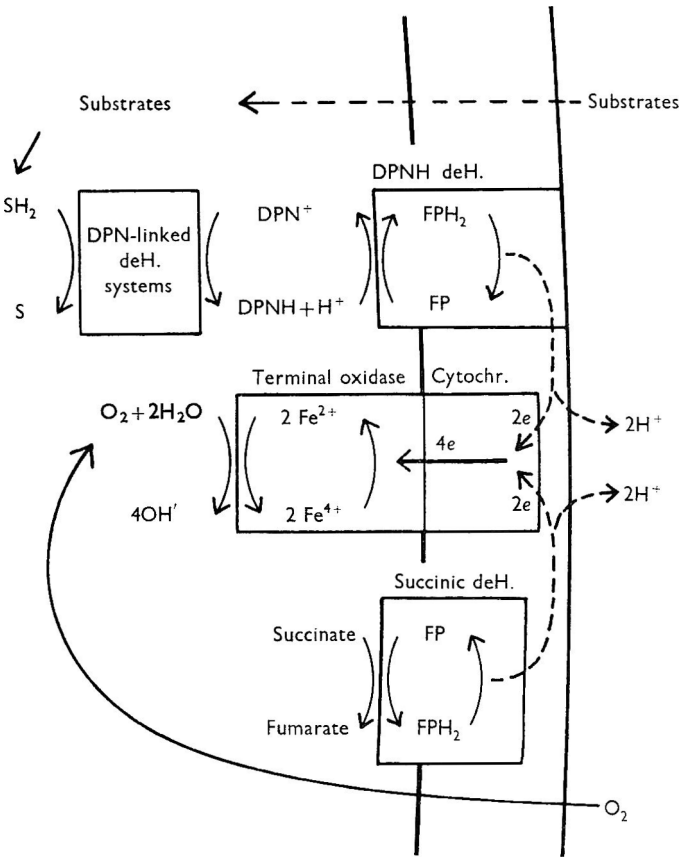

Fig. 6. Suggested arrangement of dehydrogenase systems to account for shift of 'acid' across the plasma membrane of *Micrococcus lysodeikticus* on aeration. Oxidizable substrates in cell are represented as SH_2, and flavoprotein as FP. The dotted lines may represent quinol/quinone systems. The main feature of the system is that hydrogen atoms pass to outer surface of membrane, where they give up electrons that flow back, down to oxygen on the inside. It is like the system suggested by Lundegardh (1945), but bent back on itself.

unbuffered suspensions of *Micrococcus lysodeikticus* containing endogenous substrates. Figure 5 shows a typical group of observations. In normal cell suspensions, the admission of air causes a rapid depression of the pH of the suspension medium, and when, two minutes later, anaerobic conditions are reimposed by bubbling with pure nitrogen, the pH rises somewhat and then comes almost to rest about 0·8 units

lower than it started. The addition of *n*-butanol at this point to give a final concentration of 5 % (v/v) allows the pH to rise to approximately 6·6—the same pH as that obtained if butanol is added at the beginning of the experiment, no air having been admitted. As, on breaking the plasma membrane with *n*-butanol, the same final pH was attained whether the cell suspension had been aerated for 2 min. or not, it was evident that the depression of the pH of the suspension medium could not be due to net acid production, but must be caused by the development of an asymmetry of the electrochemical potential of H^+ across the membrane. This was confirmed by the addition of 2:4-dinitrophenol at the beginning of the aeration experiment, for, as would be expected, the dinitrophenol decreased the asymmetry without affecting the final equilibrium pH. It was significant that in the normal cell suspensions, the initial rate of effective proton transfer during aeration corresponded to approximately 50 μequiv. H^+/g. cell dry wt. min., representing the release of between one and two protons outside the membrane (and an equivalent number of hydroxyl ions inside) per oxygen atom consumed. Figure 6 shows a tentative scheme to account for these observations. Experiments now in progress are providing similar evidence in the case of *Escherichia coli* (strain ML30).

CONCLUSIONS

Let me conclude my paper by asking: why is the oxido-reduction system anisotropic, causing the molecular oxygen and substrates that diffuse from the medium to liberate OH' ions in the cytoplasm and H^+ ions outside? I believe the answer to be that this primary type of anisotropy has evolved because it causes, by processes of exchange, other secondary asymmetries that underlie the activities of assimilation and growth. One directed or vectorial chemical process tends to cause a primary asymmetry that may, in turn, lead to other secondary vectorial or transport processes and corresponding asymmetries. The oxido-reduction asymmetry is probably the cause of the membrane potential, it may well cause the asymmetry of distribution of Na^+ and K^+, and of other substrates (such as sugars and amino acids) across the membrane; and it may, as I have recently suggested (Mitchell, 1961*c*) give rise to oxidative phosphorylation by allowing a hydroxyl-translocating ATPase reaction (Fig. 1) to run backwards across the membrane. I would like to mention, in passing, that Dr R. E. Davies has drawn my attention to the fact that a suggestion, formally akin to the latter, was tentatively put forward some ten years ago by Davies & Ogston (1950) and by Davies & Krebs (1952).

We must not lose sight of the fact that transport processes in biology are integral with the activities of growth and survival. When we separate transport from morphogenesis, growth, and movement in living organisms, the separation is not real, but abstract. Thus, it emerges that the activities of biological transport represent the elusive directiveness of the phenomena of life. Scalar (or directionless) biochemistry is a subject of dead things, like crystals. To describe the flame-like properties of living things, we have to represent the metabolic processes as projections in space as well as in time. This requires the recognition and development of a new subject: the subject of vectorial chemistry. Perhaps, once again, the exact sciences are to receive inspiration from biology!

I am indebted to many colleagues, and especially to Dr Jennifer Moyle, for helpful discussion during the preparation of this paper. It is also a pleasure to acknowledge the receipt of grants from the Nuffield Foundation in aid of this work.

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The Bacterial Cytoplasmic Membrane

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Bacteria are normally regarded as being extremely tolerant to osmotic changes in their external medium (Stuart, Frey & James, 1933). Nevertheless, some particularly Gram-negative organisms harvested in the log phase (Sherman & Albus, 1924) undergo either plasmolysis or plasmoptysis when placed in media containing varying salt concentrations (Winslow & Walker, 1939; Mitchell & Moyle, 1953). Osmotic activity in bacteria is also displayed by their ability to concentrate certain amino acids against concentration gradients (Gale, 1943) and in their selective permeability, particularly towards organic acids (Davis, 1956; Ajl, 1959).

The shrinkage of cytoplasm away from the cell wall, which may be seen by light microscopy during plasmolysis, early led to the prediction that there was a permeability barrier between the cell wall and the inner cytoplasm (Fischer, 1903). From the sensitivity of this barrier to organic solvents and the penetration of lipotrophic material (Eisenberg, 1910) it was also predicted that this barrier consisted of a membrane containing a high lipid content (Overton, 1899). This prediction has been amply confirmed both by the demonstration of a plasma membrane by electron microscopy and from direct chemical analysis of isolated membranes.

Electron microscopy. In ultrathin sections of numerous types of bacteria, a thin osmiumphilic layer is seen sandwiched between the cell wall and the bulk of the cytoplasm (Kellenberger & Ryter, 1958). Where the knife cut is at right angles to this layer it is revealed as a membrane of two dense outer layers each 20–30 Å. wide enclosing a less dense layer 50–80 Å. wide. This double membrane is often more clearly revealed in permanganate- or formalin-fixed material which has been stained with uranyl nitrate after fixation (Pl. 1, fig. 1) (North, 1961; Conti & Gettner, 1962). The dimensions of the cytoplasmic membrane in bacteria are thus similar to membrane structures in animal and plant cells associated with osmotic or secretory functions (Palade, 1956). Measurements of sections of artificially prepared lipoprotein suggest that the clear inner layer is lipid. This is also supported by the heavy staining with uranyl salts. The 50–80 Å. spacing agrees well with that expected from two fatty acid chains each of 15–18 carbons in length (Trurnit & Schidlovsky, 1960; Stockenius, 1960). As seen by electron microscopy dimensions of the bacterial cytoplasmic membrane agree well with the dimensions and structure suggested by Davson & Danielli (1943), for a generalized type of osmotically active cell membrane. That it is the main osmotic barrier is amply confirmed by the osmotic properties of protoplasts and spheroplasts where the cell wall is either wholly or partially removed (Mitchell & Moyle, 1956; Britt & Gerhardt, 1958; McQuillen, 1958).

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Although in most sections the cytoplasmic membrane appears to be firmly attached to, or associated with the cytoplasm, and to separate from the cell wall, this is not always the case. Even in Gram-negative organisms where plasmolysis is more readily demonstrated, the membrane may often remain partly attached to the cell wall and thus detached from the cytoplasm (Chapman, 1959). It is true that cell-wall preparations prepared by prolonged shaking with glass beads (Salton, 1961) are devoid of the enzymic activities and lipoprotein associated with the cytoplasmic membrane. However, when prepared by other methods such as the Hughes press (Hughes, 1951) or French press (Milner, Lawrence & French, 1950) fractions containing both cell wall and the bulk of the membrane are readily obtained both from Gram-negative and Gram-positive organisms, essentially free from cyto-

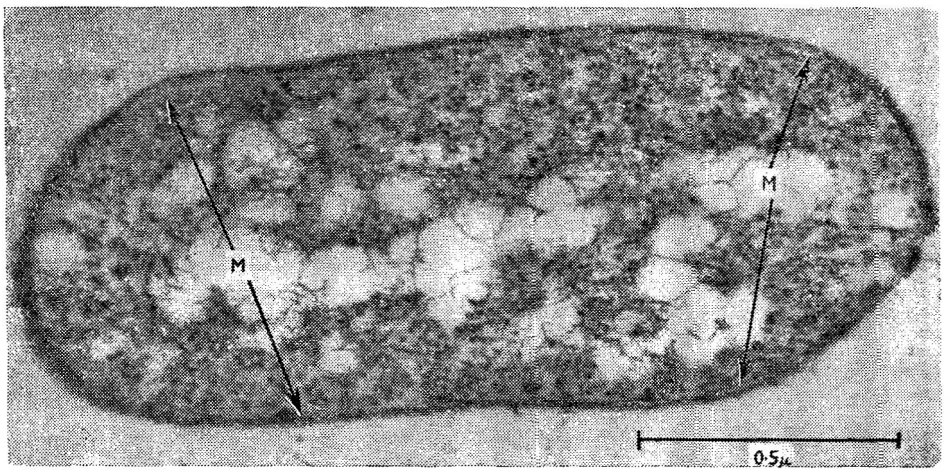

Fig. 1. Ultrathin section of *Escherichia coli*. An exponentially growing culture of *E. coli* 15T was harvested and fixed 25 hr. by suspension in 10% formalin in phosphate buffer (pH 7·4). After dehydration in ethanol the cells were placed in 1% uranyl nitrate in ethanol overnight, washed in ethanol and embedded in butyl methacrylate. Polymerization was carried out at −70° by radiation from a Cobalt-60 source. Electron micrographs were taken in a RCA EMUZB microscope.

plasmic contamination (Hunt, Rodgers & Hughes, 1959). Such preparations when further fragmented, for instance by ultrasonics, yield particulate fractions in which the carbohydrate of the cell wall is still associated with lipoprotein (Hughes, unpublished), suggesting that the membrane may in fact be more firmly associated with the cell wall than the separation during plasmolysis suggests (Mitchell & Moyle, 1956).

Preparation of isolated membranes. Lipoprotein-rich fragments of membranes are commonly prepared by osmotically shocking or bursting by other means sucrose-stabilized protoplasts of organisms such as *Micrococcus lysodeikticus* and *Bacillus megaterium* (Wiebull, 1953). In these organisms, lysozyme removes the bulk of the cell wall (Wiebull, 1958). The isolated fragments (so-called 'ghosts') appear in metal-shadowed specimens to be approximately 500 mμ in cross section, about 100 Å. thick, and to be free from adhering cell wall and cytoplasm; they nevertheless may contain up to 20% of their dry weight as carbohydrate, the rest being

mainly lipoprotein. Fractions rich in lipoprotein but containing various amounts of cell wall may also be prepared by similar methods from spheroplasts prepared by lysozyme treatment in the presence of versene (Repaske, 1956). The action of autocatalytic enzymes (Mitchell & Moyle, 1956), unbalanced growth produced by the action of penicillin or other antibiotics (Lederberg & St Clair, 1958; McQuillen, 1958) or the omission of an essential cell-wall constituent such as lysine or diaminopimelic acid (McQuillen, 1958) have also been used to prepare spheroplasts from which membrane-enriched fractions may be isolated. Protoplast preparations of certain yeasts have been prepared by the action of carbohydrases from *Helix pomatia* (Eddy & Williamson, 1957).

In addition to these chemical and enzymic methods for isolating cytoplasmic membranes, cell fractions can be prepared by mechanical rupture of the cell wall followed by procedures to remove the bulk of the cytoplasm; these contain the bulk of the cell wall and cytoplasmic membrane (Hunt, Rodgers & Hughes, 1959). Such cell-wall membranes prepared from *Pseudomonas fluorescens* were made by rupture of the cells in the Hughes press, treatment with deoxyribonuclease, subsequent emptying by shaking with glass beads and extraction with phosphate buffer. The present author has used this procedure more recently with a wide range of micro-organisms to yield preparations which typically contain the bulk of the cell wall and have an enzymic constitution similar to membrane preparations from protoplasts. Similar preparations may be obtained from cells ruptured in the '*x*' press (Edebo, 1960) or the French press (Milner *et al.* 1950) although these presses appear to give greater fragmentation of the wall (Hughes, unpublished). In some cases varying amounts of cell-wall material may be removed from cell-wall membrane preparations by subsequent treatment with lysozyme (Hunt *et al.* 1959) or other carbohydrases (Hughes, unpublished); usually this leads to fragmentation and yields preparations which appear similar to 'ghosts' by electron microscopy but which still consist of particles which contain both lipoprotein and cell-wall carbohydrates.

Although it is obviously of advantage especially for chemical analysis to prepare the cytoplasmic membrane free from cell wall, it is often also advantageous to be able to isolate cell-wall membranes. The membrane in these appears to be structurally intact and easily freed from supernatant fractions containing cytoplasm particles such as ribosomes. These in turn are relatively free from enzymes and other material associated with particles, for instance so-called 'oxidosomes', which are now generally assumed to be comminuted cytoplasmic membrane.

Chemical analysis of cytoplasmic membrane. The most constant feature of cytoplasmic membrane and cell-wall membrane fractions is their high concentration of lipid, up to 30 % of dry weight, which is mostly present as phospholipid. Carbohydrate is also generally present but this depends on the proportion of adhering cell wall. Where the cell wall is absent, amino sugars are absent and the carbohydrate is probably not derived from the cell wall. For instance, in *Micrococcus lysodeikticus*, mannose in contrast to glucose is found in the membrane ghosts. The carbohydrate of cell-wall membrane preparations appears indistinguishable from that of purified cell walls (Hughes, unpublished). The amino acid composition of purified membranes appears similar to cell protein and is unlike that of wall polypeptide in that it contains all the common L-amino acids and no D-amino acids

(Gilbey, Few & McQuillen, 1958). The lipid composition of membranes from *M. lysodeikticus* has recently been analysed more fully by McFarlane (1961*a*, *b*). About 80 % of the total lipids is phospholipid, the remaining 20 % being neutral lipid, mainly a diglyceride. Of the phospholipid, about 80 % is diphosphatidyl glycerol, the remainder consisting of phosphatidyl inositol and a complex which yields glycerol, mannitol and fatty acids on hydrolysis. The fatty acids in all these fractions were mainly C_{15} anteiso and iso-acids, an observation which fits well with the 50–60 Å. spacing seen in the electron micrographs. The lipid content of these preparations represented approximately 80 % of that of the intact cell. Cell-wall membranes from *Pseudomonas fluorescens* and *Lactobacillus arabinosus* contain at least 90–95 % of the total cell lipid. The main fatty acids from these organisms are similar to those from *M. lysodeikticus*, and staining reactions suggest they are also present mainly as phospholipid (Hunt *et al.* 1959). In *L. arabinosus* lactobacillic acid can be detected in the membrane extracts and in *P. pseudomonas* an unidentified C_{17} branched acid was detected (Hughes, unpublished).

Table 1. *Enzymic activities of cytoplasmic membranes and particulate fractions from bacteria*

Organism	Cell fraction	Enzyme activity	Reference
Bacillus megaterium	Membrane and particles	Succinic, malic, lactic oxoglutarate dehydrogenases, DPNH oxidase	Storck & Wachsman (1957); Wiebull (1956)
B. stearothermophilus	Membrane and particles	—	Georgi, Militzer & Decker (1955)
Staphylococcus aureus	Membranes	Succinic, lactic, formic and α-glycerophosphate dehydrogenases, acid phosphatase	Mitchell & Moyle (1956)
Pseudomonas fluorescens	Cell-wall membrane and particles	Nicotinic hydroxylase, succinic dehydrogenase, DPNH oxidase, ATPase, and malic oxidase	Hunt *et al.* (1959), Hughes (unpublished)
Azotobacter vinlandii	Membrane and particles	Hydrogenase, DPN oxidase, oxidative phosphorylation	Cota-Robles, Marr & Nilson (1958)
Mycobacteria sp.	Particles	TPNH and DPNH oxidase, oxidative phosphorylation, succinic dehydrogenase	Brodie & Ballantyne (1960)
Escherichia coli	Cell-wall membranes	Numerous dehydrogenases, ATPase	Hughes (unpublished)
Lactobacillus arabinosus and other lactobacilli	Cell-wall membranes	ATPase? Hexokinase	Hughes (unpublished)
Alkaligenes faecalis	Membrane and particles	Oxidative phosphorylation, ATPase and dehydrogenases	Shibko & Pinchot (1961); Hughes (unpublished)

The enzymatic function of the cytoplasmic membrane. The cytoplasmic membrane was first established as the site of respiratory enzymes and cytochrome-linked electron transport by studies on highly purified 'ghost' preparations from *Bacillus megaterium* (Storck & Wachsman, 1957; Wiebull, 1953). Since then a similar localization of enzymes concerned with respiration has been found in many other aerobic organisms (Table 1). In addition to these enzymes in membranes, numerous particu-

late fractions which have a similar enzymic constitution to the membranes have been isolated from mechanically broken cells (Table 1). These particulate enzyme fractions usually consist mainly of lipoprotein granules, are often heterogeneous in size and contain ribonucleoprotein, which, when it can be separated, is usually without enzymic activity (Hunt *et al.* 1959). The enzymic activity associated with the lipoprotein particles and membranes is often difficult to solubilize without destroying their structure. However, by treating them with detergents or other surface-active reagents such as deoxycholic acid (Hunt, 1959), which disassociate lipid and protein, the enzymes sometimes may be brought into solution and further purified. Granules which reduce tetrazolium in whole cells have been regarded as equivalent to animal and plant mitochondria. These have been identified with the isolated lipoprotein granules as 'bacterial mitochondria' (Mudd, Kawata, Payne, Sall & Takagi, 1961). There seems little doubt now, however, that most if not all of such preparations have been derived by comminution of the cytoplasmic membrane from which they can be readily prepared by, for instance, treatment with ultra-sound (Hunt *et al.* 1959). If analogies for bacterial structures are to be sought in the higher cells, it would seem more correct to regard the cytoplasmic membrane as equivalent to the 'christae mitochondriales' of animal or plant mitochondria (Green, 1961; Marr, 1960). It would be expected in this case that intact membrane preparations or cell-wall membranes would carry out oxidative phosphorylation. So far this has only been demonstrated in particulate fractions from micro-organisms (Brodie & Ballantyne, 1961; Shibko & Pinchot, 1961). It is possible that this failure to phosphorylate oxidatively is due to either the lack of essential factors present in supernatant fractions. Crude supernatants from disrupted bacteria cannot always be added to the particulate fractions because they contain material and enzymes such as adenosine triphosphatase (ATPase) which interfere with the measurement of oxidative phosphorylation. In the case of cell-wall membranes, the effect of freezing and thawing may also be deleterious.

In addition to enzymes associated with energy production many others have been shown to occur in the cytoplasmic membrane or in particles derived from it. These include enzymes such as formic hydrogenlyase, nicotinic acid and other aromatic hydroxylases and many polyol-dehydrogenases which occur exclusively in microorganisms (Table 1).

Most of the work on membrane and particulate-bound enzymes has so far been done with aerobic organisms, and apart from the demonstration of particulate enzymes such as hydrogenase (Gest, 1954) in *Clostridium pasteurianum*, little work has been reported on the membrane of facultative or obligate anaerobes. Recently cell-wall membranes have been prepared from a number of lactobacilli. These organisms, although obtaining the bulk of their energy by anaerobic glycolysis, nevertheless can utilize oxygen to oxidize substrates such as pyruvate and lactate at low rates. This oxidation is mediated through riboflavin electron-transport systems; cytochromes are normally completely absent. Neither the glycolytic enzymes, with the exception of some weak hexokinase activity, nor the flavo-enzyme systems were found in the cell-wall membranes. The only activity so far found consistently is ATPase. The ATPase in these cell-wall membranes has an unusually low pH optimum (5·2–5·3), is Mg^{2+}-activated but is not activated by *o*-dinitrophenol. It has not so far been found possible to free it from the cell-wall

membrane nor to show definitely that it is associated with the lipoprotein portion of this fraction. However, similar ATPase activity has been demonstrated in membranes prepared from *Pseudomonas fluorescens*, *Escherichia coli*, and *Aerobacter aerogenes*. The enzyme from all these organisms does not liberate on orthophosphate from a wide range of organic phosphates including AMP; a low rate of hydrolysis is found with other nucleoside triphosphates and ADP. The consistent occurrence of an ATPase in the bacteria is of interest in connexion with the suggested role of this enzyme in ion transport and permeability (Whittam, 1961).

The possible role of the cytoplasmic membrane in protein synthesis is discussed by Hunter in this symposium. It is of significance in this connexion that most purified membrane preparations contain about 2 % ribonucleic acid (Hunter, Brookes, Crathorn & Butler, 1959; Nisman & Fukuhora, 1961), and that this also forms a constant feature of cell-wall membrane preparations (Hunt *et al.* 1959). It is difficult to remove this without chemical treatment or disruption of the membrane (Hunt *et al.* 1959).

It seems possible that the cytoplasmic membrane is the site of synthesis as well as of activity of permeases (Kepes, 1960) and other inducible membrane-bound enzymes such as the nicotinic hydroxylase system (Hunt *et al.* 1959). Also of interest in this connexion is the apparent lability of respiratory cytochromes which appear to be firmly bound and almost exclusively in the membrane but yet alter concentration rapidly when oxygen tension in the growth medium is changed (Lenhoff & Kaplan, 1956). Such problems, which are connected with the synthesis of the membrane itself, are open to an experimental approach now that methods for isolating the membrane from a wide range of microorganisms are available.

SUMMARY

A membrane sited between cytoplasm and cell wall forms the permeability barrier in bacteria. It is rich in lipoprotein and in the electron microscope is seen to consist of two parallel outer protein layers between which there is a lipid layer. In aerobic organisms the membrane is the site of cytochrome-mediated substrate oxidation. Oxidative phosphorylation has not been shown to take place in the membrane. In lactobacilli, neither anaerobic glycolysis nor flavine-linked oxidation takes place in the membrane. The membranes from most organisms so far examined have a firmly bound characteristic ATPase. The intact cytoplasmic membrane may thus be considered as similar in function, structure and chemical composition to the christae of animal and plant mitochondria. Particulate fractions isolated from mechanically disrupted cells often mislabelled 'bacterial mitochondria' are probably comminuted membrane.

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The Chromatophores of Photosynthetic Bacteria

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In 1952, Schachman, Pardee & Stanier discovered that the entire complement of photoreactive pigments (bacteriochlorophyll and carotenoids) in the photosynthetic organism, *Rhodospirillum rubrum*, was bound to large particles, known as chromatophores. These were readily separable from cell-free extracts by centrifuging and had a sedimentation constant of about 190 Svedberg units. Since then similar particles have been shown in many other photosynthetic bacteria including Chromatium (Newton & Newton, 1957) and *Chlorobium thiosulphatophilum* (Gibson, 1957; Hulcher & Conti, 1960). Preparations of chromatophores in relatively pure form have been made by differential and gradient centrifugation (Newton & Newton, 1957; Anderson & Fuller, 1958; Frenkel & Hickman, 1959; Cohen-Bazire & Kunisawa, 1960). The particles are antigenic, which might provide another possible method for their purification (Newton & Levine, 1959; Newton, 1960).

Structure as revealed by electron microscopy. The chromatophores of *Rhodospirillum rubrum* isolated by Schachman *et al.* (1952) were revealed by electron microscopy as flattened disks, about 110 mμ in diameter. The appearance of the chromatophores in ultrathin sections of cells is in accord with that of the isolated particles (Vatter & Wolf, 1958; Hickman & Frenkel, 1959; Frenkel & Hickman, 1959). Their size varies with the organism, from 50–100 mμ (*Rhodospirillum rubrum*) to 15–25 mμ (*Chlorobium limicola*). In photosynthetically active Athiorhodaceae and in Chromatium the chromatophores are seen as discrete vesicles bounded by a single membrane but in *C. limicola* they appear as opaque granules. The lamellar structures revealed in electron micrographs of plant chloroplasts have been demonstrated only in the chromatophores of *R. molischianum*, an exceptionally large organism (Drews, 1960). The chromatophores of other photosynthetic bacteria do not exhibit any fine structure, though systems of paired lamellae appear together with chromatophores in cells from old cultures of *R. rubrum* (Hickman & Frenkel, 1959). The budding organism, *Rhodomicrobium vannielii*, differs completely from other photosynthetic bacteria in that it has a basic lamellar structure similar to that of the blue-green algae; chromatophores are not discernible (Vatter, Douglas & Wolfe, 1959).

There is evidence that the chromatophores of Chromatium (Newton & Newton, 1957) and *Rhodospirillum rubrum* (Frenkel & Hickman, 1959) are composed of subunits which are functionally complete and which are released by sonic oscillation of whole chromatophores. Electron microscopy of preparations of 'subchromatophores' from *R. rubrum* shows them to be homogeneous and about one-fiftieth the size of a normal chromatophore. These particles are also found in preparations from very young cultures (Frenkel & Hickman, 1959).

The electron micrographs of Vatter & Wolfe (1958) show the chromatophores of the Athiorhodaceae to be packed throughout the cell while those of Hickman &

Frenkel (1959) suggest that they are most numerous at the periphery. This discrepancy might be due to differences in the method of cultivation of the organisms (e.g. light intensity).

The structures to be seen in sections of photosynthetically grown *Rhodospirillum rubrum* vary considerably with the age of the culture (Hickman & Frenkel, 1959). Chromatophores are not present in cells from young cultures (though 'subchromatophores' are present) while lamellar structures occur in sections of cells from old cultures. These observations probably account for the conflicting reports in the literature about the fine structure of photosynthetic bacteria.

Athiorhodaceae can grow aerobically in the dark but do not form the photosynthetic pigments under these conditions. No chromatophores can be seen in sections of organisms which have been grown in this way, suggesting that the particles are formed only under conditions which allow pigment synthesis (Vatter & Wolfe, 1958; Frenkel & Hickman, 1959).

Table 1. *Composition of chromatophores of* Rhodopseudomonas spheroides*

All values except for phosphorus are expressed as % of the dry weight.

Protein	58·4	Bacteriochlorophyll	4·3
Total lipid	24·6	Carotenoid	1·1
Carbohydrate	4·2	Haem	0·015
Nucleic acid	0·94	Acid-soluble iron	< 0·02

Total phosphorus 0·22 μmole/mg. dry wt.
Lipid phosphorus 0·14 μmole/mg. dry wt.

* Unpublished work by M. J. Bull of this laboratory.

Composition. The most detailed analyses have been made with chromatophores from Chromatium (Newton & Newton, 1957; Bergeron, 1959). Besides bacteriochlorophyll and carotenoids (4·2 and 1·5 % respectively of the dry weight) they are rich in protein and phospholipid (61 and 22·3 % respectively). In addition, the chromatophores contain polysaccharide, probably composed of galactose, together with small amounts of ribonucleic acid. The phospholipid fraction contains ethanolamine as the only nitrogenous base. Chromatophores from *Rhodopseudomonas spheroides*, purified by centrifugation on a sucrose gradient, have a similar composition to those of Chromatium (Table 1). They contain, however, considerably less haem and acid-soluble iron. Ethanolamine, but not serine, has been demonstrated in the phospholipid fraction (Dr N. G. Carr, personal communication).

The chromatophores of *Rhodospirillum rubrum* contain choline phospholipids and are rich in cardiolipin (diphosphatidyl glycerol) and galactosyl diglycerides which are major components of chloroplast lipids (Benson, Wintermans & Wiser, 1959; Benson, 1961).

Chromatophores are complete with respect to components of the electron transport chain, containing various cytochromes, flavin and pyridine nucleotide (Newton & Newton, 1957; Hulcher & Conti, 1960; Kamen, 1961); in addition they are exceptionally rich in coenzyme Q derivatives (Lester & Crane, 1959; Rudney, 1961).

Ratios of bacteriochlorophyll and carotenoid to protein and cytochrome have been calculated but these estimates are possibly not of great significance since the

amount of photosynthetic pigment per mg. of chromatophore protein (at least in the Athiorhodaceae) varies considerably according to the conditions under which the cultures are grown (Stanier, 1959; Cohen-Bazire & Kunisawa, 1960). A constant amount of chromatophore protein is apparently formed to which is bound variable amounts of pigment. Hypothetical models of chromatophores must therefore allow a considerable degree of flexibility.

The composition of chromatophores is strikingly similar to that of the electron transport particles of non-photosynthetic bacteria, which may be derived from the cytoplasmic membrane (Marr, 1960). There is indeed evidence that chromatophores are closely associated with this membrane. Thus, the photosynthetic pigments of Chromatium are not released immediately, as are soluble enzymes, upon rupture of whole cells by sonic oscillation (Newton & Newton, 1957). Also, the pigments in a functionally active form are associated with membranous structures ('ghosts') formed by osmotic lysis of lysozyme-protoplasts of *Rhodospirillum rubrum* (Tuttle & Gest, 1959). These observations might, however, be attributed to trapping of the particles in the ruptured membranes.

Biochemical reactions in chromatophores. The discovery by Frenkel (1956) that crude extracts of *Rhodospirillum rubrum* catalyse an anaerobic, light-dependent phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) was soon followed by the demonstration of this reaction in extracts of other photosynthetic bacteria (Frenkel, 1959). Fractionation of the crude extracts has shown that the entire photophosphorylation activity is catalysed by the chromatophores. Purified preparations from *R. rubrum* and Chromatium esterify inorganic phosphate at rates of up to 150 μmoles per hour per mg. of bacteriochlorophyll; the reaction is strictly light-dependent and occurs anaerobically (Newton & Kamen, 1957; Anderson & Fuller, 1958; Frenkel & Hickman, 1959; Geller & Lipmann, 1960). The rate of photophosphorylation is dependent on the protein rather than the bacteriochlorophyll content of the particles (Cohen-Bazire & Kunisawa, 1960). Besides the substrates, inorganic orthophosphate and ADP, the washed chromatophores need magnesium ions and catalytic amounts of a reducing agent such as reduced diphosphopyridine nucleotide (DPNH) or succinate. The reducing agent is apparently required to poise the oxidation-reduction system only when traces of O_2 are present; under completely anaerobic conditions photophosphorylation proceeds in the absence of an external reductant (Vernon & Ash, 1960). Flavine adenine dinucleotide stimulates the activity of chromatophores of *R. rubrum* (Baltscheffsky, 1960) and coenzyme Q derivatives are also required by depleted chromatophores from this organism, grown with diphenylamine to inhibit synthesis of isoprenoid compounds (Rudney, 1961). The reaction is considerably stimulated by the oxidation-reduction dye, phenazine methosulphate, which has a similar effect with plant chloroplasts (Geller & Lipmann, 1960; Arnon, 1961*a*). Photophosphorylation is inhibited by many of the compounds which inhibit oxidative phosphorylation, including antimycin *A* and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (Geller & Lipmann, 1960; Baltscheffsky, 1961).

On the basis of these observations Arnon (1961*a*) has proposed that illuminated chromatophores esterify inorganic phosphate by a mechanism of cyclic photophosphorylation similar to that postulated for chloroplasts. This scheme requires the expulsion of an electron at high energy potential from a molecule of chlorophyll,

at the expense of energy contained in an absorbed quantum of light. The excited chlorophyll thus becomes electropositive and creates a flow of electrons along the electron transport chain; coupled phosphorylation is presumed to occur at sites along this chain.

Recent reports from Arnon's laboratory (Arnon 1961*b*; Arnon, Losada, Nozaki & Tagawa, 1961) have shown that chromatophores also carry out non-cyclic photophosphorylation in which there is net formation of a reductant as well as ATP. Particles from *Rhodospirillum rubrum* form stoichiometric amounts of ATP and DPNH ('trapped' as lactate by the addition of lactic dehydrogenase and pyruvate) when illuminated in the presence of ADP, inorganic phosphate, diphosphopyridine nucleotide (DPN) and an artificial electron donor system, ascorbate plus 2:6-dichlorophenol indophenol.

There is abundant evidence for the proposed coupling of the phosphorylation with light-induced oxidation-reduction reactions, but the actual site(s) and the mechanism of coupling are unknown. The observation that oxidation of cytochrome c_2 in extracts of *Rhodospirillum rubrum* occurs upon illumination only if phosphorylation is proceeding simultaneously suggests that an esterification site is at this step of the electron transport chain (Smith & Baltscheffsky, 1959).

Numerous oxidation-reduction reactions have been observed upon illumination of extracts and purified chromatophores of photosynthetic bacteria. These have been indicated by changes in the absorption spectrum of components of the electron transport chain *in situ* (see, for instance, Smith & Ramírez, 1959; Olson & Chance, 1960; Chance & Nishimura, 1961) or by following the oxidation or reduction of externally added natural cofactors and dyes. Illuminated chromatophores of *Rhodospirillum rubrum* catalyse the reduction of DPN with concomitant oxidation of succinate or reduced flavine mononucleotide (Frenkel, 1958; Vernon & Ash, 1959, 1960). With purified chromatophores only DPN is active; this is in sharp contrast to chloroplasts in which triphosphopyridine nucleotide is the active cofactor Chromatophores of *R. rubrum* also catalyse the light-dependent, anaerobic oxidation of ferrocytochrome *c* or reduced dyes in the presence of an acceptor such as fumarate or DPN (Vernon, 1959; Vernon & Ash, 1960). All these observations provide strong support for Arnon's proposed mechanisms of cyclic and non-cyclic photophosphorylation in bacterial chromatophores.

Enzyme activities detected in chromatophores, which are not light-dependent, include succinic dehydrogenase (Woody & Lindstrom, 1955; Cohen-Bazire & Kunisawa, 1960). Unlike chloroplasts, the bacteria preparations do not contain ribulose diphosphate carboxylase which appears to be a soluble enzyme in these organisms (Fuller & Gibbs, 1959).

Formation of chromatophores. The absence of structures resembling chromatophores in electron micrographs of aerobically grown Athiorhodaceae (Vatter & Wolfe, 1958; Frenkel & Hickman, 1959) suggests that the particles are formed only under conditions that permit pigment synthesis. However, Stanier (1959) has pointed out that the presence of the photosynthetic pigments could alter profoundly the physical properties of a preformed lipoprotein matrix.

There is evidence that pigment formation is closely associated with protein synthesis (Lascelles, 1959; J. Lascelles and M. J. Bull, unpublished). Formation of bacteriochlorophyll by suspensions of *Rhodopseudomonas spheroides* occurs only

under conditions that permit protein synthesis. It is dependent on a nitrogen source and is inhibited by amino acid and nucleic acid analogues; suspensions of mutant strains requiring purines and amino acids require the addition of these metabolites for pigment formation. In addition, ^{14}-C-labelled amino acids are incorporated into chromatophore protein at 2 to 3 times the rate of incorporation into soluble protein during the adaptive synthesis of photosynthetic pigments. These observations as yet provide only slender support for the *de novo* synthesis of chromatophore protein linked to pigment formation and more extensive investigation is necessary before the origin of the structure is understood.

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Bacterial Ribosomes and Protein Synthesis

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Bacterial cells, like other living organisms, contain ribonucleic acid (RNA) both free and in combination as ribonucleoprotein (RNP). The free, or soluble, RNA has a composition different from, and a molecular weight (*c.* 30,000) lower than, that of combined RNA. Neither has a composition obviously related to that of the deoxyribonucleic acid (DNA). In addition to the usual four nucleotides, soluble RNA contains pseudo-uridylic acid, and much of this RNA is thought to function in the transfer of activated amino acids to the site of synthesis of protein. This fraction is known as 'S-RNA' or 'transfer-RNA' or 'acceptor-RNA'. Most of the RNA, however, can be isolated from disrupted cells as *ribosomes* which are RNP-particles of sedimentation constant in the range 20 *S* to 100 *S*. There is controversy as to whether such particles exist *in vivo* and, if so, which sizes occur within living cells. The proportions of the different sizes obtainable from bacteria depend on various factors such as the cultural conditions (growth medium, phase of growth, presence of antibiotics) and the nature of the medium in which the preparation is made. The 'fundamental particles' appear to be 30 *S* and 50 *S* and combination of one of each of these yields a 70 *S* ribosome. Dimerization of the latter gives 100 *S* particles. These changes can be brought about by altering the Mg^{2+} concentration in the medium:

$$100S \rightleftharpoons 2(70S) \rightleftharpoons 2(30S + 50S) \rightarrow \text{Disintegration.}$$
$$Mg^{2+} \text{ concentration decreasing} \longrightarrow$$

Isolated ribosomes appear by electron microscopy to be 100–200 Å. in diameter (Hall & Slayter, 1959; Huxley & Zubay, 1960) and ultrathin sections of bacterial cells if suitably fixed show the cytoplasm to contain electron-dense particles of similar size. There is no indication of an endoplasmic reticulum to which these particles are attached as in some mammalian tissue cells but the particles are generally believed to be ribosomes. On disrupting the cell they are released and, after removing the large cell fragments, can be sedimented by centrifugation for 1–3 hr. at 100,000 *g*. The various sizes can be separated by differential centrifugation or by spinning down through a solution of increasing density, e.g. a sucrose gradient from 5 to 20 % (w/v) (Britten & Roberts, 1960).

When highly purified, ribosomes consist of 50–65 % RNA and 50–35 % protein, and contain cations such as Mg^{2+} or possibly polyamines (putrescine, spermidine, spermine) but no detectable lipid or DNA. The protein is thought to be largely 'structural' and can be separated into more than two dozen basic proteins of average molecular weight about 25,000 (Waller & Harris, 1961). In *Escherichia coli* 80–90 % of these protein chains have *N*-terminal alanine or methionine but whether each ribosome contains all kinds or only one kind is not known. The total protein in 30 *S* and 50 *S* particles would correspond to molecular weights of about 300,000 and 600,000 respectively.

Nucleotides of adenine, guanine, uracil and cytosine are the major constituents of the RNA of ribosomes and the small amounts of pseudo-uridylic acid which are sometimes found may be due to contamination or transient association with transfer-RNA. 30*S* ribosomes contain a single unit of RNA (molecular weight *c.* 500,000, sedimentation constant 14–16*S*); the 50*S* particles contain either two such units or one unit of twice the size (*c.* 23*S*). These high-molecular-weight units can be degraded into subunits by heat, controlled dialysis and other treatments. The relative dispositions of the RNA and protein in ribosomes are not known with certainty but the particles may be sponge-like in form, carrying several times their own weight of water and with the RNA more exposed than in the small RNA viruses.

In addition to containing RNA and structural protein, ribosome preparations frequently exhibit enzymic activity. Much of this may be due to non-specific binding but there is reason to believe that some is inherent. Most of the ribonuclease activity of bacterial cells is demonstrable in purified ribosomes but is latent until the particles have been disrupted, e.g. by urea or chelating agents (Elson, 1961; Bolton *et al.* 1959). Unmasking results in digestion of ribosomal as well as added RNA. Latent deoxyribonuclease activity (Elson, 1961) and non-latent peptidase activity (Bolton *et al.* 1959) have also been reported. Finally, traces of all kinds of proteins may be present as nascent material at its site of synthesis (McQuillen, Roberts & Britten, 1959; Roberts *et al.* 1960).

There is abundant evidence from *in vivo* and *in vitro* experiments that ribosomes are concerned in the synthesis of protein in animals, plants and micro-organisms (McQuillen, 1962). When radioactive precursors to proteins (amino acids or inorganic sulphate) are fed to a growing culture of bacteria, the radioactivity is incorporated in 'nascent protein' bound to ribosomes before appearing in the soluble protein fraction of the cells (McQuillen *et al.* 1959). This is an extremely rapid process and to demonstrate the higher specific radioactivity of the nascent protein it was necessary to carry out *in vivo* experiments lasting only a few seconds. The ribosomes became maximally labelled in 5–10 seconds and the radioactivity was 'chased out' by unlabelled substrates equally rapidly. The radioactivity associated with ribosomes was present in amino acids linked as polypeptides or proteins. Even when $^{35}SO_4^{2-}$ was used for only a few seconds, radioactive cysteine and methionine were found in a number of peptides on partial hydrolysis of the ribosomes. Although this association is transient *in vivo*, it is quite stable *in vitro* and nascent protein has only been removed by treatments which disrupt the ribosomes, e.g. 4 M-urea (McQuillen, 1961).

Preparations were usually made in media containing Mg^{2+} (0·005–0·01 M) such that most of the ribosomes were in the 70*S* or larger form. Reduction to 0·0001 M Mg^{2+} caused dissociation of the bulk of these to 50*S*+30*S* particles which could be separated by centrifugation through a sucrose gradient. Nevertheless, a great deal of the nascent protein still occurred in the 70*S* region, which now contained only a few ribosomes (McQuillen *et al.* 1959). It may be that a small fraction of the ribosomes exists in a form which does not readily dissociate and on which the nascent protein is synthesized. Studies with cell-free fractions of *Escherichia coli* support this possibility. Lamborg & Zamecnik (1960) devised a system containing ribosomes and soluble factors which converted amino acids to peptides or proteins.

This was investigated further by Tissières, Schlessinger & Gros (1960) who showed that the newly synthesized polypeptides were principally associated with the 70*S* region even when reduction of Mg^{2+} concentration had caused the dissociation of the majority of the 70*S* ribosomes to 50*S* + 30*S* particles. It was estimated that 5–10 % of the ribosomes might be in a non-dissociating, 'active 70*S*' form. 30*S* and 50*S* particles had been previously shown to reassociate to 70*S* when the Mg^{2+} concentration was restored to 0·01 M but this procedure did not yield 'active 70*S*' ribosomes.

Until recently it was generally assumed that the RNAs of ribosomes acted as templates for organizing the synthesis of the many different kinds of protein. Each bacterial cell contains 10,000 or more ribosomes and makes perhaps 1000 different proteins, and it is important to know whether or not different proteins can be made on the same ribosome or whether a specific kind of ribosome is needed for each specific protein. If DNA in the nucleus transmits 'information' to RNA which then specifies the sequence in which amino acids are joined to form proteins, it is reasonable to hypothesize that the information might involve the sequence of the nucleotides in the DNA and RNA and, therefore, that there might be expected some relation between DNA and RNA composition. Although there is no apparent correlation between DNA and ribosomal RNA, in several systems there has been found an RNA component which resembles DNA in nucleotide composition. The concept of 'messenger-RNA' has been developed (Brenner, Jacob & Meselson, 1961; Jacob & Monod, 1961). 'Ribosomes are non-specialized structures which synthesize, at a given time, the protein dictated by the messenger they happen to contain' (Brenner *et al.* 1961). This 'messenger-RNA' or 'tape-RNA' (Bonner, 1961) is said to be made in association with DNA and to have a related composition; to be heterogeneous in molecular weight (this is necessary since it is to 'code' for proteins of different sizes) but averaging not less than 500,000; to associate with ribosomes in order to 'programme' them; and to have a turnover rate such that each 'messenger-RNA' is used only once or a few times (Jacob & Monod, 1961). Much of the experimental evidence on which this theory is based comes from investigations of organisms infected with bacteriophage (Volkin & Astrachan, 1956; Volkin, 1960; Brenner *et al.* 1961; Spiegelman, Hall & Storck, 1961). Infection may halt the synthesis of the many proteins previously made by the host, initiate synthesis of 20–30 new proteins concerned in phage production, and lead to the synthesis and turnover of an RNA fraction with properties like those mentioned above. Formation of completely new ribosomes is not, apparently, involved but the new RNA may associate with pre-existing ribosomes. A similar mechanism (i.e. transient association of specific 'messenger-RNA' with ribosomes) has also been suggested for uninfected *Escherichia coli* (Gros *et al.* 1961; Jacob & Monod, 1961) but the evidence is not conclusive. A puzzling feature is that the material identified with the postulated 'messenger-RNA' has kinetic properties required of a precursor of ribosomal-RNA—amount, rate of synthesis, etc. Moreover, there are other systems (e.g. reticulocytes) in which protein synthesis occurs without concomitant turnover of a component corresponding to 'messenger-RNA'. Roberts and his colleagues (McCarthy & Britten 1962; Britten & McCarthy 1962; McCarthy, Britten & Roberts, 1962; Britten, McCarthy & Roberts, 1962) in a very detailed investigation have shown the necessity for, and have demonstrated the occurrence of, a precursor of ribosomal RNA in uninfected *E. coli*. This material sediments in the same region

as the 'messenger-RNA' (8–14*S*), amounts to the same fraction of the total RNA (*c.* 3%), becomes labelled equally rapidly (maximally in 1–2 min.) and is not conserved in this form, i.e. it is transferred elsewhere or broken down. The evidence presented suggests that after synthesis this RNA becomes incorporated into new ribosomes rather than being degraded. The possibility that the 'information' or 'message' may be conserved or 'buried' in ribosomes together with additional nucleotides which alter the overall composition cannot yet be excluded. Moreover, there must be explanation of the whereabouts of the necessary ribosomal RNA precursors which are not apparent in the scheme of Gros *et al.* (1961) and an indication of the function of ribosomal RNA if it is not mainly 'information'. It is, after all, 60–90% of the total RNA and is conserved during growth.

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The Effect of Various Drugs and Inorganic Ions on Bacterial Ribonucleoprotein

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In bacteria, protein synthesis is usually accompanied by synthesis of ribonucleic acid (RNA); but in the presence of the antibiotics chloramphenicol (Gale, 1953) and puromycin (Yarmolinski & de la Haba, 1959; Takeda, Hayashi, Nakagawa & Suzuki, 1960), or the purine analogues 8-azaguanine (Chantrenne & Devreux, 1960; Roodyn & Mandel, 1960) and 8-azaadenine, protein synthesis can be suppressed while RNA continues to be made. Such RNA has often been thought to be abnormal and the cessation of protein synthesis attributed to the fact that it could not function in metabolism. Undoubtedly the RNA formed by *Bacillus cereus* in the presence of azaguanine is not normal since 28 % of its guanine may be replaced by the analogue (Matthews & Smith, 1956) mainly at the ends of polynucleotide chains (Mandel & Markham, 1958). But in the case of chloramphenicol the evidence that the accumulating RNA is abnormal is entirely indirect: thus, this material is unstable while most bacterial RNA is not (Horowitz, Lombard & Chargaff, 1958); it is broken down by the cells when they are freed from antibiotic and at the same time material absorbing at 260 mμ appears in the medium, re-entering the cells when growth resumes (Hahn, Schaechter, Ceglowski, Hopps & Ciak, 1957); and in ultracentrifuge diagrams for cell-free bacterial extracts it appears as large peaks due to ribonucleoprotein (RNP) sedimenting at 14–18 *S*, whereas in extracts of untreated cells only small peaks are seen in this region of the pattern (Nomura & Watson, 1959; Dagley & Sykes, 1959). Chantrenne & Devreux (1960) have pointed out the similarity between the action of chloramphenicol and that of azaguanine which has been proved to cause accumulation of abnormal RNA: both compounds inhibit synthesis of cytoplasmic protein but not of RNA and neither interfere with the synthesis of cell walls of *B. cereus*. Nevertheless, chemical analysis does not support the view that 'chloramphenicol RNA' is abnormal since the base ratios are those of normal bacterial RNA (Horowitz *et al.* 1958; McQuillen, 1961). Moreover, if we regard 'chloramphenicol RNA' as a species of RNP which is normally present at much lower concentrations in bacteria as an intermediate stage in ribosome synthesis, most of its abnormal properties can be accounted for; and we shall show that in the analytical ultracentrifuge although it is quite different from 'azaguanine RNA' it does in fact resemble the RNP formed by a mutant which appears unable to stabilize ribosomes due to its inability to synthesize the protein required.

The work of Horowitz *et al.* (1958) shows that it is not correct to regard the cells as accumulating a type of RNA which, as soon as the antibiotic is removed, they break down and eject. When 'chloramphenicol RNA' was labelled with ^{14}C-adenosine it was shown to break down at the same time as it accumulated since the label

could be 'chased out' with excess of non-labelled nucleoside; no label was lost from cells that had not been treated with chloramphenicol. These and other experiments show that the accumulation of RNA must be regarded kinetically: as soon as chloramphenicol is added, the rate of breakdown is reduced below that of synthesis and RNA accumulates; and when chloramphenicol is removed, breakdown outstrips synthesis and the additional RNA disappears. In tending to stabilize the RNA it is clear from the results of Horowitz *et al.* (1958) that the chloramphenicol does not act alone, because the RNA which accumulates when it is first added is more stable than that accumulating later, as though some material which is involved in stabilization is in limited supply and becomes progressively exhausted. Since chloramphenicol inhibits protein synthesis, it may well be that this material is protein of the type associated with ribosomes; this may confer stability on ribosomal RNA either by coating it or by imposing upon it a configuration less vulnerable to enzymic attack. Nomura & Watson (1959) and Dagley & Sykes (1960) isolated 'chloramphenicol RNA' and showed it to be ribonucleoprotein containing less protein than ribosomal RNP. On this view, accumulation of RNA is a consequence of, and not the reason for, cessation of protein synthesis. When formed inside uninhibited cells this RNA would immediately take part in further reactions, including union with protein, that would lead to stable ribosomes. For a reason which is not evident the antibiotic appears to inhibit breakdown of RNA when the amount of protein available is not sufficient for this purpose. Direct experimental evidence for these views was obtained by Aronson & Spiegelman (1958) who reported that breakdown of 'chloramphenicol RNA' did not occur when amino acids were present in the culture: instead, this RNA appeared to be converted straight into ribosomal RNA because it was found that ^{14}C-amino acids were incorporated into particle-bound protein without any simultaneous incorporation of labelled nucleic acid precursors into RNA. Although few details were given of this important experiment, its interest increases as more attention is focused upon intermediate steps in the bacterial synthesis of RNP. By examination of cell-free extracts in the analytical ultracentrifuge we have shown that peaks at 14–18 *S* due to 'chloramphenicol RNP' disappear within 30 min. when cells are suspended in a medium containing amino acids which is free from antibiotic, and that this disappearance is accompanied by a marked enhancement of the peak due to 30 *S* ribosomes. A precursor–product relationship is also suggested by the fact that 30 *S* ribosomes disappear as 'chloramphenicol RNP' accumulates (Pardee, Paigen & Prestidge, 1957; Dagley & Sykes, 1959).

Three main stages are discerned in current theories of protein biosynthesis: (1) conversion of an amino acid to an adenylate, (2) its reaction with a suitable 'transfer RNA' molecule to form an aminoacyl—transfer RNA, (3) reaction of this ester, in the presence of guanosine triphosphate and a labile 'transfer factor' (Nathans & Lipmann, 1961), with a ribosome which may contain 'messenger RNA' whereby the amino acid becomes incorporated into protein. Demoss & Novelli (1956), Lacks & Gros (1960) and others have shown that chloramphenicol does not inhibit stages (1) and (2) and may block stage (3) at some point. Yarmolinski & de la Haba (1959) have drawn attention to the close similarity in structure between the antibiotic puromycin and aminoacyl—transfer RNA—and they showed experimentally that the antibiotic also inhibits stage (3). This was confirmed by Nathans & Lipmann (1961) who found that neither transfer RNA nor transfer factor were

affected by puromycin but that ribosomes were irreversibly poisoned for *in vitro* experiments. Like chloramphenicol, puromycin inhibits the synthesis of protein but not of bacterial RNA which therefore accumulates but is said to differ from 'chloramphenicol RNA' in being stable, i.e. it is not broken down when the bacteria are freed from puromycin and suspended in mineral salts medium (Takeda *et al.* 1960). We have examined cell extracts of *Escherichia coli* inhibited with puromycin and find changes in the ultracentrifuge patterns similar to those due to chloramphenicol. Thus, as RNA accumulates large peaks appear, due to material sedimenting at 14–18*S*; but by contrast, when cells were resuspended in a growth medium free from antibiotic the peaks disappeared much more slowly from ultracentrifuge diagrams of cell extracts and there was no rapid increase in concentration of 30*S* ribosomes. However, chloramphenicol and puromycin evidently act in similar fashions; and the observation that accumulating RNA may be rapidly degraded by the cells in one case and not in the other is not convincing evidence that there are two types of RNA which differ in structure. We found that resumption of protein synthesis in a puromycin-free growth medium was initially very slow and gradually accelerated. By virtue of its similarity in structure, puromycin may become firmly attached to sites normally occupied by transfer RNA and it may be displaced from them only when the cell is able to build up certain concentrations of metabolites. If the material sedimenting in cell extracts is precursor RNA for ribosomes, stabilized at this point by combining with puromycin, it may differ from 'chloramphenicol RNA' only in the rate at which the antibiotic is displaced from combination. If this should occur slowly, the RNA may be incorporated directly into stable ribosomes rather than suffer degradation before they are synthesized.

We have examined cell-free extracts of *Bacillus cereus* prepared 50 min. after addition of 40 μg. azaguanine/ml. culture; this caused marked inhibition of growth as well as an increase in the ratio RNA/protein, determined chemically for 1 ml. of extract, from 0·28 to 0·37. Extracts were also prepared 45 and 85 min. after guanosine had been added to reverse the action of the analogue and allow growth to resume. For none of the extracts was there evidence that materials had been produced similar to that giving rise to the 14–18 *S* peaks which result from poisoning the cells with chloramphenicol or puromycin. The only changes observed in patterns were due to a marked increase in concentration of 30*S* ribosomes when azaguanine had been displaced. Similar results were obtained with cultures of *Escherichia coli* treated with aza-adenine. This difference in rates of sedimentation of the RNA which is synthesized in the presence of chloramphenicol or azaguanine stands in contrast to various similarities in action already noted (Chantrenne & Devreux, 1960); however 'azaguanine RNA' has been proved to be chemically abnormal whereas 'chloramphenicol RNA' has not. Mandel & Markham (1958) found that the polynucleotides built during inhibition with azaguanine are relatively short, and from our work we conclude that they sediment slower than 14*S*; nevertheless, after azaguanine has been ejected 30*S* ribosomes may be synthesized from these additional polynucleotides at a speed sufficient to cause an increase in ribosome concentration above the normal value.

Borek & Ryan (1958) showed that RNA continued to be made by a mutant of *Escherichia coli* requiring methionine when protein synthesis had ceased due to

lack of methionine. Dr W. Hayes kindly supplied us with a similar strain of *E. coli* which showed this effect. This mutant synthesized little or no protein when cells were suspended in a methionine-free medium and after 180 min., synthesis of additional RNA also ceased. Cell-free extracts made at this time showed additional peaks in about the same region of the ultracentrifuge pattern as those due to 'chloramphenicol RNP', namely at 14*S* and 16*S*. When methionine was added to restore protein synthesis these peaks diminished and there was a simultaneous increase in area of the peak due to 50*S* ribosomes: this occurred over a period in which the increase in cell mass was equivalent to less than a third of a generation. These peaks at 14*S* and 16*S* may thus be due to ribosomal precursor material stabilized by the protein known to be 'turning over' in starved cells (Mandelstam, 1960); but proof of this must await the results of tracer experiments designed to decide whether the material is incorporated directly into the 50*S* ribosomes, as the ultracentrifuge results suggest, or whether it is first broken down and the ribosomes then resynthesized. Meanwhile it has been observed that the RNA accumulated by the mutant resembles 'chloramphenicol RNA' in being converted by Mg^{2+} ions to larger, or at least more rapidly sedimenting, ribosomes and also in being rapidly broken down to smaller particles on treatment with sonic vibrations.

For all our experiments the cell-free extracts were made with water or phosphate buffer (0·015 M or 0·066 M) or tris buffer (0·001 M) and all gave essentially the same ultracentrifuge pattern when used to extract disrupted bacteria from the same crop. We did not make a practice of extracting with solvents containing 0·01 M-magnesium acetate, such as other workers have used, for the following reasons: (1) there is no evidence that the 70*S* ribosomes which are formed at this elevated concentration, and which readily dissociate when it is lowered, are those ribosomes which take part in protein synthesis (Tissières, Schlessinger & Gros, 1960) or indeed in any metabolic process; (2) on the other hand there is direct evidence that, in the intact bacterial cell, readily-dissociated 70*S* ribosomes are normally present at much lower concentrations than other RNP particles (Bowen, Dagley, Sykes & Wild, 1961); (3) added magnesium acetate would have aggregated most of the material that we suggest may be precursors of ribosomes, and changes in ultracentrifuge patterns which appear to be meaningful would not have been observed.

There are two other features of this work which appear relevant to current problems. First, the rapid disappearance of additional RNA following removal of chloramphenicol, azaguanine or azaadenine was accompanied by a rapid rise in concentration of 30*S* ribosomes but not, in the first instance, of 50*S* ribosomes. Resumption of protein synthesis by the methionine-requiring mutant took place with a loss of the 14–16*S* peaks and a rise in concentration of 50*S*, but not 30*S* ribosomes. Although, therefore, 50*S* and 30*S* ribosomes may have common precursors they appear to be formed from them by separate and distinct metabolic routes. Second, if the peaks at 14–16*S* shown by extracts of the mutant are indeed due to ribosome precursors then this RNA would become labelled rapidly in cells exposed to a suitable radiotracer; and on raising the Mg^{2+} ion concentration of the extract it would sediment at 30*S* or adhere to larger ribosomes. Such material would therefore behave similarly to the 'messenger RNA' found in uninfected bacteria by Gros *et al.* (1961) and is clearly not readily distinguished from it solely by sedimentation studies.

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The Final Stages of Protein Synthesis and the Role of Lipids in the Process

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It is now a well-known fact that protoplasts prepared by the action of lysozyme on *Bacillus megaterium* can under suitable conditions carry out many of the functions of the intact cell. Thus they can respire, grow, form spores and synthesize nucleic acids and proteins when incubated in media of high osmotic pressure. In more dilute media, the protoplasts can readily be lysed with the liberation of about 70 % of their dry weight in a soluble cytoplasmic fraction. The remainder of the protoplast can be completely sedimented under moderate centrifugal forces as a membrane complex which appears to consist of two principal components when viewed in the electron microscope (Godson, Hunter & Butler, 1961). One component consists of large empty membranous vesicles; many sections seen, however, are almost entirely populated by smaller vesicles which appear to be studded with electron-dense particles of approximately 150 Å. diameter. While the larger vesicles are almost certainly derived from the cytoplasmic membrane of the cell, the smaller ones may partly originate from internal structures.

When protoplasts are incubated with labelled amino acids for short periods of time up to 2 min. and then lysed, the bulk of the labelled protein formed is found in this membrane complex. The isolated membrane complex is itself capable of incorporating labelled amino acids into protein *in vitro*, although the observed incorporation rate is linear with time and can continue in this way for several hours. Degradative experiments have clearly shown that the bulk of the label is bound in polypeptide form (Hunter, Brookes, Crathorn & Butler, 1959). Similar results have been reported by Spiegelman (1959) and by Zillig and his co-workers (Schachtschabel & Zillig, 1959; McCorquodale & Zillig, 1959), using *Escherichia coli*, and by Connell, Lengyel & Warner (1959) using Azotobacter.

The appearance of the membrane complex in the electron microscope led us to suspect that it contained bound ribonucleoprotein particles of the type that have been implicated in other systems as being directly concerned in protein synthesis, and our more recent work (Godson *et al.* 1961) has been directed towards the further fractionation of this complex.

The most effective method used has involved the dispersal of the complex in a dilute buffer under the impact of ultrasonic vibrations. The dispersed material can now be readily resedimented in two distinct layers, leaving about 24 % of its dry weight in the supernatant fraction. The two layers can be separated quite simply by mechanical means, the lower heavy white layer consisting largely of non-

proteinaceous material which need not concern us here. The upper layer, which analyses chemically as mainly phospholipoprotein with small amounts of RNA, appears in the electron microscope as small, rather ghostly, vesicles. No dense particles are visible, such as, for instance, were seen in the preparations of the original membrane complex or as can be seen clearly in electron micrographs of the whole cell. However, density gradient centrifugation of the supernatant fraction from the ultrasonic treatment readily yields ribonucleoprotein particles of sedimentation constants 45 and 30, leaving finally in solution material containing protein and nucleic acids of very low sedimentation constant.

Thus, if labelling experiments are carried out in the intact protoplast, four significant protein fractions can subsequently be obtained by this procedure: the initially formed cytoplasmic fraction; and from the membrane complex, ribonucleoprotein particles, the phospholipoprotein fraction and protein solubilized by the ultrasonic treatment (5*S* protein). Rather surprisingly, such experiments clearly show that the bulk of the labelled protein resides at early times in the phospholipoprotein fraction. The level found in the ribonucleoprotein particles is somewhat variable from experiment to experiment, but always less during the first minute than that found in the phospholipoprotein. The cytoplasmic fraction and the probably related 5*S* protein acquire label at later times, and 'chaser' experiments, where the dose of labelled amino acid is followed after a few seconds with a much larger dose of ^{12}C-amino acid, indicate that its label is derived from the phospholipoprotein fraction. We were, however, unable to obtain any evidence from these 'chaser' experiments indicating that the labelled protein in the phospholipoprotein fraction was derived from the ribonucleoprotein particles. In this connexion, it is of interest that Mitsui (1961) has recently described the incorporation of labelled amino acids into cytochrome *c* in a related cell-free system from a pseudomonad. The protein was isolated in a pure state, and Mitsui (personal communication) finds no labelled cytochrome *c* bound to free ribonucleoprotein particles.

However, recent work from other laboratories has indicated that only a small proportion of 70*S* ribonucleoprotein particles are directly concerned in protein biosynthesis, and our phospholipoprotein fraction does contain up to 2% of RNA. It is possible that the ribonucleoprotein particles isolated elsewhere are obtained in our delicate fractionation procedure in a bound form attached to the phospholipoprotein surface. This surface might then be concerned merely in the rapid removal of newly synthesized protein from the ribonucleoprotein. This could account for the fact that the protein synthetic abilities of isolated ribonucleoprotein particles are, in general, rather small.

On the other hand, the phospholipid surface might have a rather more direct role to play. One of the difficulties of the hypothesis of protein synthesis as expounded by Zamecnik, Hoagland and others (Zamecnik, 1960) is that the transfer RNA molecules when transferred to the ribosomal surface appear to be too long and complex to serve in their entirety as suitable transfer agents. It was this difficulty, combined with our experimental results, that led us to propose a mechanism for the final stages of protein biosynthesis which has been described in more detail elsewhere (Hunter & Godson, 1961). We assume that the specific ordering of the transfer RNA molecules, and hence of the amino acid residues, is determined by specific hydrogen bonding with the ribosomal RNA, by some such mechanism as that out-

lined by Crick, Griffith & Orgel (1957). If this linkage takes place at the same distance down the transfer RNA chain for all types of transfer RNA molecule, then a surface with a sequence of charged phosphate groups placed at approximately that distance from the ribosomal surface could make contact with each transfer RNA chain at relatively closely defined points. It was suggested that a phospholipid surface spatially orientated in this manner could serve to hydrolyse the amino acids from their binding to the terminal nucleotide residues of the transfer RNA molecules with the preservation of their bond energy, so that the formation of the peptide chain would proceed spontaneously and rapidly.

The same type of considerations, however, might apply if 'messenger-RNA' should prove to be of general occurrence. In this case, it could be postulated that the transfer RNA molecules bind initially not to the ribosomal RNA but to the messenger RNA, with the ribosomal RNA taking the place of the phospholipid surface. This would account for the finding of 'nascent' protein directly on the ribosomal surface in the experiments described by Dr McQuillen and others. However, another possibility is that most methods of cellular fractionation tend to destroy delicate phospholipid surfaces and membranes, in which case any protein originally fixed between phospholipid and ribonucleoprotein surfaces, would then be found on the ribosomes alone.

To turn to another aspect, one of the consequences of our own hypothesis would be the transient existence of lipo-amino acid complexes in the course of the transfer of the amino acid residues from transfer RNA to the phospholipid surface, and these might accumulate in special circumstances. Lipo-amino acid complexes have been detected by several groups of workers in recent years (Hendler, 1959, 1961; Gaby, Naughten & Logan, 1959; Gaby & Silberman, 1960; Gaby, Wolin & Zajac, 1960; Wallach, Soderberg & Bricker, 1960; Bogoch, Belval & Winer, 1961; Fukui & Axelrod, 1961), and rapidly metabolizing compounds of this type are best isolated from protoplasts of *Bacillus megaterium* by acetone extraction which leaves the bulk of the phospholipids undissolved. It is a pity that so little progress has been made towards a more precise chemical definition of these complexes. However, complexes have been separated from free amino acids by chromatographic (Hunter & Goodsall, 1961), electrophoretic (Gaby *et al.* 1960) and counter-current methods (Hendler, 1961). In the case of *B. megaterium* the complexes can be separated from free amino acids on a semi-preparative scale by paper chromatography. On a larger scale, the complexes were separated from other lipids by successive chromatography on silicic acid and DEAE-cellulose, the last fractionation effecting a separation of the complexes into phosphorus-free and phosphorus-containing fractions. Both the principal fractions gave fatty acids on hydrolysis, but very little serine, ethanolamine or choline was detected. Small amounts of glycerol and carbohydrates were found, but it is not as yet possible to propose any chemical structures for these materials.

Whatever the precise chemical nature of these lipo-amino acid complexes, there seems to be little doubt that the amino acid residues bound to them can readily be used for protein biosynthesis (Hunter & Goodsall, 1961). The role of these compounds is, however, by no means established, and they may perhaps serve as a store of activated amino acids. Gaby, on the other hand, has recently suggested (Gaby *et al.* 1960) that the complexes studied in his laboratory are primarily concerned in amino acid transport.

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Further Studies on the Isolation of Proteolytic Bacteria from the Sheep Rumen

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SUMMARY

A survey of the proteolytic bacteria present in the rumens of sheep on different kinds of diets has been made, with special emphasis on the isolation of anaerobic types. The results suggest that proteolytic activity is not confined to a single kind of rumen bacterium, but that it is a variable property possessed by strains of many kinds of bacteria which can be active in the breakdown of other feedstuff constituents. The properties of the bacteria isolated are given.

INTRODUCTION

In a previous paper (Blackburn & Hobson, 1960*a*), it was shown that the micro-organisms of sheep rumen contents readily hydrolysed casein and that this activity was associated both with the protozoa and the large and small bacteria. The results of attempts to isolate actively proteolytic bacteria, however, resulted in the isolation of only a limited number of types, most of which were facultative anaerobes (Blackburn & Hobson, 1960*b*). Although these were present in numbers which seemed to be quite comparable with the highest dilutions of rumen contents from which a mixed proteolytic flora could be grown, it was felt that they represented only a part of the proteolytic bacteria present in the rumen. The isolated bacteria did not include representatives of the strictly anaerobic rumen bacteria which have been shown to be active in the breakdown of feedstuff carbohydrates and lipids and which were present in the proteolytic suspensions of bacteria, but it was shown that mixed cultures containing these types of bacteria could be grown in a casein-containing medium and that the casein was hydrolysed in some of the cultures (Blackburn & Hobson, 1960*b*, *c*). The present work describes the results of experiments directed towards the isolation and classification of types of proteolytic bacteria other than those previously described.

METHODS

Media

In the previous work different kinds of media were tested for growth of proteolytic bacteria and it was on the basis of these results that the media used here were prepared. All media, except those used to detect growth without CO_2, were thoroughly gassed with oxygen-free CO_2 during preparation and dispensing, and all manipulations were carried out under a stream of oxygen-free CO_2 using techniques based on those of Hungate (1950). Media used during the preliminary tests with cysteine as

reducing agent, and resazurin as oxidation-reduction indicator, were reduced to below the Eh value needed for production of the colourless form of resazurin and generally had an oxidation-reduction potential measured between platinum and calomel electrodes of *c.* −370 mV. The more reduced media (using cysteine and dithionite as reducing system) were reduced to the point where phenosafranine was just colourless. The measured reduction potential of these media was about −410 to −470 mV.

General constituents of the media

Salts solutions. Solution (*a*) contained (g./l.): KH_2PO_4, 3·0; $(NH_4)_2SO_4$, 6·0; NaCl, 6·0; $MgSO_4$, 0·6; $CaCl_2$, 0·6. Solution (*b*) contained (g./l.): K_2HPO_4, 3·0. Rumen fluid was prepared by straining rumen contents (freshly obtained from a hay and grass-fed sheep) through gauze and centrifuging at 62,000 *g* for 10 min. The clear liquid was kept for not more than a few days at 2° before use.

Bicarbonate–dithionite solution. Dithionite was added, as a small volume of a concentrated solution (6 %, w/v) in boiled water, to a sterile 10 % (w/v) solution of $NaHCO_3$. The mixed solution was gassed with CO_2 and a portion added to the medium to bring the final concentrations to 0·5 and 0·003 % respectively of $NaHCO_3$ and dithionite.

'*Sugars*' (including carbohydrates, alcohols and acids) were the usual commercially available reagent grades, the xylan was the xylan no. 3 used by Howard, Jones & Purdom (1960). Cellulose was prepared from Whatman no. 1 filter-paper ground in a ball mill to a fine powder. Carboxymethyl cellulose was Cellofas B, low viscosity (I.C.I. Ltd). For isolation of bacteria, cellulose, xylan, carboxymethyl cellulose and malt extract were incorporated into the medium before autoclaving. Other carbohydrates were added as concentrated sterile filtered solutions, except for starch which was an autoclaved solution.

Casein was Glaxo casein C (Glaxo, Ltd, Greenford, Middlesex). This was acid precipitated, washed with water and dissolved in dilute NaOH, neutralized and the solution freeze-dried to give an easily soluble powder, which was added as a solution to the medium before autoclaving.

Other constituents. Yeast extract was 'Difco' brand (Bacto Laboratories, Detroit, U.S.A.). Tryptose was 'Bacto' brand (Bacto Laboratories, Detroit, U.S.A.). Malt extract was 'Bacto' brand (Bacto Laboratories, Detroit, U.S.A.). L-Cysteine hydrochloride was from L. Light, Ltd, Colnbrook, Bucks.

Media for isolations. The media used for the isolations described here were basically similar and the constituents and preparation of the medium for isolations from sheep 74 and 190 only will be described in detail. This medium contained per 100 ml.: salts solution (*a*) 15 ml.; salts solution (*b*) 15 ml.; phenosafranine, 0·0001 g.; cysteine hydrochloride, 0·05 g.; casein, 0·5 g.; rumen fluid, 10 ml.; tryptose, 0·3 g.; agar, 2·5 g.; water, 55 ml. These constituents were added as solids or solutions, mixed and gassed with CO_2 and autoclaved at 120° for 15 min. under CO_2 in a flask fitted with a bunsen valve and a stoppered side tube. For addition of other medium constituents or dispensing of medium the bunsen valve was removed and a syringe inserted through the tubing to which the valve had been attached. A brisk stream of CO_2 was meanwhile passed through the side tube and flowed out around the syringe ensuring anaerobic conditions. After addition of the bicarbonate–dithionite

solution (5 ml., see above) and any carbohydrate solution (final carbohydrate concentration was usually 0·5 % (w/v)) necessary, the medium at 50° was dispensed into tubes under CO_2 by means of hypodermic syringes or a laboratory-made dispensing apparatus for use under CO_2. Tenfold dilutions of rumen fluid were made under CO_2 in a solution prepared exactly like the medium, but omitting the agar and carbohydrate. One half millilitre portions of the diluted rumen fluid were added to 4·5 ml. of medium in $6 \times \frac{5}{8}$ in. test tubes under CO_2, the tubes tightly stoppered with rubber bungs and rolled under cold water. For liquid media the agar was omitted, but the cultures were still incubated in test tubes as above, at 38°.

Media for biochemical reactions. For determination of fermentation and other reactions of the bacteria the isolation medium was appropriately modified. Unless the degree of hydrolysis of casein was to be determined this was omitted from the media and yeast extract (0·3 %, w/v) was added. Growth without CO_2 was tested in media prepared and dispensed under oxygen-free nitrogen and with bicarbonate replaced by phosphate.

Motility was looked for in young cultures containing only 0·1 % glucose or maltose, and films for flagella staining made from these same cultures.

Analytical methods. The degree of proteolysis in liquid media was determined with biuret reagent in a manner similar to that used in the previous work (Blackburn & Hobson, 1960*b*). Less than about 10 % hydrolysis of casein could not be determined very accurately by this method. Urea hydrolysis was determined by estimating the ammonia liberated in suitable portions of the cultures using the method of Conway (1957). Volatile fatty acids (VFA) and succinic acid were identified by paper chromatography by suitable methods, uninoculated media being used as controls.

Sheep and sampling procedures. The two sheep used for the main isolations, 74 and 190, were fed 700 g. hay + 350 g. concentrates (fish meal, linseed meal, oat bran, maize meal) and 900 g. hay + 450 g. grass cubes, respectively, per day, fed in two lots at 7 a.m. and 4 p.m. Sheep 100 was fed 900 g. hay and 450 g. concentrates per day in two feeds. Sheep 6 and 47 were given the partially synthetic soluble casein-containing diet previously described. (Diet *a*, Blackburn & Hobson, 1960*c*.) Sheep 5 was fed 900 g. hay per day in two feeds.

Samples were taken via a rumen cannula, rapidly strained to remove coarse debris, and diluted and cultured as soon as possible after this. Generally, samples were taken about 3 hr. after the morning feed.

RESULTS

During a series of initial experiments many variations in the constituents and methods of reduction of the media were tested, as was the time of incubation needed to detect proteolysis. The results obtained, which relate to the problems of the best medium for growth of rumen bacteria, are not given here but may be obtained from the authors. Liquid and solid media were also compared. Although proteolysis on solid media was not always easy to detect and did not always correspond with the activity in corresponding liquid media, it proved difficult to isolate pure proteolytic strains from mixed liquid cultures and so most of the isolations were made by culturing dilutions of rumen fluid in solid media, picking off a selection of colonies

which seemed to show some change in the medium, and testing these for proteolytic activity in liquid media initially and at intervals after purification by dilution and subculture. In order to culture as many types of bacteria as possible it was finally decided to use the same basic medium with a wide variety of carbohydrates as energy sources and to survey the proteolytic bacteria in two sheep (74 and 190) on two basic types of diet, as well as in the other sheep available. The isolations were carried out over a period of some months, but the types of bacteria in the rumens seemed to be stable over this time. All the bacteria isolated could thus be taken to be stable constituents of the flora and not transient types introduced, for example, with the feed. Only the proteolytic bacteria growing in the highest dilutions in each test were subcultured and examined.

A wide range of properties of the bacteria were tested as detailed information on the properties of rumen bacteria from other than a few sources (principally cattle) is lacking. Also, since the properties of the individual strains of the bacteria which have been tentatively classified often seem to be very variable, a large amount of data on the bacterial properties would seem to be necessary before a comprehensive classification can be made. However, to economize in space complete fermentation reactions of the isolates are not given in the tables (full details may be obtained from the authors). Apart from the properties mentioned in Table 1, fermentation of the following 'sugars' was tested: arabinose, rhamnose, mannose, galactose, mannitol, dulcitol, sucrose, trehalose, melibiose, raffinose, α-methylglucoside, aesculin, dextrin, glycogen, sorbose, sorbitol, inositol, succinate, acetate. The method of numbering the isolated strains is as follows. Number of host animal, carbohydrate in isolation medium, log. 10 dilution of rumen contents from which isolation was made (number of colonies of this organism in the dilution), i.e. 74 cell 7 (2) was an organism from sheep 74, isolated in a medium containing cellulose and occurring as two colonies in the $1/10^7$ dilution of rumen contents. The abbreviations used for the carbohydrates in the isolation media are given with the tables. A letter '*a*' or '*b*' has been added to the designation in some cases merely to distinguish between two isolates otherwise bearing the same number.

Properties of bacteria isolated from sheep fed different diets

The isolates have been grouped according to their properties and some tentative classifications made on the basis of the named groups of bacteria isolated from cattle by American workers. As only a few strains of some of the named organisms have been described in detail and the properties of individual strains often vary considerably, it has been found difficult to classify accurately some of the isolates described here, but connexions with the named bacteria are mentioned. Since similar bacteria were isolated from all animals they have not been grouped according to the source. Unless otherwise mentioned, all isolates were strict anaerobes.

Group 1. *Bacteroides amylophilus*. This group includes isolates 6malt7, 74maltE6, 74gluc8(2), 5xmc7, 5xmc8, 47gluc8, 47cellob9 47gluc8a, 47cellob8 (malt = maltose; maltE = malt extract; glu = glucose; xmc = mixture of xylose, maltose, cellobiose; cellob = cellobiose). These were all Gram-negative pleomorphic coccoid or rod-shaped bacteria, sometimes with very irregular shapes. All fermented dextrin, glycogen, maltose and starch. Isolate 5xmc8 also fermented fructose. The main

Table 1. *Properties of Butyrivibrio and presumptive Butyrivibrio isolates*

Property†	Organism number																				
	100bran9	100maltE8	100cellob8	100cellobb8a	100malt8	6cellob8	100maltE8	190malt7(1)	190xyl7(6)	190gluc7(1)	190cell8(1)	74cellob7(4)	74cellob7(4)a	74xyl7(2)	74cell8(2)	47xyl9	74gluc6(2)	190xan8(6)	74xan7(2)	74xan7	190cellob7(4)	47CMC8
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+ −
Glucose	+	+	+	+	+ −	+	+	+	+	−	+	+	+ −	+	+	+	+	+	+	+	+	+
Glycerol	+	−	−	−	−	−	+	+ −	−	+	−	+	−	−	−	−	+	−	+	+	−	−
Adonitol	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Lactose	−	+	+	+	+	+	−	−	−	+	−	−	+	+	−	−	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	−
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
Salicin	−	+	+	+	+	+	+	−	−	+	+	−	+ −	−	−	+	−	+	+	+	+	+
Xylan	−	+	+ −	+	−	+	+	+	−	+	+	+ −	−	−	−	+	+	+	+	+	+	+
Inulin	−	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Starch	−	−	+ −	+	−	+	−	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+
CMC	−	−	−	+ −	−	−	−	+ −	−	−	−	−	−	+	−	−	−	−	+	−	+	+ −
Lactate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
V.P.	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	+ −	−	−	−	−	−	−
Gelatin	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	−	+	+	+	+
Hydrolysis of casein (%)	48	10	38	*	6	94	10	39	37	19	8	30	36	0	36	32	4	0	19	26	11	32

No isolates fermented cellulose. All fermented fructose. None produced indole, catalase or reduced nitrate. None except 74xan7(2) grew at 30°, none grew at 50°, all except 190gluc7(1), 190xan8(6) grew at 45°. 190cell8(1), 74cellob7(4), 190xan8(6), 74xan7(2), 74xan7 grew without CO_2. 190malt7(1), 190gluc7(1), 47CMC8 did not grow without rumen fluid. Final pH in glucose or maltose medium was 5·5 to 6·4, generally about 5·8.

Abbreviations used for sugars in isolation media: bran = bran extract; maltE = malt extract; cellob = cellobiose; malt = maltose; xyl = xylose; gluc = glucose; xan = xylan; CMC = carboxymethyl cellulose. The other constituents of the media are given in the text.

Casein hydrolysis was measured after a 4-day incubation. * No test. † This includes fermentation, hydrolysis or formation of the substance noted.

VFA product of maltose fermentation was acetic acid, and the final pH *c.* 5·7. The bacteria in this group can be classified as *Bacteroides amylophilus* (Hamlin & Hungate, 1956). All isolates except 74gluc8(2) were similar to the type species in requiring CO_2 for growth, and all except 47gluc8 would grow without rumen fluid. Isolate 74gluc8(2) formed H_2S, and 47gluc8 reduced nitrate. No strains grew at 30° and only 5xmc8, 47gluc8, and 47cellob9 at 45°. All isolates except 74maltE6 hydrolysed gelatin and the hydrolysis of casein by the isolates (in the order given initially) was 49, 78, 85, 33, 60, 47, 62, 54 and 84 % respectively.

Group 2. Butyrivibrio. The bacteria grouped in Table 1 were all presumptively identified as Butyrivibrio (Bryant & Small, 1956*a*). With the exceptions mentioned

Table 2. *Properties of presumptive Selenomonas isolates*

	Organism number						
Property	6cellob8	6malt7	6malt7a	6cellob8a	74star-7(2)	190malt-E8(1)	190star-8(3)
Sorbose	−	−	−	−	+	−	−
Glycerol	+	+	+	−	−	−	−
Sorbitol	−	−	−	−	+	−	−
Lactose	+	+	+	+	+	+	−
Cellobiose	+	+	+	+	+	−	+
Xylan	+−	−	−	+	+	−	−
Inulin	+	−	−	−	+	+	+
Starch	+−	+−	+	−	+	+	+
Lactate	+	+	+	−	−	−	−
Succinate	−	−	−	−	+−	−	−
H_2S	−	+	+	−	+	−	−
V.P.	−	−	+	−	−	−	−
NO_3	−	+	+	−	+	−	*
Indole	−	−	−	−	−	+	−
Growth-rumen fluid	+	−	+	+	+	+	−
Growth-CO_2	−	−	−	−	+	−	−
Gelatin	+	−	*	+	+	−	−
Hydrolysis of casein (%)	95	90	90	95	84	47	46

Symbols as in Table 1, with star = starch. All isolates fermented xylose, glucose, fructose, maltose, salicin. No isolates fermented CMC, adonitol, cellulose. Only 190maltE8(1) and 74star7(2) grew at 45°, the latter also at 50°. Final pH in glucose was about 5·2.

* No test.

later all were Gram-negative curved rods, about 0·6μ × 1·5μ in average size, sometimes in chain formation. A number of isolates were motile with a polar flagellum. The principle product of fermentation of glucose, or in one case maltose, was butyric acid. Isolate 47cmc8 is probably a Butyrivibrio, although in morphology it tended to be a stouter and more coccoid rod. Isolate 190cellob7(4) was similar in many properties to Butyrivibrio, but it was more pleomorphic and at times formed straighter rods than the usual Butyrivibrio and these rods formed chains. In morphology and growth it somewhat resembled the two strains of group 4, but differed from them in forming butyric acid, and is best classified here. Isolates 190xan8(6), 74xan7(2), 74xan7 were all broader curved rods than the others with one side straighter, 190xan8(6) was also capsulated, but their general properties fit in with the rest of this group.

Group 3. Selenomonas. The organisms in this group (Table 2) all usually exhibited

a characteristic Selenomonas morphology and flagellation, varying in size from about 2 to 5μ in length and from 0·4 to 1μ in maximum width. However, all isolates tended to form chains at times and the cells in the chains were often straighter rods with rounded ends. The fermentation reactions were generally similar to those of *Selenomonas ruminantium* (Bryant, 1956), and the three lactate- and glycerol-fermenting isolates could be classified as *S. ruminantium* var. *lactyliticas* (Bryant, 1956). In VFA fermentation products of glucose the four isolates labelled 6cellob8 and 6malt7 resembled the type cultures of Bryant in forming mainly propionic and acetic acids. The other three isolates produced butyric acid, with little acetic, and in this respect resembled the atypical *S. ruminantium* strain B385 (Bryant, 1956), which is probably a species of Butyrivibrio.

Group 4. Lachnospira. Two isolates were presumptively identified with the new genus *Lachnospira* described by Bryant & Small (1956*b*). These two isolates (74malt-7(1), 74cellob6(2)) were similar in being Gram-negative or -variable rods, about 3μ long, sometimes slightly curved, motile with a polar flagellum and often occurring in chains. They formed a 'woolly' colony in agar and in liquid medium a zoogloeal type of growth which sedimented out. They formed acetic acid from glucose and grew without rumen fluid. In all these properties they resembled *L. multiparus* (Bryant & Small, 1956*b*). They differed from the type culture in some reactions. The other main properties of the two isolates were as follows. Both fermented cellobiose, glycerol (74malt7(1) only weakly), glucose, inulin, lactose, maltose and starch. Neither fermented adonitol, inositol, salicin, cellulose, carboxymethyl cellulose, pectin nor lactate. Isolate 74cellob6(2) fermented melibiose, xylose and xylan; 74malt7(1) formed H_2S; neither grew without CO_2, reduced nitrate nor formed indole; both gave a positive V.P. test and both grew at 45°. The final pH in glucose medium was 4·7. Isolate 74malt7(1) hydrolysed casein to the extent of only about 2 %; 74cellob6(2) to about 9 % in 4 days; both hydrolysed gelatin.

Group 5. The properties of group 5 are given in Table 3. These bacteria showed similarities in being Gram-negative rods, 0·8–1$\mu \times$ 3–5μ, sometimes curved with pointed ends, either singly or in chains, with granulations or polar staining, but often forming chains of straighter cells. In some cases motility, with peritrichous flagella, was seen, but in most cases only an oscillatory movement, or no motion, was found. All formed mainly butyric acid, with some acetic, from glucose or maltose. Growth in liquid medium was zoogloeal or granular. Isolates 190nil7(6) and 190nil7(6)a differed to some extent from the others in being more crescentic, with more definite bipolar staining and also in not hydrolysing gelatin and being only weakly proteolytic on casein. 5xmc7, 5xmc7a and 5xmc8 were generally less curved than the others and had more rounded ends. The bacteria of this group had some similarity to Lachnospira in growth characteristics in liquid media but formation of butyric acid excludes them from this classification. They are similar to Bacteroides. However, the curved shape and pointed ends of the cells of some of the isolates might preclude them from this group and suggest relationships with the Spirillaceae (in morphology and growth they somewhat resemble *Myconostoc gregarium*) although *B. succinogenes* (Bryant & Doetsch, 1954) can have pointed ends. In the formation of butyric acid from glucose they also differ from all but one strain of rumen Bacteroides so far reported, this latter was found in young calves by Bryant, Small, Bouma & Chu (1958).

Group 5a comprised three isolates (190malt7(4), 74starch6(2), 47nil9), and their properties are given in Table 3. Isolates 190malt7(4) and 74starch6(2) were similar in being pleomorphic Gram-negative curved rods, sometimes long and thin with more than one coil ($0{\cdot}3\mu \times 9\mu$), at other times stouter, straighter and shorter ($0{\cdot}6\mu \times 2\mu$) with rounded ends. Chain formation was common and the cells in some cultures of 74starch6(2) had a twisting movement. Isolate 47nil9 were generally straighter rods about $1\mu \times 4\mu$, more variable in Gram staining. The one isolate

Table 3. *Properties of groups 5 and 5a*

	Group 5. Organism number										Group 5a organism number		
Property	190nil7(6)	190nil7(6)a	190CMC8(1)	190CMC8(1)a	74CMC7(1)	74CMC7(1)a	74CMC7(1)b	5xmc7	5xmc7a	5xmc8	190malt7(4)	74star6(2)	47nil9
Xylose	+	+	+	+	+	+	+	−	+	+	+	−	−
Glucose	+	+	+	+	−	−	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	−	+	+
Glycerol	+−	−	+	+	−	+	+	+	−	−	−	−	−
Adonitol	+	−	−	−	+	−	+	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	+	−	−
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	−	+	+	+	+	+	+	+	+	+−	+	+
Cellobiose	+	+	+	+	+	+	+	+	−	+	−	+	+
Salicin	+	+	+	+	+	+	+	−	+	+	+	+	+
Xylan	+	+	+	+	+	+	+	+	−	+−	−	−	−
Inulin	−	+	+	+	+	+	+	+	+	+	+	+	−
Starch	+−	−	+	+	−	−	+−	+	+	+	+	+	+
CMC	−	−	+	+	−	−	+	−	+−	+−	−	−	−
Cellulose	+−	−	+	−	−	−	−	−	−	−	−	−	−
Lactate	+	−	+	−	−	−	+	−	−	−	+	−	−
Succinate	−	−	−	−	−	−	−	+	+−	+−	−	−	−
H_2S	+	−	−	+	−	−	+	+	−	−	−	−	−
Indole	−	−	−	−	−	−	−	−	−	−	−	+−	−
Gelatin	−	−	+	+	+	+	+	+	+	+	−	+	−
Hydrolysis of casein (%)	4	14	45	40	51	65	40	62	57	69	68	0	0

Symbols as in Table 1 and 2 with nil = no carbohydrate.

Group 5. V.P. and nitrate reduction tests were all negative. No isolate grew at 30° or 50°, strains 190nil7(6)a, 190CMC8(1)a, 74CMC7(1), 74CMC7(1)a grew at 45°. All except 190CMC8(1) and 74CMC7(1) grew without CO_2 and 5xmc7 grew without rumen fluid. All produced butyric acid from glucose or maltose. Final pH varied from 4·6 to 5·9 with different isolates and was generally about 5·0.

Group 5*a*. V.P. and nitrate tests were negative. No isolate grew without CO_2 and 190malt7(4) would not grow without rumen fluid. None grew at 30° or 50°, all grew at 45°. All produced acetic acid from glucose, final pH was 5·8 to 6·4.

tested (190malt7(4)) produced succinic acid from glucose and all three produced mainly acetic amongst the VFA products of glucose fermentation. These isolates were tentatively classified as *Bacteroides ruminicola* (Bryant *et al.* 1958).

Groups 6, 6*a*, 6*b*. The organisms in this section are grouped under one main heading as they were all rather pleomorphic, Gram-negative or -variable, oval cocci or coccobacilli. They have been subdivided on the basis of morphology and also on their fermentation products. However, fermentation reactions and to some

Table 4. *Properties of Gram-negative or -variable cocci and coccobacilli*

Property	Group 6. Organism number					Group 6*a*. Organism number							Group 6*b*. Organism number			
	6cellob8	6maltE8	100malt7	74nil7(6)	74cellob7(4)	6malt9	6cellob9	6malt9a	74nil7(6)a	74malt7(6)	190gluc9(24)	74malt7(6)a	5xmc7	5xmc7a	5xmc7b	190xyl8(2)
Xylose	+	+	+	−	+	+	+	+	+	+ −	−	−	−	−	−	+
Glucose	+	+	+	+ −	+	+	+	+	+ −	−	+ −	+	+	+	+	+
Fructose	+	−	+	+	+	+ −	+	+	+	+	+	+	+	+	+	+
Glycerol	−	−	+	−	−	−	−	−	−	+ −	+	+	−	−	−	−
Maltose	+	+	+	−	+	+	+	+	−	+ −	−	−	+	+	+	+
Lactose	+	+	+	−	+	+	+	+	−	+ −	+ −	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+
Salicin	+	+	+	+ −	+	+	+	+	+	+	−	−	+	+	+	+
Xylan	+	+	+	−	+	+ −	+ −	+ −	+ −	−	−	−	−	−	−	+
Inulin	+	+	−	−	+	+	+	+	−	+	+	+	+	+	+	+
Starch	+	+	+	−	+	+ −	+ −	+	−	−	−	−	+	+	+	−
CMC	*	*	*	+ −	+ −	−	−	−	+ −	−	−	−	−	−	−	−
Cellulose	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−
Lactate	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−
H_2S	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Indole	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−
NO_3	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
Gelatin	−	−	+	−	+	+	+	+	−	+	−	+	−	−	−	+
Hydrolysis of casein (%)	88	88	32	0	22	85	91	86	0	11	26	9	*	8	7	25
Growth-CO_2	−	−	−	−	−	+	+	−	−	−	+	+	+	−	−	−
Growth-RF	−	+	−	−	+	+	+	−	−	+	+	+	+	+	+	−

Symbols as in Tables 1–3, with RF = rumen fluid. * No test.
No isolates fermented adonitol. V.P. test was negative in all cases.
Group 6. Produced mainly acetic acid from glucose or maltose with sometimes a little propionic.
Group 6a. Produced acetic and propionic acids from glucose or maltose, with some butyric in the case of 190gluc9(24).
Group 6b. Produced acetic and butyric acids from glucose.
All isolates of group 6*b* grew at 45°, and 190xyl8(2) also at 30°, as well as 38°.

Table 5. *Properties of Gram-positive cocci*

Property	Organism number								
	5xmc7	74gluc6(2)	74cellob6(2)	5xmc7a	47xylan8	47cell9	47cellob8	47gluc9	47CMC9
Xylose	−	−	−	−	−	+	+	−	−
Glucose	+	+	+	+	+	+	+	−	+
Mannose	+	+	+	+	+	+	+	−	+
Fructose	+	+	+	+	+	+	+	−	+
Glycerol	−	−	−	−	−	+	−	−	−
Adonitol	−	−	−	−	−	+−	−	−	−
Lactose	+	+	+	+	+	+	−	−	−
Cellobiose	+	+	+	+	−	+	−	−	−
Salicin	+	+	+	+	−	+	+	−	−
Xylan	+	−	−	−	+−	+	+	−	−
Inulin	+	+	+	+	−	+	+	−	−
Starch	+	+	+	+	+	+	+	+	+
CMC	−	−	+−	−	+	+−	−	−	−
Cellulose	−	−	+−	−	+−	+	−	−	−
Lactate	−	−	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−	−	−
V.P.	+	−	−	+	−	−	−	+	−
Indole	−	+−	+−	−	+	+−	−	−	−
NO_3	−	−	−	−	+	+	−	−	+
Gelatin	+	+	+	−	+	+	+	+	+
Hydrolysis of casein (%)	10	0	3	7	42	50	*	58	11
Growth-CO_2	+	−	−	+	−	+−	−	−	−
Growth-RF	+	+	+	+	+	−	−	+	−
Growth temp.†	30, 45, 50	30, 45, 50	30, 45, 50	30, 45	30	−	50	45	45

Symbols as in previous tables. RF = rumen fluid.

No isolates produced H_2S.

All isolates fermented maltose. All isolates except 5xmc7 and 74gluc6(2) produced mainly acetic acid from glucose or maltose, these two isolates produced acetic and butyric acids.

* No test. † Other than 38°. Final pH of fermented sugars varied from 4·2 to 6·2 but was generally about 5·5.

extent morphology, varied amongst members of the groups and it is probable that a number of species are represented in each group. Their properties are given in Table 4.

Group 6 were small Gram-negative or -variable oval cocci 0·5–1 μ long, sometimes occurring in pairs, but generally singly. They formed mainly acetic acid from glucose or maltose.

Group 6*a*. This was a group of Gram-negative or -variable cocci or coccobacilli, all exhibiting some pleomorphism and variation in size, but in general, being about 0·5–1 μ long. All produced propionic as well as acetic acid from glucose or maltose.

Group 6*b*. The first three members of this group were all similar Gram-variable coccobacilli, rather larger than those of groups 6 and 6*a*, being about 0·9 $\mu \times 2\mu$. They were also facultatively anaerobic. Isolate 190xyl8(2) was predominantly Gram-negative, had more tendency to chain formation and the cells were about 0·9 $\mu \times 1{\cdot}3\mu$. All the isolates in this group produced a mixture of acetic and butyric acids from glucose, and all were motile with a polar flagellum.

Some of the isolates of groups 6, 6*a*, 6*b* resembled *Eubacterium ruminantium* (Bryant, 1959), but there seems in general to be no complete basis for including these groups in any so far named groups of rumen bacteria.

Group 7. These were all small Gram-positive cocci, about 1 μ diameter occurring singly or in groups and, apart from the three mentioned above, they were the only facultatively anaerobic bacteria amongst those described in this paper. Their properties are given in Table 5. As these isolates differed in reactions it seems likely that this group could be further subdivided.

Urea hydrolysis. All the bacteria isolated were tested for ammonia production in a medium containing urea but no ammonium salts. Except for one isolate (Gram-positive coccus, 47cell9) there was little change in the ammonia concentration in the media (there was always a small amount of ammonia present from the rumen fluid and other constituents) and any increase in concentration was very small compared with the amount of urea present. In some cases there was an uptake of ammonia. There was thus no evidence for any definite hydrolysis of urea by any of the strictly anaerobic bacteria isolated.

Zones of proteolytic activity. Proteolytic activity of colonies growing in roll tubes of medium containing casein was shown by a clear zone surrounded by a more opaque ring in the somewhat cloudy medium and this area gradually spread with growth of the colony.

DISCUSSION

It was noted in earlier experiments that the carbohydrates included in the medium had an effect on the types of proteolytic bacteria growing and the apparent degree of proteolysis, but more extended observations showed that this was not as specific as was at first thought. In some cases the influence was due to provision of a suitable substrate for growth, as in the case of *Bacteroides amylophilus* which grew well in a maltose-containing medium. Some of the carbohydrates used may have also aided the growth of recognizably proteolytic bacteria by somewhat suppressing the rapid growth of other bacteria which occurred on more easily utilized substrates such as glucose.

In many cases the extent of hydrolysis of casein to trichloroacetic acid-soluble

products was not high, and in some cases casein hydrolysis seemed to be an unstable property, decreasing on continued subculture. There was also evidence that the enzymes were to some extent adaptive as rapid subculture in a casein-containing medium in some cases led to a greater degree of hydrolysis of the protein. It is thus probable that the comparatively limited hydrolysis of casein exhibited by many of the bacteria is not completely indicative of their proteolytic activity in the rumen, where a constant amount of protein is added to what is, in effect, a continuous culture of the organisms.

In the earlier work (Blackburn & Hobson, 1960*b*), facultatively anaerobic Gram-positive cocci occurred in almost all the experiments and they have also been isolated in the present tests, although attention was focused in these experiments on the anaerobic types. The bacteria other than cocci isolated here were all found over a long period in the different sheep and must also be a stable part of the rumen proteolytic flora. *Bacteroides amylophilus* was especially prevalent in sheep being fed a ration high in starch. The types isolated in many cases belong to groups of bacteria already shown to be of importance in the ruminal breakdown of other feedstuff constituents and to be widely distributed in ruminants. These results would support the general findings that the proteolytic activity of rumen contents does not vary greatly with variations in diet.

Fulghum, King & Moore (1958) reported the isolation of non-sporeforming anaerobic bacteria from the bovine rumen which caused clearing in a medium containing milk as casein source, and Bryant *et al.* (see review by Bryant, 1961) have noted that a number of their isolates of bovine rumen bacteria, especially *Bacteroides ruminicola*, some Butyrivibrio strains and some Eubacterium strains, hydrolysed casein and/or gelatin during biochemical tests. These results fit in with the present data, and all the results so far indicate that there is no one specific type of proteolytic bacterium in the rumen, but that proteolytic activity is a variable property associated with particular strains of species generally active in other feedstuff breakdown. It is possible that some non-proteolytic strains may contain peptidases which will act during the complete breakdown of feedstuff protein in the rumen.

The fact that no definite hydrolysis of urea was found is again in accord with results of attempts to isolate anaerobic urease-producing bacteria from the rumen. Only one type has so far been isolated and this was a Gram-positive rod related to *Lactobacillus bifidus* (Gibbons & Doetsch, 1959).

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Cellular Division and Reproduction of Bacteriophage in Synchronized Cultures of *Escherichia coli*

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SUMMARY

Bacteriophage T2*r* inhibits the division of the host cell *Escherichia coli* B, immediately after adsorption. Synchronized cells, when infected by phage at the moment of cell division, cannot carry this process to completion. The same effect is exhibited by phage inactivated by ultraviolet light. In contrast, bacteriophage ϕx-174 allows infected cells to divide before lysis. Determination of the rate of synthesis and final yield of virus showed that synchronized cells from the middle period between two divisions produced the highest burst sizes, whereas the lowest burst sizes were observed in dividing cells.

INTRODUCTION

It is generally accepted that infection of bacterial cells by bacteriophage may inhibit growth and further cell division. However, methods such as microscopic observation or turbidity measurements of randomly dividing bacteria cannot decide whether a cell dividing at the moment of infection can complete this process or whether it is completely inhibited immediately after the phage is adsorbed. The inhibitory effect of virulent bacteriophage on bacterial division was studied in the light microscope by Luria & Delbrück (1942) but these authors concluded that their method did not give any definite answer to this question. On the other hand, it has been concluded that certain temperate phages allow the infected cell to divide once or twice before lysis (Lwoff, Siminovitch & Kjeldgaard, 1950).

Cell division, although closely dependent upon growth, is believed to involve some more or less specific mechanisms (Swann, 1957). It is, therefore, not self-evident that the growth-inhibiting action of virulent bacteriophage must necessarily be accompanied by simultaneous inhibition of cell division. Synchronization of bacterial division facilitates the study of this interesting problem. In addition it enables one to relate the final yield of virus to the stage in the cell-division cycle at which the cells are infected. The effect of bacteriophage on synchronously dividing *Escherichia coli* was reported in a previous communication (Stárka & Koza, 1958). When cells were infected immediately before an expected division, a slight increase in the intensity of scattered light was observed, indicating that probably some cells divided before lysis.

Virus synthesis in bacteria dividing synchronously was first studied by Barner & Cohen (1956) using *Escherichia coli* 15T$^-$ and bacteriophage T2r^+. The results indicated that bacteriophage adsorption and yield were essentially independent of

the stage of host development at the time of infection, at least under the special conditions of their experiments. On the other hand, Yanagita, Maruyama & Takebe (1958), using a filtration method of synchronization, observed significant differences in the amount of phage adsorbed by synchronized cells depending on the increase of cell surface during the cell-division cycle. The burst sizes in one-step growth experiments with bacteria infected at different phases of synchronized growth also varied significantly and the highest values were obtained with cells midway between two divisions. In agreement with the conclusions of Yanagita *et al.* (1958), the importance of the actual state of the growing cell at the time of infection by phage for the yield of virus was also described in our preceding report (Stárka & Koza, 1958) where cold shock treatment was used as a method of synchronization. The apparent contradiction between the results of Barner & Cohen (1956) and these later observations might be explained by the different methods of synchronization, i.e. by withdrawal and addition of an essential metabolite in the first case, and by filtration or cold shock in the second.

The present study was begun in an attempt to throw more light on two problems, namely whether phage inhibits the division process of already dividing bacteria; and how the rate of synthesis and final yield of virus varies in synchronized cultures.

METHODS

Organisms and growth methods. A streptomycin-resistant strain of *Escherichia coli* B was used and the division cycle was synchronized by a single temperature shift as described previously (Stárka & Koza, 1959). The growth medium was Bacto Tryptose Broth (Difco) 0·5 % (w/v) and 0·085 M-NaCl, pH 7.

Single-step growth curves and single cell bursts were determined at 30° with bacteriophage T2*r* using the techniques described by Adams (1950, 1959) except that all dilutions were made in tryptose medium instead of in broth, and the temperature for phage growth was maintained at 30°. The burst size in non-synchronized cells at 30° was found to be 90, with a latent period of 40–45 min. At 37° the burst size was 100, latent period 26 min. The multiplicity of infection in all burst-size determinations was between 0·08 and 0·1.

Phage T2*r* inactivated by ultraviolet light (u.v.) was prepared according to Luria & Delbrück (1942). After irradiation, the phage suspension contained 2×10^4 plaque-forming particles and 3×10^9 inactivated phages per ml.

In experiments with phage ϕx-174, *Escherichia coli* C was used as bacterial host. The growth medium contained tryptose 0·5 % (w/v), 0·085 M-NaCl and $2{\cdot}5 \times 10^{-3}$ M-$CaCl_2$.

Optical method of bacterial count. Bacterial counts were determined using a Pulfrich nephelometer. In this instrument the incident light is scattered by suspended bacteria and collected by a converging lens at an angle of 45°. The intensity of the light scattered by the sample is compared with the intensity of light passing through an opal glass diffusing plate and can be adjusted by means of a diaphragm. A scale on the diaphragm ranges from zero (slit closed) to 100 (slit fully opened). In the range 0–70 of the nephelometer readings (N.R.) the number of bacteria per ml. determined by plating and by counting in a counting chamber is directly proportional to the intensity of the scattered light. Using selected Pyrex tubes (internal

diameter 15 mm.) as cuvettes, 1 division (N.R.) represents 5×10^5 bacteria/ml. Under the experimental conditions described here the intensity of the scattered light is considered to depend only on the particle count and not to be related to increase in cell mass. Moreover, change in volume of the particles within certain limits (e.g. dividing bacteria) has little effect on the relation of the particle count to the nephelometer reading (Koch, 1961).

RESULTS

Inhibition of cellular division by phage. The influence of bacteriophage infection on cellular division was followed directly in synchronized cultures of bacteria. Phage was added to 10 ml. samples of cultures in different phases of the division cycle at an input ratio of phage:bacteria of 20:1. These samples were shaken separately in

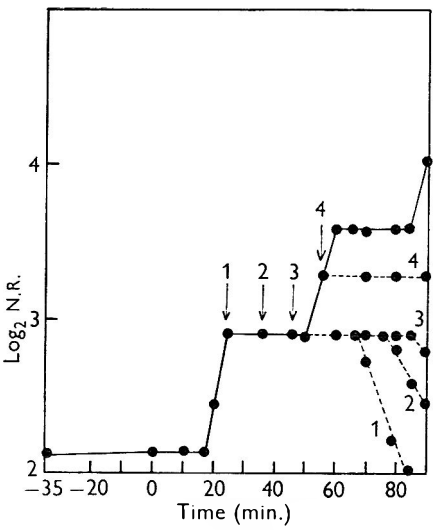

Fig. 1. The effect of bacteriophage T2*r* on cell division in a synchronized culture of *Escherichia coli* B. Phage was added to non-dividing cells (1, 2, 3) and to already dividing cells (4). Dashed curves show the nephelometer readings (N.R.) of samples after infection. The solid line represents the light scattering of non-infected control. Phage/bacteria ratio = 20:1; temperature 30°.

tubes. In non-infected controls the growth rate and division time were identical with the parameters of the stock culture. Five min. after addition of phage, essentially all bacteria were infected.

Figure 1 shows a typical growth curve of synchronized bacteria and the effect of added phage on bacterial division. The time of one division cycle was approx. 35 min. In repeated experiments it varied between 30 and 35 min. This is in good agreement with the generation time of 34 min. for a non-synchronized control. It appears that the growth rate of synchronized bacteria is not altered by cold treatment. If the time of completed division was considered as 0, then infection of bacteria at 0, 10 and 20 min. (Fig. 1, points 1, 2 and 3) resulted in lysis after approx. 40 min. without even a transient increase in the intensity of scattered light. Thus the division of bacteria was completely arrested by the infecting virus even if it was

added only 5 min. before the next expected division. A remarkable property of phage in connexion with dividing bacteria is illustrated by the fact that addition of phage to a culture of already dividing cells (Fig. 1, point 4) stopped further increase in the intensity of scattered light. This shows that the division process was immediately inhibited by adsorbed phage. When the input of phage was 100:1 or more, a transient increase in intensity of scattered light was previously observed when cells were infected 5 min. before expected division (Stárka & Koza, 1958). Phase-contrast microscopy showed that loss of rigidity of the cell wall was accompanied by swelling of paired cells and by their separation. This, and the transient increase in light scattering, were probably caused by the action of the lytic enzyme of the phage tail. Fifteen minutes after addition of phage (input 100:1) complete lysis was observed in all samples ('lysis from without'—Delbrück, 1940).

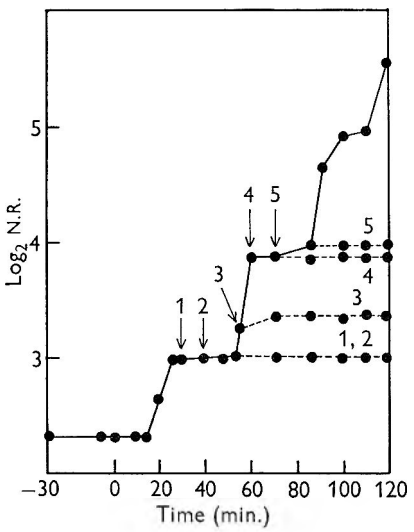

Fig. 2

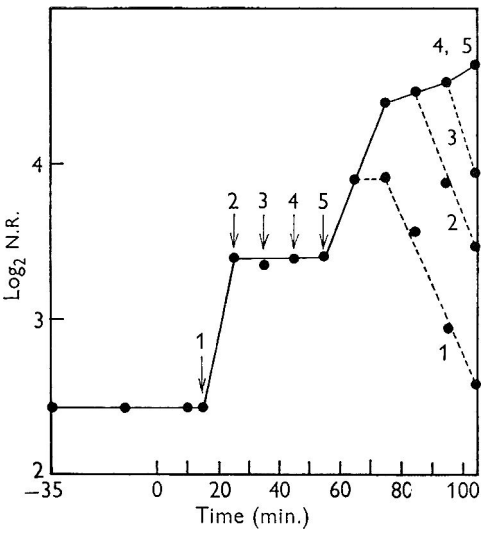

Fig. 3

Fig. 2. The effect of u.v.-inactivated phage T2*r* on cell division in a synchronized culture of *Escherichia coli* B. Inactivated phage/bacteria ratio = 4:1. (Compare with Fig. 1.)

Fig. 3. The effect of bacteriophage ϕx-174 on a synchronized culture of *Escherichia coli* C. Phage/bacteria ratio = 5:1. (Compare with Fig. 1.)

Similar experiments were performed with u.v.-irradiated phage containing a negligible fraction of plaque-forming survivors ($1:1{\cdot}5 \times 10^5$). Inactivated particles of T2*r* phage were added at intervals to the samples of synchronized bacteria. Input ratio of inactivated phage:bacteria was 4:1. The results of these experiments show that the irradiated phage did not differ from intact virus in its immediate action on dividing cells. In Fig. 2, point 3 represents the effect of inactivated phage on already dividing cells. The slight increase in nephelometer reading indicates division of a small fraction of bacteria during adsorption. When the input ratio was increased to 20:1, no such increase was recorded.

Závada (personal communication) demonstrated that *Escherichia coli* C infected by bacteriophage ϕx-174 was still able to synthesize β-galactosidase when induced

during the latent period. Turbidity measurements indicated that the host cells continued to grow until the first signs of lysis. In our studies, *Escherichia coli* C was synchronized as described above. Bacteriophage ϕx-174 was added to the samples of synchronized cells in a phage/bacteria ratio of 5:1. Figure 3 shows that the division of infected cells was not affected by phage during the whole of the latent period. Considering that at 30° this lasts approx. 55 min. it is possible that some cells may divide twice after infection. Thus, bacteriophage ϕx-174 behaves similarly to temperate phages (Lwoff, Siminovitch & Kjeldgaard, 1950).

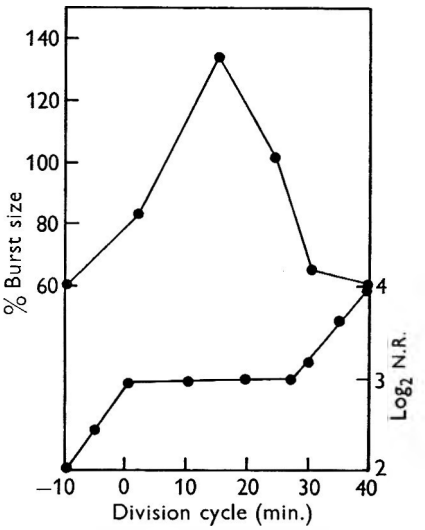

Fig. 4. Burst size of synchronized cells of *Escherichia coli* B. Phage yields from bacteria withdrawn at different phases of the division cycle (lower curve) were determined by single-cell method and by single-step growth experiments and plotted (upper curve) as percentage of burst size in a randomly dividing culture.

Phage yields of synchronized bacteria

Figure 4 shows the burst sizes obtained by means of single-cell determinations of phage yields from synchronized bacteria. The average burst size from non-synchronized cells was 92. Synchronized cells which had just completed division (0 min.) gave a value of 62 and cells from between two subsequent divisions (15 min.) gave an average burst size of 145. The differences were statistically significant. The distribution of burst sizes of individual bacteria from samples of synchronized cells is shown in Fig. 5. It appears that the burst size distribution of randomly dividing bacteria is wider than that of synchronized cells.

These findings were checked with burst size determinations obtained by means of single-step growth experiments. Samples of synchronized host cells were withdrawn at different phases of the division cycle and infected by phage. The latent period preceding liberation of vegetative phage was determined using bacteria from various points of the division cycle which was found to vary between 40 and 45 min. It appears that the rate of synthesis of new phage particles is not related to the division cycle of the host. On the other hand, the burst size depended significantly

on the time at which the bacteria were infected. Assuming that variations of adsorption rate during division cycle (Yanagita *et al.* 1958) did not play an important role in these experiments, the input ratio of phage:bacteria was always the same. Burst size determined by this method varied in the same range as in the single-cell burst determinations. The average burst size from randomly dividing cells was 87. Synchronized cells in 0 min. gave a burst of 51 and cells from the middle period between two divisions (15 min.) gave a value of 139 (see Fig. 4).

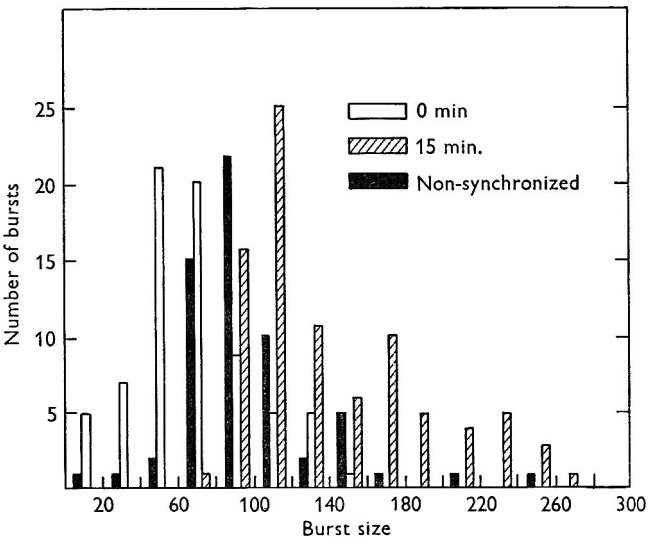

Fig. 5. Distribution of burst sizes from synchronized and non-synchronized cultures of *Escherichia coli* B infected with bacteriophage T2*r*. Data labelled 0 min. are from cultures in which cells had just completed division; data labelled 15 min. from culture in which cells were mid-way between divisions. Data are derived from 5 independent experiments with approx. 20 % samples showing bursts. (See Fig. 4.)

It appears, therefore, that during cell division the extent to which the cell can reproduce the virus is lowered and reaches a maximum between divisions. In non-synchronized cultures growing exponentially the burst size represents a composite of that from dividing and non-dividing cells.

DISCUSSION

The killing effect of T2 bacteriophages on bacterial cells is complex and involves the inhibition of the division of infected bacteria. The use of a nephelometric technique allows precise determinations to be made of the actual number of bacterial cells irrespective of size, and has demonstrated that infection of *Escherichia coli* by phage T2*r* prevents division even of those cells which were on the point of dividing. It may be that the lytic enzyme present in the phage tail causes some structural alterations in the cell wall the integrity of which is essential for division. The almost instantaneous inhibition of cellular division by phage could also be explained by an abrupt inhibition of synthesis of bacterial ribonucleic acid, which is well known to participate in cellular division (Swann, 1957). It has been established that the

synthesis of nucleic acids and proteins by the cell is drastically altered shortly after infection by phage (Cohen, 1947; Monod & Wollman, 1947; Brenner, Jacob & Meselson, 1961). T2 'ghosts' and phages irradiated by u.v. or X-rays and unable to multiply have the same killing properties as have intact phage (Garen & Kozloff, 1959) and one of the important properties of phage 'ghosts' is that they inhibit the synthesis of host ribonucleic acid (Herriot & Barlow, 1957). It cannot, however, be excluded that inhibition of cell division involves both the phage lytic enzyme and interference with nucleic acid metabolism. It is, perhaps, relevant that temperate phages do not interfere as greatly with the metabolism of the host as does the virulent phage T2 (Jacob & Wollman, 1959). This may be why they do not affect the division mechanism of the host cell. Bacteriophage ϕx-174 resembles the temperate phages in allowing the host cell to divide during the whole latent period.

In his paper Delbrück (1940) showed that the number of phage particles released from infected host cells depended on the physiological state of the host bacterium. With actively dividing bacteria the burst size was 170 and with resting cells it was only 20. The latent periods were 17 and 30 min., respectively. Another observation indicating the importance of the physiological state of the host cell was made by the same author (Delbrück, 1945). In experiments employing the single-cell technique, burst sizes varied from less than 20 to over 1000. This large fluctuation was explained by some feature in the mechanism of intracellular virus growth. In our experiments the variation of burst sizes was in the range from 10 to 280 only, but there was a significant difference in fluctuation range between non-dividing and dividing cells from synchronized cultures. It seems that the large fluctuation of burst sizes of randomly dividing bacteria could be explained at least in part by the variation in burst size depending on the division cycle of the host.

The observations of Yanagita *et al.* (1958) on burst size variations during the division cycle are confirmed and extended by our results despite the use of a completely different method of synchronization. But our findings differ from those of Barner & Cohen (1956) who used a third method—that of 'unbalanced growth' resulting from temporary withdrawal of an essential metabolite. Considering the fact that the balanced growth is affected only slightly by cold shock and to an even lesser extent by filtration, it may be assumed that the final yield of virus particles from an infected bacterium growing under normal conditions is influenced both by its physiological state and its position in the cell division cycle.

Why the cell synthesizes fewer bacteriophages when infected at the time of division as yet remains unknown, since our understanding of the dual problem of cellular division and virus multiplication remains incomplete.

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With 1 *plate*
Printed in Great Britain

Evidence that *Streptobacillus moniliformis* is an Intermediate Stage between a Corynebacterium and its L-form or Derived PPLO

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SUMMARY

Morphological and serological evidence is presented to demonstrate that an organism morphologically identical with *Streptobacillus moniliformis* is a stage intermediate to the L-form of a strain of *Corynebacterium cervicis*, that it is serologically closely related to a type culture of *S. moniliformis*, as well as to strains of *Mycoplasma hominis*; thus supporting the conclusion that L1, derived from *S. moniliformis*, and *M. hominis* have a similar origin, and suggesting that *S. moniliformis* is an intermediate stage between a corynebacterium resembling *C. cervicis* and an L-form.

INTRODUCTION

Streptobacillus moniliformis is commonly found in the nasopharynx and infected middle ear of rats (Strangeways, 1933; Klieneberger, 1936; Nelson, 1930, 1931, 1933); it causes rat-bite fever (Blake, 1916; Blake, Horstmann & Arnold, 1944; Brown & Nunemaker, 1942; Dolman, Kerr, Chang & Shearer, 1951; Hamburger & Knowles, 1953; Lominski, Stewart-Henderson & McKee, 1948; Scharles & Seastone, 1934) and similar conditions without a history of rat-bite (Hazard & Goodkind, 1932; Levaditi, Nicolau & Poincloux, 1925); epidemics of Haverhill fever (Parker & Hudson, 1926; Place & Sutton, 1934); a disease of mice (Freundt, 1956; Levaditi, Selbie & Schoen, 1932; van Rooyen, 1936); and calf pneumonia (Smith, 1918). It has also been isolated from brain abscesses (Oeding & Pederson, 1950) and subacute bacterial endocarditis (Blake, 1916; Hamburger & Knowles, 1953; McDermott, Leask & Berrit, 1945; Peterson, McCullough, Eisele & Goldinger, 1950; Stuart-Harris *et al.* 1935). It has generally been regarded as an unusual organism and difficult to classify, especially since it appears not only in its typical, Gram-negative, filamentous phase, with irregular swellings associated with the production of its L-form (first described by Klieneberger, 1935, 1936, as a symbiotic mycoplasma) but also in the form of short, regular, Gram-positive rods (Dubos, 1948). In its filamentous phase, and in the phenomenon of L-form production, *S. moniliformis* simulates the appearances produced in other bacteria by the influence of adverse conditions, notably the presence of penicillin. When in this state, *S. moniliformis* is impossible to separate from its L1 phase (Klieneberger, 1935, 1936). In fact, the difference between a culture of *S. moniliformis* and of L1 is simply that the latter lacks the filamentous stages (Heilman, 1941*a*; 1941*b*).

The purpose of this paper is to show that *Streptobacillus moniliformis* represents a semi-stable form intermediate between the L-stage and the Gram-positive, bacillary phase of a corynebacterium. Corynebacteria have been shown to produce

streptobacillus-like variants in cases of subacute bacterial endocarditis (Wittler *et al.* 1960) and when grown in the presence of fresh blood (Bisset, 1938); they are also known to produce variants resembling L-forms and mycoplasma (Minck, 1953; Wittler, Cary & Lindberg, 1956; Smith, Peoples & Morton, 1957; Pease & Laughton, 1962), and an antigenic relationship between *S. moniliformis* and certain mycoplasma is known to exist (Brown & Nunemaker, 1942).

The present investigation arose by the isolation of a micro-organism possessing the characters of *Streptobacillus moniliformis* from a culture of *Corynebacterium cervicis* derived from the human genital tract. This organism and its parent bacterium were compared with a standard strain of *S. moniliformis* and with cultures of *Mycoplasma hominis*.

METHODS

The strain of *Corynebacterium cervicis* (strain P; Pease & Laughton, 1962) from which the streptobacillus variant was obtained, and two strains of mycoplasma (Pease A and B) serologically identical with Edwards Type I, were isolated from vaginal swabs in this laboratory; other strains of mycoplasma were obtained by courtesy of Dr D. G. ff. Edward. The standard strain of *Streptobacillus moniliformis* was NCTC 8069.

Media used were Difco PPLO medium, sometimes with 3·5 % (w/v) sodium chloride added, nutrient agar + 10 % (w/v) glucose and Hartley's broth + 10 % (v/v) horse serum or human plasma and 0·5 % (w/v) yeast extract.

Gel-diffusion tests were made by double diffusion precipitation in agar/phosphate gel at pH 7·4. The antigens and the antisera were prepared according to the techniques given by Pease & Laughton (1962).

RESULTS

The vaginal corynebacterium (strain P) was grown upon Difco PPLO medium containing 3·5 % sodium chloride, and a strip of filter-paper soaked in penicillin solution (300 units/ml.) was placed on the agar. After 48 hr. incubation at 37°, L-form colonies appeared between the normal bacterial growth and the zone of complete inhibition. Between the L-colonies and the bacterial forms there were some colonies of intermediate appearance. These consisted of bacteria in the centre and a thin transparent periphery of L-organisms. Subculture of the pure L-colonies and the mixed colonies upon the same medium without the penicillin usually resulted in the growth of corynebacteria only.

The pure L-colonies were not stable. If inoculated upon medium without penicillin, they grew in the form of corynebacteria, and if upon medium containing penicillin, they died out progressively in the course of one or two subcultures.

However, subculture of the pure L-colonies sometimes resulted in the growth of filaments resembling *Streptobacillus moniliformis*. This form remained antigenically unaltered when subcultured upon medium without penicillin, but was somewhat less filamentous in morphology.

The colonies of the streptobacillus form consisted of filaments (Pl. 1, fig. 1) and chains of bacillary elements (Pl. 1, fig. 2), sometimes containing swellings (Pl. 1, fig. 3). There also occurred small colonies of flat disk-like elements (Pl. 1, fig. 4) similar to the X colonies of Brown & Nunemaker (1942). These forms were usually

Gram-positive, but mixed with some of them were Gram-negative L-forms (Pl. 1, fig. 5), which were morphologically and serologically identical with *M. hominis* (Pease & Laughton, 1962). The entire appearance was markedly similar to that of *S. moniliformis* (Pl. 1, fig. 6) except that the latter was usually Gram-negative in the filamentous phase.

The streptobacillus form grew well in Hartley's broth with serum and yeast-extract. No growth was obtained in this medium or in nutrient broth without serum. Growth was, however, obtained on nutrient agar without serum.

The results of the serological tests are shown in Table 1. The antisera were prepared against the corynebacterium and its streptobacillus variant, *Streptobacillus moniliformis* and a strain of *Mycoplasma hominis* Type 1 (Pease A) isolated in this laboratory. Antigens were made from the same organisms and also from a strain of L1, which was isolated from *S. moniliformis* with the aid of penicillin, and from *M. hominis* Types 1 (H26) and 2 (Campo) supplied by Dr Edward, and Type 1 (Pease B).

Table 1

	Antigens						
					M. hominis 1		
					Pease		
Antisera	Coryne-bacterium	Strepto-bacillus	*S. monili-formis*	L	A	B	H26
Corynebacterium	2	1	1	0	0	0	0
Streptobacillus	1	1	1	1	1	1	1
S. moniliformis	1	1	2	2	1	1	1
M. hominis 1 A	1 (weak)	1	0	0	1	1	1

Note: figures indicate number of precipitate lines appearing at junction.

The corynebacterium tested against its homologous antiserum showed two lines of precipitation, whereas with antiserum for the streptobacillus, only one line was formed indicating the loss of one antigen. A test in which the corynebacterium antigen was placed in the centre cup and alternating streptobacillus and *S. moniliformis* antisera in the outer six cups resulted in the formation of a hexagon (Fig. 1), showing that the corynebacterium, the streptobacillus and *S. moniliformis* have an

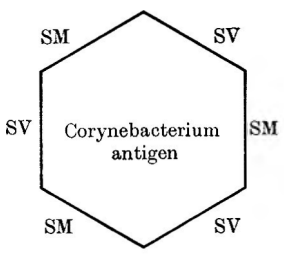

Fig. 1

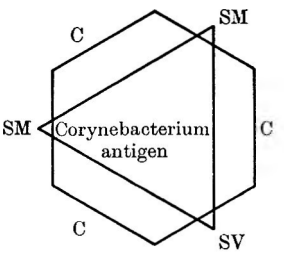

Fig. 2

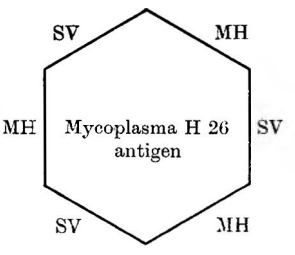

Fig. 3

Figs. 1–3. Diagrams of gel diffusion precipitin reactions. SV = Streptobacillus variant serum; SM = *S. moniliformis* serum; C = Corynebacterium serum; MH = *Mycoplasma hominis* Type 1 (Pease) serum.

antigen in common. In a similar test with three alternating antisera including the corynebacterium antiserum, a hexagon and a triangle (Fig. 2) were formed, thus showing that the streptobacillus and *S. moniliformis* both lack one antigen possessed by the corynebacterium.

The relationship between the streptobacillus and *Mycoplasma hominis* (Type 1) was shown in a test in which *M. hominis* (H26, Edward) antigen was placed in the centre cup and alternating streptobacillus and *M. hominis* antisera in the outer six cups. A hexagon (Fig. 3) was formed, showing that the streptobacillus and *M. hominis* have an antigen in common. *M. hominis* Type 2 (Campo) antigen did not react with any of the antisera.

DISCUSSION

The foregoing observations show that a corynebacterium of vaginal origin is capable of giving rise to a filamentous variant resembling *Streptobacillus moniliformis*, both morphologically and serologically, which further dissociates to give an L-form resembling *Mycoplasma hominis* both morphologically and serologically. The process of variation entails the loss of an antigenic component by the original corynebacterium, which presumably represents some portion of the cell wall material. This simultaneous production of a filamentous variant and loss of a surface antigen is exactly analogous with the process of S–R variation, as shown in *Corynebacterium diphtheriae* by Bisset (1938) and leads to the rather surprising suggestion that *Streptobacillus moniliformis*, of which the corynebacterium-like rod phase is, in fact, well known (Smith, 1918; Heilman, 1941*a*; Oeding & Pederson, 1950; Parker & Hudson, 1926) and is illustrated by Dienes (in Dubos, 1948), is both a stage intermediate to the L-form and in its morphology and antigenic structure simulates a filamentous R variant of one or more corynebacteria. It is by no means certain that all strains described as *S. moniliformis* do, in fact, have a single origin. The serological relationship of these variants, and of classical *S. moniliformis* and L1 to *Mycoplasma hominis* serves to reinforce the view of Pease & Laughton (1962) that mycoplasma are, in fact, L-forms of corynebacteria, in many cases.

These observations were foreshadowed, in a notable manner, by Hadley, Delves & Klimek (1931) who recognized the progression S–R–G (gonidial) in several types of bacteria, and remarked upon the resemblance of the G-form, now called the L-form, to the organism of bovine pleuropneumonia. Hadley's studies were, however, curiously neglected, and when the same resemblance between the L-colonies of *Streptobacillus moniliformis* and the pleuropneumonia organism was again noticed by Klieneberger (1935), the earlier observations were forgotten by subsequent workers. Klieneberger at first believed that the L-form was a symbiote of the bacillus, but was later convinced of its bacterial origin. Rather curiously, it appears to have been generally accepted that recognition of the bacterial origin of L1 had the effect of disproving the inferences drawn from its very strong resemblance to the pleuropneumonia organisms, or mycoplasma, and this has delayed recognition of what now appears to be the truth: that the L1 of *S. moniliformis* is synonymous with *Mycoplasma hominis*, and that both have their origin in strains of corynebacterium.

I wish to thank Dr K. A. Bisset for help and advice, and Dr Nancy Laughton for help in preparing the antisera.

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EXPLANATION OF PLATE

PLATE 1

Figs. 1–5. Streptobacillus-like variant of *Corynebacterium cervicis*, showing various appearances previously described as characteristic of *Streptobacillus moniliformis*. 48 hr. growth on Difco PPLO medium, at 37°.

Fig. 1. Filaments. × 3000.

Fig. 2. Chains of bacillary elements. × 2000.

Fig. 3. Streptobacilli showing swellings. × 3000.

Fig. 4. Streptobacilli showing a collection of flat disk-like elements, appearing as circular masses. × 3000.

Fig. 5. Gram-positive bacillary forms mixed with Gram-negative L-forms. × 2000.

Fig. 6. *Streptobacillus moniliformis* showing filamentous and swollen forms. × 2000. 48 hr. growth on Difco PPLO medium, at 37°.

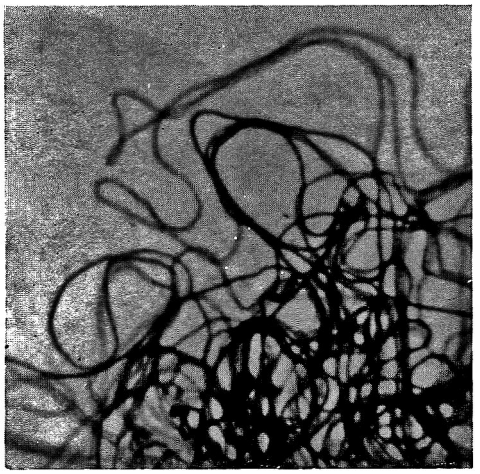

Fig. 1

Fig. 2

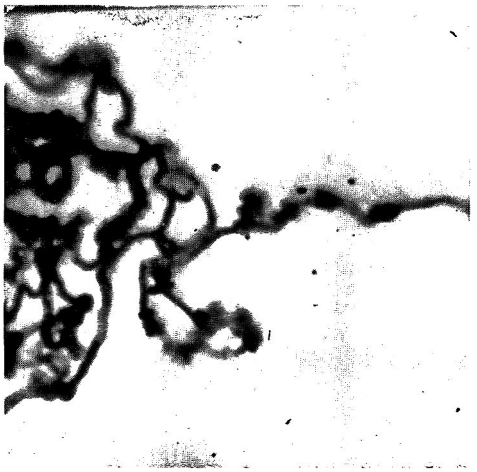

Fig. 3

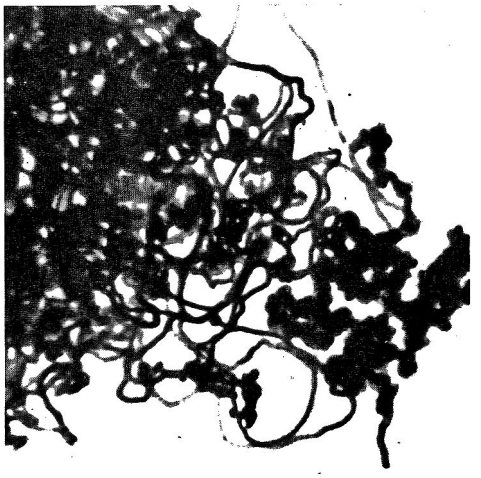

Fig. 4

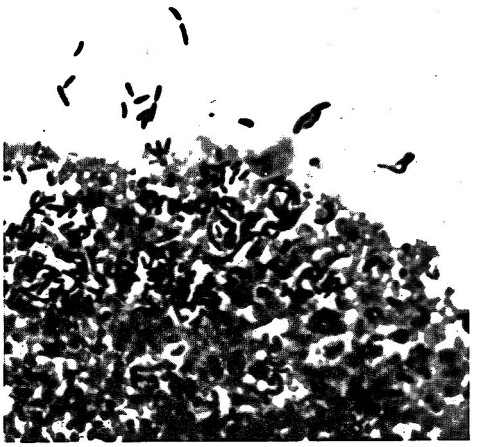

Fig. 5

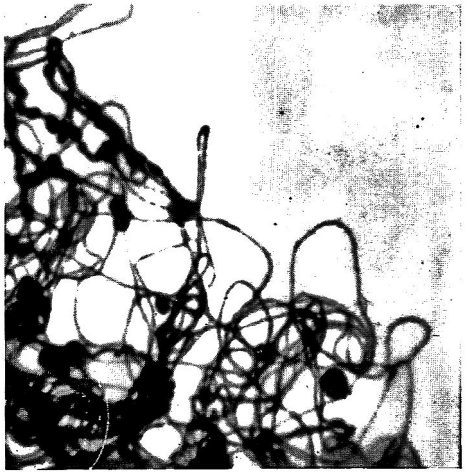

Fig. 6

P. PEASE

(*Facing. p.* 96)

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Total Counts of Bacterial Spores using Counting Slides

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(*Received* 15 *November* 1961)

SUMMARY

Total counts were made of bacterial spore suspensions using counting slides with chamber depths of 0·02 mm. and of 0·10 mm. Sources of error were analysed. In addition to an inherent sampling error, significant variation between counts on replicate slides may result from: (i) variation in the depth of different counting chambers, (ii) variation in the fit of cover-glasses with counting chambers. The use of 0·10 mm. rather than 0·02 mm. depth slides results in a lower estimate of the total count of a spore suspension. The former slides give more reproducible counts; evidence for their greater accuracy has been obtained by a comparison of 'percentage viability' of spores determined by counting and by slide-culture techniques.

INTRODUCTION

The need to assess the accuracy of total counts arose at the outset of an investigation of factors which may affect the viability of bacterial spores. When spores are plated on a nutrient medium production of a colony depends on (1) germination of the spore and emergence of a vegetative cell, (2) survival and multiplication of the vegetative cell. These two phases may require different optimal conditions (Halvorson & Church, 1957; Hyatt & Levinson, 1957, 1959; Demain, 1958; Demain & Newkirk, 1960). A determination of the total count of a spore suspension would enable calculation of the percentage of spores giving colonies on a nutrient medium. When this percentage is low the reason may be (*a*) because a proportion of spores are not viable, (*b*) they may be viable but fail to germinate in the growth conditions supplied, or (*c*) germination may be initiated, but the environment may fail to support outgrowth and multiplication.

Microscope counts of bacteria in chambers of known depth have generally been accepted and used as the most accurate total count technique available (Glynn, Powell, Rees & Cox, 1913–14; Wilson, 1922; Wilson & Kullman, 1931; Jordan & Jacobs, 1944; Semenitz, 1951). Recently an electronic method (Kubitsehek, 1958), used widely for counting blood cells, has been applied to counts of bacteria; this technique might effect a considerable saving in time and labour, but a detailed assessment of its accuracy when applied to bacteria does not appear to have been published.

In preliminary experiments using several slides (depth 0·02 mm.) the variation of replicate counts was greater than had been expected. The possible sources of variation and of error were analysed and include: (1) invisibility of spores; (2) movement of spores; (3) inaccuracy of counting the spores in a chamber and personal bias in counting; (4) sampling error due to distribution of spores in the

counting chamber; (5) technique in filling the chambers; (6) difference in depth of counting chambers having the same nominal depth; (7) variation of the *actual depth* of liquid enclosed in the counting chamber by the cover-glass. The actual depth of liquid enclosed in counting chambers with a nominal depth of 0·02 mm. has been reported to vary from about 0·0229 mm. to about 0·0297 mm. (Norris & Powell, 1961), depending mainly on the attention paid to closeness of contact between the cover-glass and the slide. Previous workers have failed to appreciate the magnitude of this error. Norris & Powell (1961) described an interferometric method for measuring the depth of liquid each time a chamber is assembled for a count.

The importance of the error due to the fit of cover-glass and slide is confirmed in this paper.

METHODS

Spore suspensions. The organisms used were several strains of *Bacillus subtilis*: Strain A, a rough variant derived from *B. subtilis* NCTC 8236; strain B, a smooth variant derived from *B. subtilis* NCTC 8236; Strain D, a laboratory strain supplied by the late Dr J. F. Powell, Microbiological Research Establishment, Porton, Wiltshire. Cultures were grown at 37° for 7 days on the surface of peptone agar containing 1 mg. $MnSO_4.4H_2O$/l. Organisms were harvested in sterile distilled water, washed several times on a refrigerated centrifuge and suspended in sterile distilled water to give a total count of about 10^{10} spores/ml. Suspensions were cooled in ice-water during this treatment, and finally stored at 0–4°. They contained about 90% of highly refractile spores; the remaining 10% consisted of spores which appeared dark by phase contrast, and some apparently 'empty' cells. 'Bright spores' only were counted.

Dilutions of stock suspensions. 100- to 500-fold dilutions of stock suspensions were prepared in distilled water sterilized by filtration through a 5/3 sintered glass filter, and shaken for 5 min. to ensure adequate dispersion.

Counting slides. Two types of slides were obtained from Messrs Hawksley and Sons Ltd., 17 New Cavendish Street, London, W. 1.

(1) Helber slide with central annulus and Thoma ruling. Nominal depth of chamber 0·020 mm. ± 0·001 mm ('0·02 mm. slide').

(2) Haemocytometer slides according to the British Standard Specification 748:1953 with Improved Neubauer ruling ('0·1 mm. slide'). Nominal depth of chamber 0·100 mm. ± 0·001 mm. The dimensions of one slide of each type were checked at the National Physical Laboratory. Using Johnson's (1952) procedure the mean depth of the 0·02 mm. slide was 0·0215 mm. ± 0·0003 mm., that of the 0·1 mm. slide was 0·1001–0·1003 mm. ± 0·0003 mm. (depth measured over two rulings). The observed volume of the counting chamber was 7·5% greater than the nominal volume of the 0·02 mm. slide and 0·25% less than the nominal volume of the 0·1 mm. slide. Twelve slides of type 1 and six of type 2 were used.

Cover-glasses for 0·02 mm. slides were 19 × 23 mm. area, 0·5 mm. thick, those for 0·1 mm. slides were 22 × 23 mm. area, 0·45 mm. thick.

Filling the counting chambers. 0·02 *mm. slides.* A dropping pipette (Cook & Yousef, 1953) was rinsed out with the suspension, and a drop placed on the central plateau of the slide. The coverglass was then lowered into position. The

effect of pressing down the edges of the cover-glass to ensure contact with the slide was significant, and is considered later. 0·1 *mm. slides.* The cover-glass was wrung to the supporting surfaces of the slide, a drop of spore suspension from the pipette was allowed to flow under the cover-glass.

Storage of filled counting slides. Sedimentation of spores and counting five replicate slides could require up to 2 hr. Evaporation was prevented by sealing the edges of the 0·1 mm. slides with DPX mounting fluid (British Drug Houses, Ltd.) and by storage of all slides in the presence of moist cotton wool.

Counting technique. A phase-contrast microscope was used (C. Baker Ltd.), with phase plate type TA-0·25λ, $\times 40$ objective, $\times 10$ binocular eyepieces and blue filter. Spores in 50 or 100 small squares (area 0·0025 mm.^{2}) were counted.

RESULTS

Error due to invisibility of spores. Their refractile nature makes spores readily visible by the phase-contrast microscope. Using a $\times 40$ objective (numerical aperture 0·7) the depth of field is less than 0·001 mm.; the counting chambers were 20 or 100 times this depth, therefore sedimentation of spores is essential. It was observed to occur within half an hour in both types of slide. Counts were made after sedimentation, and the error due to invisibility of spores was judged to be negligible.

Movement of organisms. Spores are non-motile, Brownian movement practically ceased after sedimentation.

Error in counting spores on a slide. Three experiments were made with 0·02 mm. slides stored for up to 7 hr., and four with 0·1 mm. slides stored for up to 3½ hr. Five counts were made during the storage, and the coefficient of variation of replicate counts of spores in a single, filled chamber, was calculated. For 0·02 mm. slides, coefficients of variation ranged from 0·79 to 1·81 %: for 0·1 mm. slides the values ranged from 0·54 to 1·14 %.

Error due to personal bias in counting. Replicate counts of the same slides were made by direct microscopic examination and from photomicrographs. The photographic count was 98 % of the visual count, and had a lower coefficient of variation.

Sampling error due to distribution of spores in the counting chamber. 'Student' (1907) showed that under ideal conditions the occurrence of yeast cells on the squares of a haemocytometer slide followed a Poisson distribution. Conditions which must be met before the distribution of the bacterial cells will be random are (1) the cells should not repel one another, or else there must be sufficient space so that the repelling effect will be negligible; (2) the volume of the cells, relative to the volume of the liquid in which they are suspended, should be small; (3) there should be no clumping of the cells (Stearman, 1955).

Agreement with a Poisson distribution was tested by calculating the Index of Dispersion (χ^2) of numbers per square on each slide (Fisher, 1948). The χ^2 values were grouped and the observed frequency of occurrence compared with that expected from a Poisson distribution. The results for strain D spores are shown in Table 1. For 60 counts of strain D spores total $\chi^2 = 3008{\cdot}3$ (60×49 degrees of freedom). Determination of $\sqrt{2\chi^2} - \sqrt{(2n-1)}$ and reference to the Normal Deviate table showed that the probability (P) of obtaining a total χ^2 equal to or greater than

this observed value was 0·185. In counts of strain A spores closer agreement between expected and observed frequencies was obtained. For 60 counts (60×49 degrees of freedom) the corresponding probability was 0·742. There was no evidence

Table 1. *The distribution of χ^2 values for counts of spores of* Bacillus subtilis *(strain D)*

Counting chambers 0·10 mm. depth, or 0·02 mm. depth. Each value of χ^2 is derived from counts of spores in 50 squares on one slide. (χ^2 distribution for 49 degrees of freedom from Fisher & Yates, 1955).

χ^2	Expected frequency	Observed frequency
< 28·942	0·6	0
29·942 –30·872	0·6	0
30·872 –33·931	1·8	2
33·931 –36·819	3·0	3
36·819 –40·530	6·0	2
40·530 –43·3665	6·0	6
43·3665–48·335	12·0	13
48·335 –53·6695	12·0	15
53·6695–57·0785	6·0	5
57·0785–62·037	6·0	7
62·037 –66·338	3·0	5
66·338 –71·405	1·8	1
71·405 –74·9185	0·6	1
> 74·9185	0·6	0
	60	60

Table 2. *Variation between counts on replicate slides*

Number of replicate slides for each experiment, $\geqslant 5$, Series 1, cover-glass *pressed down* on to slide. Series 2, cover-glass *lowered* on to slide.

	Spore suspension *B. subtilis* strain	Mean number of organisms per square	Coefficient of variation (%)
Series 1. Depth of slides = 0·02 mm.	D	1·305	23·1
	A	2·395	12·2
	A	4·058	14·0
	A	4·186	22·0
	B	4·292	14·4
	A	5·136	14·5
	D	5·676	16·5
Series 2. Depth of slides = 0·02 mm	D	2·372	12·1
	D	2·936	11·2
	B	4·752	15·0
	D	5·992	8·6
	A	6·030	7·7
	A	6·423	6·2
	A	6·920	8·3
	A	7·580	8·6
	A	10·550	10·9
Series 3. Depth of slides = 0·10 mm.	D	4·578	5·8
	D	5·471	5·6
	A	6·236	10·3
	D	7·304	5·5
	A	7·364	9·2
	D	7·825	6·2
	A	9·033	3·6

that distribution of spores in the counting chambers differed significantly from a Poisson distribution.

Variation in technique of filling a slide. A single 0·02 mm. slide was used to count five replicate samples from a spore suspension. With an average of 4·56 spores per square the coefficient of variation was 4·9 %. A comparable coefficient of variation, 5·1 % is shown by five replicate counts recorded in his table 1 by Wilson (1922).

Between slides variation. Coefficients of variation of counts on replicate slides are recorded in Table 2. The highest coefficients of variation were obtained when 0·02 mm. slides were used with the cover-glass pressed firmly down on to the slide. Use of 0·1 mm. slides gave the lowest values; the variation between counts on these slides was therefore compared with that attributable to sampling errors.

Seven groups of replicate counts (four of strain D spores, three of strain A spores) on 0·1 mm. slides were analysed. χ^2 for each group of replicates was combined, $\Sigma\chi^2 = 47{\cdot}1846$ (35 degrees of freedom) corresponding to a probability of 0·05–0·10. This is comparable with the variation between corrected counts in replicate 0·02 mm. slides reported by Norris & Powell (1961). Since the probability of Poissonian distribution of strain D spores was 0·185 the variation between counts on replicate 0·1 mm slides was probably not significantly greater than the variation due to sampling. Using *Bacillus stearothermophilus* spores and 0·1 mm slides, seven groups of replicate counts were made by a second operator. For this series of counts $\Sigma\chi^2 = 30{\cdot}752$ (31 degrees of freedom) corresponding to a probability of 0·3–0·5.

For the second series of experiments in Table 2 $\Sigma\chi^2 = 121{\cdot}77$ (38 degrees of freedom), corresponding to a probability less than 0·001. Clearly when either method of fitting the coverglass was used the variation between counts on replicate 0·02 mm. slides was significantly greater than that attributable to sampling.

Variation in fit of cover-glasses to slides. The 0·1 mm. slide has the following advantages over the 0·02 mm. slide, (1) the former is an 'open' type of chamber, to which the cover-glass can be firmly fitted before the spore suspension is introduced, whereas the 'closed' type of Helber chamber requires introduction of the suspension before application of the cover-glass; (2) the error introduced by variable fit of the cover-glass will be proportionally less in the case of the slide with the greater depth.

Using 0·02 mm. slides, counts with the cover-glass pressed firmly down on to the supporting edges of the slide were compared with those on slides with the cover-glass lowered into position. Four to eight slides were prepared by each method and the experiment was repeated 6 times. Typical results were:

mean count with cover-glass pressed down (method 1)—

5·136 spores/square (95 % fiducial limits 4·212–6·059);

mean count with cover glass lowered (method 2)—

6·924 spores/square (95 % fiducial limits 6·209–7·693);

$$\frac{\text{count by method 1}}{\text{count by method 2}} = 74{\cdot}2\,\%.$$

From the whole series of experiments the average ratio of these counts was 75·5 %.

Comparison of counts by 0·02 mm. and by 0·1 mm. slides. Six slides of each type were used to estimate the count of a spore suspension. Four such experiments were

performed. In three of the experiments the same spore suspension was counted in both types of slide, therefore the mean number of spores per square of the 0·1 mm slide would be expected to equal 5 times the mean number per square of the 0·02 mm. slide. In the 4th experiment the concentration of the suspension counted by the 0·02 mm. slide was 5 times that counted on the 0·1 mm. slide. When the count by 0·1 mm. slide was expressed as a percentage of the count by 0·02 mm. slide the values obtained were 69·9 %, 85·6 %, 75·4 % 63·4 %; mean 73·6 %.

0·1 mm. slides gave more reproducible counts which were lower and likely to be more accurate than counts by 0·02 mm. slides. An attempt was made to confirm this conclusion by an investigation of the percentage viability of a spore suspension (1) by comparison of total count with viable count estimated by colony production, (2) by slide culture of the spores.

Determination of percentage viability

Method 1. Total counts were made using 0·1 mm. slides. 'Bright' (highly refractive) spores only were counted, since the small proportion of 'dark' spores were less easily visible in the counting chamber. The percentage of bright spores initially present in the suspension, determined in the slide culture experiments, was taken as 94·5 % based on the fourth experiment (Table 3). The count of bright spores was multiplied by a factor of 100/94·5 to give the total count shown in Table 3. Viable counts were performed by a spread plate technique. In the first experiment four dilution series were used, and six plates prepared from the final dilution in each series. In the second experiment eight plates were prepared from each of five dilution series.

Table 3. *The percentage viability of* Bacillus subtilis *strain D spores, as determined:* (1) *by comparison of total, and colony counts,* (2) *by slide culture.*

Method 1

Time of storage of suspension (days at 4°)	Total count per ml.	95 % fiducial limits (as % of mean)	Viable count per ml.	95 % fiducial limits (as % of mean)	% viability
135	$3{\cdot}130 \times 10^7$	±9·91 %	$2{\cdot}899 \times 10^7$	±6·90 %	87·52
175	$1{\cdot}938 \times 10^7$	±4·14 %	$1{\cdot}615 \times 10^7$	±3·46 %	83·3

Method 2

	Number of replicates	Initial % 'bright' spores	95 % fiducial limits (as % of mean)	% viability	95 % fiducial limits (as % of mean)
175	1	—		83	
176	4	—		73	
213	2	—		88·0	
221	5	94·46	±1·25	88·87	±2·07
225	2	94·42		91·7	
227	2	92·25		91·2	

Method 2. Slide cultures were prepared according to the technique described by Postgate, Crumpton & Hunter (1961). When inoculated on to peptone agar and incubated at 37°, spores of *Bacillus subtilis* strain D showed a rapid fall in refractive index followed by swelling and outgrowth of the vegetative cell. The

percentage of spores showing outgrowth or swelling after incubation at 37° for 3 hr. was recorded as percentage viability. On further incubation growth appeared to proceed to microcolony formation, and no lysis of outgrown cells was observed.

The results are shown in Table 3.

DISCUSSION

The tolerance of ±0·001 mm. quoted by the manufacturers is ±5% of the depth of 0·02 mm. slides. Norris & Powell (1961) found no serious errors in the chambers they examined; the depth of one of our slides was reported to be at least 5% greater than the nominal depth. The demonstration of a significant systematic error depending on the fit of the cover-glass with the 0·02 mm. slide agrees with the observations of Norris & Powell (1961). These workers reported that a minimum depth of 0·02288 mm. of liquid was enclosed in slides with a mean depth of 0·0204 mm., i.e. the actual depth of the slide was at least 12·4% less than the depth of liquid enclosed between slide and cover-glass.

The tolerance of 0·001 mm. is 1% of the depth of 0·1 mm. slides; the error of the slide tested was less than this. In our experiments total counts of spores by 0·1 mm. slides were 15–36% lower than those by 0·02 mm. slides with coverglasses firmly pressed into position. In view of Norris & Powell's findings, the closer fit between cover-glasses and 0·1 mm. slides and the smaller significance of this source of error in relation to the depth of the slide, it seemed likely that use of 0·1 mm. slides would give a more accurate count. This was confirmed by the correlation between percentage viability of *Bacillus subtilis* spores determined by counting experiments and by slide culture. If 0·02 mm. slides had been used the resulting estimates of viability would have been about 61, and 57%, i.e. 26% lower than estimates based on the use of 0·1 mm. slides.

The variation between counts of replicate slides was greater between 0·02 mm. slides than 0·1 mm. slides. Greater variation occurred when the cover-glass was pressed down on to the slide, than when it was merely lowered. The χ^2 test for variation between counts of *Bacillus subtilis* spores on 0·1 mm. slides gave a probability of 0·1–0·05. Since the probability of Poissonian distribution of strain D spores was 0·185 the use of replicate slides does not appear to have significantly increased the variation. Two total counts by 0·1 mm. slides are shown in Table 3; the first was based on counts of about 390 spores on each of four slides, the second on 230 spores on each of 10 slides. The replication used for the second count has reduced the 95% fiducial limits to about ±4% of the mean count. Unless systematic errors can be shown to be small in relation to this variation there is probably no value in further replication to increase the precision of this determination.

Errors due to the other factors investigated are relatively small; if there is a tendency of organisms to form clumps the distribution will not be Poissonian, and sampling errors will be increased. Norris & Powell concluded that other errors resulted from the adherence of organisms to solid surfaces, and advised the use of a detergent and a low surface/volume ratio of suspensions to minimize this effect. We have noted a drop in total count of spore suspensions after placing in clean glass bottles and storing for 24 hr. at 4°, presumably due to adsorption of spores on to the glass. For this reason a fresh spore suspension of suitable concentration was prepared for each series of counts, and replicate samples removed within a few hours.

The use of 0·1 mm. slides for total counts of vegetative bacteria has not been investigated; these organisms may not sediment sufficiently rapidly for the technique to be practicable.

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Virus–Cell Relation in Rat Polyoma Tumours

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SUMMARY

Experiments on rat tumours induced by polyoma virus indicate that, although no virus was directly demonstrable in these tumours, at least by the technique used, some of the tumours when set up as tissue cultures were still capable of synthesizing polyoma virus. This faculty disappeared, however, in most instances after transplantation *in vivo*; virus recovery by tissue culture procedures after passage in rats was exceptional. Furthermore, if one draws a parallel between the virus/cell relationship in rat polyoma tumours and the lysogenic system in bacteria, one finds that both phenomena though not exactly similar have many points in common.

INTRODUCTION

Most of the studies on the virus/cell relationship in tumours induced by polyoma virus have been made on primary and transplantable tumours in hamsters (Habel & Silverberg, 1960) and in mice (Sachs & Fogel, 1960; Winocour & Sachs, 1961). These studies suggested that differences in the virus/cell relationship might depend on the experimental animal chosen. For instance, whereas mouse tissue was shown to have not only a non-lytic but also a strong lytic response to polyoma virus *in vivo* and *in vitro* (Stanton, Stewart, Eddy & Blackwell, 1959; Sachs & Fogel, 1960), hamster tissue upon infection reacted predominantly by a non-lytic response with rapid tumour formation (Vogt & Dulbecco, 1960; Stoker & MacPherson, 1961; Roizman & Roane, 1960).

In the present work we studied the virus/cell relationship in polyoma tumours induced in our R inbred strain of rats. Previous experiments (Vandeputte, 1961) showed that no polyoma virus could be recovered from meningeal sarcomas induced by this virus in our rat strain. To determine whether this finding applied to other rat tumours, we analysed different primary and transplantable rat polyoma tumours both directly and *in vitro* for the presence of the virus or its antigen, using the fluorescent antibody technique and tissue culture procedures. We also studied the behaviour of these tumours when transplanted and when challenged by polyoma virus *in vitro*.

METHODS

Virus stock. The polyoma strain used in our experiments was obtained from Dr C. Dawe (National Institutes of Health, Bethesda, Md., U.S.A.). The virus was grown on mouse embryo fibroblast cultures in our laboratory; the stock virus used had a titre of 2×10^6 Tissue Culture Infectious Dosis 50/ml. (TCID 50/ml.).

Primary and transplanted tumours in the rat. Within 24 hr. after birth, rats of the Albino R strain (obtained from Prof. Mühlbock, Amsterdam) were inoculated intra-

cerebrally or subcutaneously with 10^5 TCID 50 doses of polyoma virus. The rats which developed tumours were bled-out and after heating the sera to 56° for 30 min. the haemagglutination-inhibition antibody titre was determined, using 10 haemagglutinating units (Rowe, Hartley, Estes & Huebner, 1959). Each tumour was removed aseptically and part of it frozen immediately either for cryostat frozen sections or for fluorocarbon extraction. Another part was trypsinized and set up as a tissue culture. When a tumour was very large, a fragment was also minced with scissors and grafted subcutaneously into isologous adult rats. Meningeal sarcomas and liposarcomas were not trypsinized; they were used only for transplantation or for fluorescent antibody studies and fluorocarbon extraction. Primary tumours that grew after grafting into isologous rats were serially transplanted and fragments from them were trypsinized for fluorescent antibody studies and polyoma virus isolation in tissue culture.

Tissue culture preparation of rat polyoma tumours. A portion of the primary tumour was trypsinized, and the cell suspension obtained after washing three times was put into tissue culture. For fluorescent antibody studies Leighton tubes were inoculated with 10^5 cells, and for virus isolation Petri dishes (60 mm diam.) were inoculated with 10^6 cells and kept in a humidified incubator supplied with a constant flow of 5 % CO_2 in air. In all tissue culture experiments Eagle's medium with 10 % (v/v) calf serum was used. After 24 hr. and after 4, 6, 8 and 10 days tissue cultures were removed to see whether viral antigen could be detected by the fluorescent antibody technique and whether virus could be isolated from the centrifuged supernatant medium, and from the remaining medium together with the cells after these had been frozen and thawed three times.

One or two Petri dish cultures were kept to establish a continuous line of the tumour cells. After two to three *in vitro* passages the cells from these Petri dishes were inoculated into young (about 1–3 days old) and adult isologous rats. The subcutaneous tumours obtained by direct grafting of the primary tumour as mentioned above, or by injecting subcutaneously cells of the 2nd or 3rd tissue culture passages from these primary tumours, were aseptically removed after bleeding-out the rat and were retransplanted (2nd passage Tables 1 and 2). A portion of each tumour was trypsinized and put in tissue culture to test for the presence of viral antigen and infectious polyoma virus.

Determination of the presence of infectious polyoma virus. Frozen pieces of primary rat polyoma tumours were made up as a 10% suspension by grinding in a mortar in phosphate-buffered saline (NaCl 9 g. + $NaH_2PO_4.H_2O$ 0·519 g. + $Na_2HPO_4.2H_2O$ 1·111 g./l.) and treated twice in the cold by fluorocarbon (Freon 113) in a 'Polytron' Omnimix (M. Wullimann, Selzach, Switzerland) at high speed (10,000 rev./min.). For polyoma virus isolation 0·1 ml. of the aqueous phase was inoculated into mouse embryo tissue culture. The tissue culture samples from primary and transplanted rat polyoma tumours were also inoculated on mouse embryo tissue culture (0·1 ml. of each sample in two tissue culture tubes). These inoculated tubes were kept for 12 days and examined every 2 days for the appearance of cytopathic effect. After 12 days, the presence of haemagglutinating activity in the medium was determined. Material from tissue culture tubes which showed no cytopathic effect or haemagglutinating activity was inoculated into new mouse embryo tissue culture tubes and the corresponding initial tumour extracts were inoculated intraperitoneally (0·2 ml.)

into adult mice. These were bled after 3 and 6 weeks for the titration of haemagglutination-inhibition antibodies, 10 haemagglutinating units being used (Rowe's MAP test, 1959).

Fluorescent antibody studies. Frozen tissue blocks were sectioned at an indicated thickness of 6μ in a $-20°$ microtome cryostat. These sections were air-dried at room temperature for 10 min. and then fixed in anhydrous acetone for 15 min. After air-drying and repeated washing in phosphate-buffered saline, antipolyoma chicken serum diluted 1/5 was applied on the sections which were then incubated for 30 min. at 37° in a moistened Petri dish. This serum had previously been absorbed three times with mouse fibroblasts and had a titre of 1/2048 as determined by the haemagglutination-inhibition test. After incubation, the slides were washed three times in phosphate-buffered saline and re-incubated at 37° for 30 min. with fluorescein-labelled antichicken goat serum (Microbiological Associates. Inc. Bethesda, Md., U.S.A.), which had previously been absorbed twice with acetone-dried mouse liver powder. As counterstain, we used Lissamine Rhodamine RB-200 conjugated with bovine albumin (Microbiological Associates). Before being mounted in buffered glycerol, the slides were washed in three changes of phosphate-buffered saline.

To test the specificity of the action, slides were treated in parallel with normal chicken serum before the application of fluorescein-conjugated antichick serum and with the fluorescein-conjugated serum alone. Mouse embryo fibroblasts not infected with polyoma virus were also used as a control to detect the presence of any non-specific fluorescence. The fluorescence procedure on tissue culture was similar, except that before air-drying and fixation in anhydrous acetone the slides were washed three times with phosphate-buffered saline.

The optical system consisted of the Zeiss fluorescence microscope equipped with a high pressure mercury lamp HBO 200; the BG 12 (3 mm. × 5 mm.) exciter filters and the OG5 and OG4 barrier filters were used.

RESULTS

Primary tumours of the rat

Primary tumours were studied for the presence of viral antigen by the indirect fluorescent antibody technique on frozen cryostat sections. As shown in Table 1, no polyoma antigen could be demonstrated in the nine tumours tested (Pl. 1, fig. 1). Tumour extracts prepared in phosphate-buffered saline, whether treated or untreated with fluorocarbon, were negative for the presence of polyoma virus even after a blind passage on mouse embryo tissue culture (Table 2). The fluorocarbon technique was used to separate the antibodies from any virus which might be present in the extract (Hummeler & Ketler, 1958). Samples of the same tumour extracts injected intraperitoneally in adult mice gave negative MAP tests. These data indicated that the infectious polyoma virus and its antigen were no longer detectable in the tumours or their extracts, at least by the techniques used.

However, when portions of those same tumours, found negative by direct examination as above for infectious virus or viral antigen, were put up as tissue cultures, the synthesis of polyoma virus was now detected in four of ten tumours (Table 1). By the fluorescent antibody technique, we found the viral antigen to be localized mostly in the nuclei (Pl. 1, figs. 2, 3) and only occasionally in the cytoplasm

(Pl. 2, fig. 4). Synthesis of the antigen reached a peak from the 4th to the 6th days and then decreased to disappear sometimes after 9 days (Table 1). This implies that the cells in which viral antigen was synthesized had died and that there was no re-infection of other cells by any released polyoma virus. From all the tumours set up as tissue cultures and yielding viral antigen as shown by the fluorescent antibody technique, infectious virus was isolated on mouse embryo tissue culture.

The appearance of viral antigen preceded the release of infectious virus in the supernatant medium (Table 2, RKS_1, RKS_4) but probably not the detection of intracellular infectious virus. Those tumours set up as tissue cultures which did not

Table 1. *Fluorescent cells in rat tumours* (%)*; indirect method*

Type of rat tumour*	Frozen cryostat sections†	Primary tissue culture				
		24 hr.	Day 4	Day 6	Day 8	Day 10
RKS_1	nt	5/1‡	7/2	6/2	3/0	1/1
RKS_2	–	5/0	4/1	1/0	0/1	0/0
RKS_3	–	–	–	–	–	–
RKS_4	–	3/0	5/4	1/0	nt	1/0
RKS_5	–	–	–	–	nt	–
RKS_6	nt	–	–	–	–	–
RKS_7	nt	–	–	nt	–	–
RFS_1	–	4/1	6/3	1/1	nt	0/0
RFS_2	–	–	nt	nt	–	–
RFS_3	–	–	–	nt	–	–
RLS_1	–	nt	nt	nt	nt	nt
RMS_1	–	nt	nt	nt	nt	nt

* RKS = rat kidney sarcoma; RFS = rat subcutaneous fibrosarcoma; RLS = rat liposarcoma; RMS = rat meningeal sarcomatosis.

† nt = not tested; – = negative.

‡ First number = nuclear fluorescence; second number = cytoplasmic fluorescence (determined on 100 to 200 cells).

Table 2. *Virus isolation from rat tumour cells*

Rat tumours*	Fluoro-carbon extract†	Phos-phate-buffer extract	Primary tissue culture					Tissue culture after passage in rats			
			24 hr.	Day 4	Day 6	Day 8	Day 10	1st pass.	HIA‡	2nd pass.	HIA‡
RKS_1	–	nt	+/–§	+/+	+/+	nt	nt	nt	–	–	–
RKS_2	nt	nt	+/+	+/+	nt	+/+	nt	–	–	–	–
RKS_3	nt	nt	–	–	nt	–	–	–	–	–	–
RKS_4	nt	–	+/–	+/+	nt	+/+	+/+	–	–	+/–‖	–
RKS_5	nt	nt	–	–	–	–	–	–	–	–	–
RKS_6	–	nt	–	nt	–	–	–	nt	nt	nt	nt
RKS_7	–	nt	–	–	–	–	–	nt	nt	nt	nt
RKS_8	–	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
RFS_1	–	nt	nt	+/+	+/+	+/+	+/+	–	–	–	–
RFS_2	nt	–	–	–	–	nt	–	nt	nt	nt	nt
RFS_3	nt	–	–	–	nt	–	–	nt	nt	nt	nt
RFS_4	–	–	nt	nt	nt	nt	nt	nt	nt	nt	nt

* See Table 1 for types.

† nt = not tested; – = negative.

‡ Haemagglutination-inhibition antibody (HIA) titre in the tumour-bearing rats (less than 1/32 is considered negative).

§ First sign = intracellular virus; second sign = virus in supernatant medium.

‖ One tumour out of three set up as tissue cultures released polyoma virus.

then yield for infectious polyoma virus did not show any specific fluorescence which could indicate the synthesis or presence of incomplete virus (Pl. 2, fig. 5). Sera from mice inoculated with samples of these negative tissue cultures gave negative MAP tests.

Transplantable rat polyoma tumours

An attempt to transplant fragments of primary tumours minced with scissors by subcutaneous grafting into isologous rats was not very successful. Indeed, two meningeal sarcomas, two kidney sarcomas and one subcutaneous fibrosarcoma failed to grow after being grafted into isologous hosts. Only two kidney sarcomas (RKS_7, RKS_8) could be serially transplanted. After one and three *in vivo* passages, portions of these transplantable tumours were put up as tissue cultures. Both failed to release infectious polyoma virus and neither contained viral antigen. The rats bearing these transplantable tumours had consistently negative HA-inhibiting antibody titres. The transplantation into rats of primary tumours after one or two passages in tissue cultures was more successful (Tables 1, 2). These tumours grew very well in all the inoculated young rats (1–3 days old) and gave tumours in 60–80 % of the adults. The young and adult rats which developed tumours at the site of inoculation of these tissue cultures (1st passage) and those bearing transplants of these 1st passage tumours (2nd passage, see Table 2) never developed haemagglutination-inhibiting antibodies against polyoma virus.

All the first passage tumours when put up as tissue cultures were found negative for the presence of viral antigen as tested by the fluorescent antibody technique and for infectious polyoma virus (Table 2).

One tumour from the second *in vivo* passage released virus in tissue culture (RKS_4). Curiously, two tumours that arose from the same inoculum (1st passage tumour) in two other rats from the same nest and grafted at the same time were found negative for polyoma virus when put up as tissue cultures. This seems to indicate that although there is generally no virus production after transplantation *in vivo* of polyoma-induced tumours of the rat, exceptionally some tumour lines are still capable of synthesizing and releasing virus when set up as tissue cultures. The reasons for this particular behaviour are still unknown. The difficulties encountered in the transplantation experiments of primary polyoma tumours into isologous hosts could be due either to factors depending on the host, such as genetic differences, or to factors determined by the tumour itself, such as the acquisition of new antigens, nutritional deficiency, etc. Our Albino R rat strain seems to be genetically homogeneous, at least for the histocompatibility antigens. This was verified by skin transplantation between animals chosen at random and by transplantation of one spontaneous mammary tumour and two methylcholanthrene-induced fibrosarcomas. This indicates that polyoma tumours in rats behave on transplantation like some polyoma tumours in the mouse (Dawe, 1960). Primary hamster polyoma tumours on the other hand seem to be easily transplantable, as shown by Habel & Silverberg (1960). Our data suggest that the difficulties encountered in transplanting primary rat tumours are determined by factors inherent in the tumour rather than in the host. These factors are slightly depressed by passage in tissue culture and are overcome by the use of newborn rats for transplantation, as previously described. Further studies are necessary, however, to determine the exact nature of this phenomenon.

Susceptibility of rat polyoma tumours to challenge infection with polyoma virus

Tissue cultures made from three primary kidney sarcomas and one subcutaneous fibrosarcoma were challenged with 2×10^5 TCID50 doses of polyoma virus. Two of these lines (RKS_1, RKS_2) were initially positive for virus production in tissue culture. In all cases, these tissue culture lines were found resistant to superinfection by polyoma virus, and no cytopathic effect or virus production was observed during an observation period of 12 days. For challenge, the same strain of polyoma virus as that which induced the tumours was used. In parallel with these experiments, we examined also the susceptibility to polyoma virus of a normal rat kidney continuous line (RN cells) and a continuous tissue culture line established from a carcinogen-induced mammary tumour (DE40 cells); these two lines were also found resistant to infection by polyoma virus. This indicates that the resistance of tissue culture lines established from rat polyoma tumours to challenge infection by this virus is not a specific character of the tumours. The polyoma tissue culture lines, like the RN and DE40 cells, were susceptible to herpes and vaccinia virus.

DISCUSSION

Sachs & Fogel (1960) showed that rat polyoma tumours, when put in tissue culture, synthesized infectious virus and viral antigen. Although our studies point in the same direction, we did not find this to be a general phenomenon. Putting a rat polyoma tumour found negative by direct examination for the presence of virus in tissue culture seems to induce only in some instances the synthesis of polyoma virus. The other tumours, which did not release infectious virus in tissue culture, likewise appeared to contain no viral antigen when analysed by the fluorescent antibody technique and the MAP test. No evidence was found for the existence of an incomplete virus in our rat tumour lines as described by Sachs & Fogel (1960) for certain mouse and hamster polyoma tumours. In our tumours, which were positive in tissue culture for the presence of virus, we found mostly a nuclear and only occasionally a cytoplasmic localization of the viral antigen. These results differ from those reported by Sachs & Fogel (1960), who always located the viral antigen in the cytoplasm of his tumour cells. We do not know yet whether this difference may result from the greater sensitivity of the indirect method as compared with the direct fluorescent antibody method used by Sachs.

The induction by tissue culture of virus production in rat tumours found negative for polyoma virus on direct examination, the rate of synthesis of the virus as shown by the fluorescent antibody technique and the disappearance of the virus on transplantation with occasional recovery in tissue culture, suggest a virus/cell relationship close to a lysogenic system, as was indicated by Winocour & Sachs (1961) for polyoma tumours in the mouse. The non-susceptibility of the polyoma tumour lines to superinfection by the same virus and their susceptibility to unrelated viruses (herpes and vaccinia virus) might be considered as another point of similarity if it had been specific for the rat tissue culture lines originated from polyoma tumours only. Also the possible acquisition of a new cellular antigen, as suggested by our preliminary transplantation experiments, has its equivalent in the bacterial lysogenic system

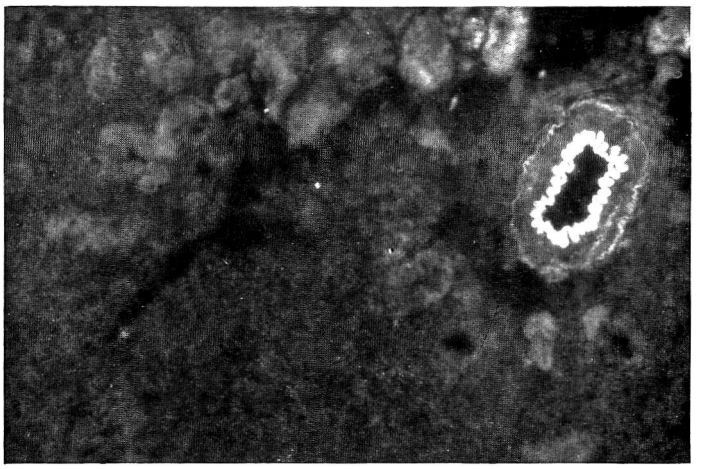


Fig. 1

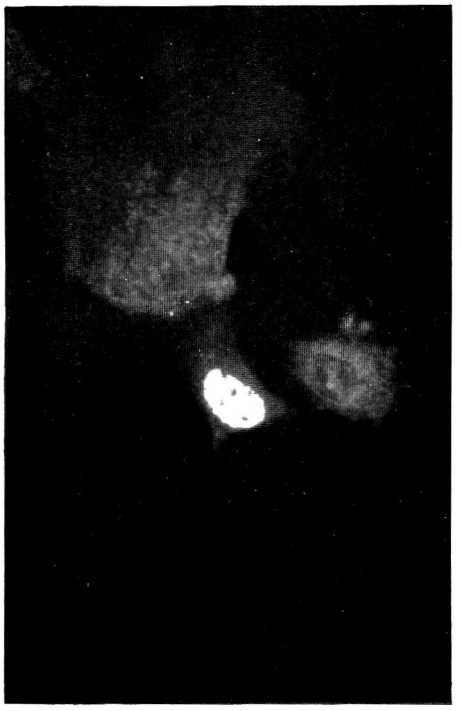
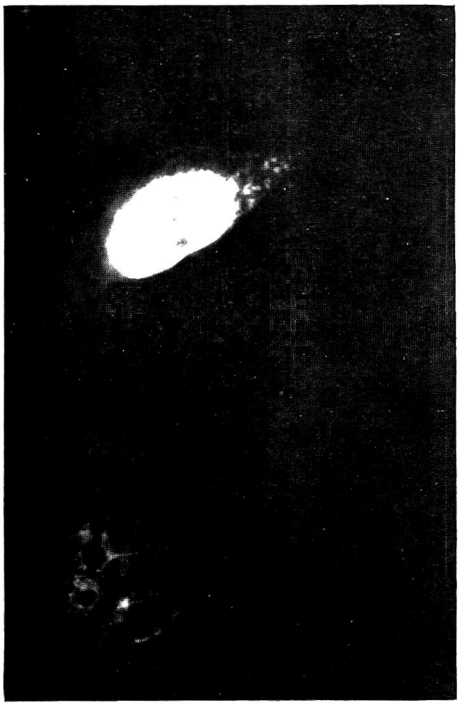


Fig. 2



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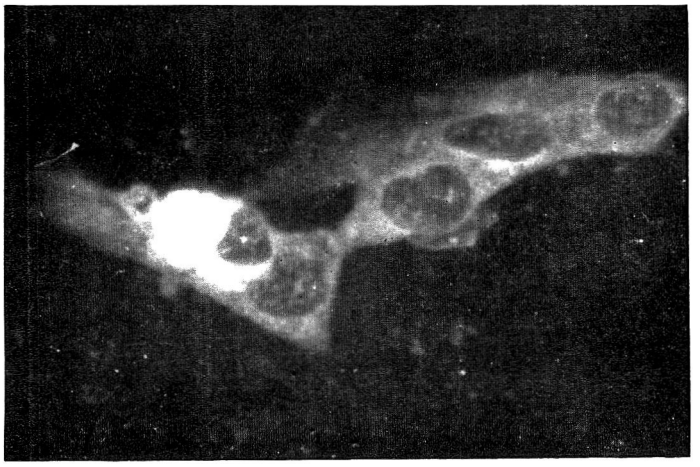

Fig. 4

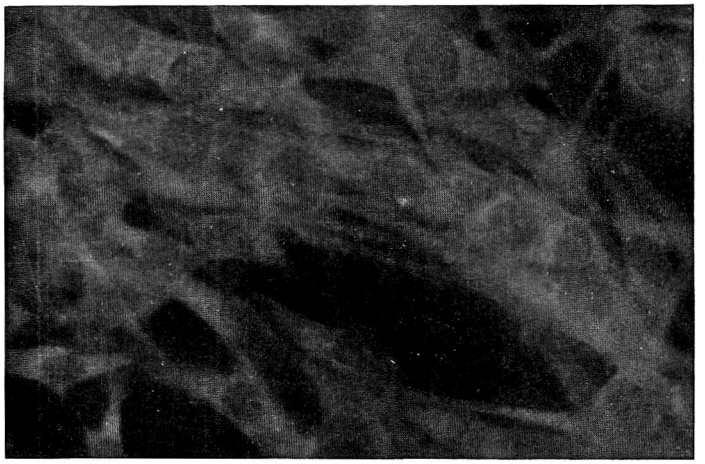

Fig. 5

(Jacob & Wollman, 1959); further studies will indicate whether our findings are analogous to those described by Sjögren, Hellström & Klein (1961), in mouse polyoma tumours.

Part of this work was presented at the Meeting of the Society for General Microbiology held in Oxford (28–30 September 1961).

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Cryostat microtome section of a primary rat kidney sarcoma induced by polyoma virus (RKS_2). Absence of detectable polyoma antigen by the indirect fluorescence method (× 80).

Fig. 2. Rat kidney sarcoma (RKS_1) grown in tissue culture (day 4). Positive nuclear fluorescence indicating the presence of polyoma antigen (× 400).

Fig. 3. Rat kidney sarcoma (RKS_4) grown in tissue culture (24 hr.). Positive nuclear fluorescence (× 400).

PLATE 2

Fig. 4. Rat subcutaneous fibrosarcoma (RFS_1) grown in tissue culture (day 4). Positive cytoplasmic fluorescence (× 400).

Fig. 5. Rat kidney sarcoma (RKS_7) grown in tissue culture (day 8). Absence of positive fluorescence (× 400).

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The Effects of Partial Pressure of Oxygen upon Respiration and Nitrogen Fixation by Soybean Root Nodules

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SUMMARY

Increased oxygen tension (pO_2) caused increased respiration by excised soybean nodules of all ages. The increase took place in two steps, the first maximum occurring at about 50 % O_2 and the second at 90–100 % O_2 for actively nitrogen-fixing nodules. With increasing nodule age the first maximum occurred at decreasing pO_2 until, when fixation ceased at about 6 weeks, this maximum had disappeared. This effect was more marked at 30° than at 23°. The respiration of bacteroids increased with increasing pO_2 with a maximum at 2–3 % O_2; the curve indicated a simple saturation of the terminal respiratory pathway with O_2. Increased pO_2 raised nitrogen fixation by excised nodules to a maximum which corresponded to the first maximum of the respiratory response to raised pO_2; higher pO_2 than this decreased nitrogen fixation. Sliced nodules showed the same effect but the stimulation of fixation at the lower pO_2 levels was not as great as with intact nodules. The Michaelis constant (K_m) for nitrogen fixation by intact excised nodules was relatively unaffected by pO_2 until this reached the pO_2 for maximum fixation when the K_m rose sharply. At external pO_2 of 80 %, oxygen was shown to be a competitive inhibitor of nitrogen fixation.

An explanation of these results is offered; it is suggested that the first part of the nodule O_2 consumption/pO_2 curves is due to O_2 consumption by plant tissue and the second part to O_2 consumption by bacteroids. The two components are separated by an O_2 permeability barrier. When this barrier permits a rise in the pO_2 at the bacteroids, nitrogen fixation is inhibited as oxygen competes with nitrogen for the reducing power of the bacteroids.

INTRODUCTION

Allison, Ludwig, Hoover & Minor (1940) concluded from studies of the effects of increased oxygen tension upon nodule respiration that oxygen tension was low within the tissue of soybean root nodules. Ebertova (1959) measured low redox potentials within nodules during the period in which nitrogen was fixed. In contrast to these findings Ferguson & Bond (1954) found that increased oxygen tension in the root medium favoured nitrogen fixation by whole nodulated plants; and Burris, Magee & Bach (1955) found with sliced soybean nodules that oxygen tensions up to 50 % O_2 stimulated fixation but that higher tensions were inhibitory. Bond (1961) reported similar effects with root nodules of non-leguminous plants.

A unified hypothesis which relates the various phases of symbiotic nitrogen fixation in legume nodules has been proposed (Bergersen, 1960*b*) in which it is suggested that reducing power generated in the bacteroids brings about the ultimate production of NH_3 from N_2. This hypothesis demands that oxygen tension at

the surface of the bacteroids be low (much less than the tension which saturates their terminal respiratory enzymes) if aerobic terminal respiration is not to compete with N_2 for their reducing power. The work to be reported here was undertaken to investigate the apparent paradox of oxygen both stimulating and inhibiting nitrogen fixation by legume nodules. In doing this the work also provided a test of one aspect of the hypothesis.

METHODS

Plant material. Lincoln variety soybeans were grown and nodule age recorded as previously described (Bergersen, 1958). Nodules were detached from the roots into beakers immersed in ice and experiments were begun within 1 hr. of picking the first nodule.

Bacteria. Rhizobium japonicum strain CC711 was used throughout and was grown on yeast-extract mannitol agar. Suspensions of bacteroids (the nodular form of the organisms) were obtained from crushed nodules as previously described (Bergersen, 1958).

Respirometry. Respiration was measured in double capillary Warburg manometers equipped with stopcocks between the fluid reservoirs and the capillaries to permit evacuation and flushing of the respirometers with various gas mixtures. In doing this the fluid was lowered to the hole of the stopcock and the respirometers evacuated and filled through both sides of the manometer. When filled, the fluid was raised and a positive pressure maintained until just before measurements began, when the excess pressure was released.

Respiration of whole and sliced nodules was measured in Warburg vessels (volume 30 ml.) containing 20 nodules and 0·25 ml. phosphate buffer (M/15, pH 7·0) with 0·2 ml. 40 % (w/v) KOH in the centre wells. Respiration of bacteroids was measured in the same vessels using 1·0 ml. bacteroid suspension and 1·0 ml. of 0·02 M-Na succinate or 1 ml. of phosphate buffer (M/15, pH 7·0).

Preliminary tests showed that little change in pO_2 due to uptake of oxygen resulted during measurement of the respiration rate during 30 min. with bacteroids or during 1 hr. with nodules; with bacteroids the pO_2 fell from 2·0 to 1·8 % and from 1 to 0·9 %. These small changes produced no detectable change of slope when the uptake of O_2 was plotted against time. With still lower pO_2 values however, a decline in rate could be detected after 20 min. In these cases only the initial rates were used.

In all cases respiration was expressed as qO_2 (μl. uptake of O_2/hr./mg. dry-weight tissue). In the first experiment a temperature of 30° was used. Later this was changed to 23°, the optimum for nitrogen fixation.

Gas mixtures. These were prepared from commercial gases through manifolds equipped with mercury manometers. The lines were evacuated by a rotary pump to 0·05 mm. Hg (McLeod gauge) and various scales graduated in percentage were used on the manometers according to the barometric pressure at the beginning of the experiment. Argon was used to bring the mixtures to one atmosphere. The following are mass spectrometric analyses of the gases used:

Nitrogen	N_2 (98·2 %), O_2 (0·1 %), A (1·7 %)
Argon	A (96·3 %), N_2 (3·2 %), O_2 (0·5 %)
Oxygen	O_2 (98·7 %), N_2 (1·3 %), A (trace)

For all purposes except the determination of the Michaelis constants, gas mixtures contained 10 % N_2.

Isotopic nitrogen (^{15}N). $^{15}N_2$ gas was prepared from NH_3 (generated from NH^+_4 labelled NH_4NO_3) by oxidation over CuO at 600–700°. The gas was passed through the oxidant for 1 hr. after constant volume was reached and was then collected over Hg after being circulated through a liquid N_2 cooled trap where nitrogen-containing impurities condensed. Mass spectrometric analyses showed less than 1 % O_2 and no trace of mass 31 (^{15}NO), 45 or 46 ($^{14}N^{15}NO$ or $^{15}N_2O$) or 47 ($^{15}NO_2$).

Measurement of nitrogen fixation. Nodules were exposed at 23° to gas mixtures containing $^{15}N_2$ in 50 ml. Erlenmeyer flasks attached to a manifold. After exposure, the nodules were crushed in 3 N-HCl and the soluble material analysed for ^{15}N after Kjeldahl digestion, distillation of NH_3 and conversion of NH_3 to N_2 by alkaline NaOBr containing 0·1 % KI (Francis, Mulligan & Wormall, 1959). The atoms % excess ^{15}N in the samples was a measure of the nitrogen fixation of the nodules during their exposure to the labelled gas mixture.

Mass-spectrometric determinations. All isotope measurements and gas analyses were done with an M86 (Atlas-Werke, Bremen) mass spectrometer. Atoms % ^{15}N were determined from the mass 29 : 28 ratios of the samples and the excess calculated from alternative readings of a sample of air N_2 contained in the other half of the double inlet system. All samples and standards were measured at the same inlet pressure (5 mm. Hg). Corrections were made for air leakage occurring during preparation of the samples by measuring the magnitude of the mass 28 and 32 peaks and applying corrections to the atoms % excess ^{15}N (Francis *et al.* 1959). In these experiments the maximum analytical error of repeated determinations on the same sample of gas was ± 2 % of the atoms % excess. The maximum analytical error of a complete determination, including digestion, distillation, conversion to N_2, measurement and correction was $\pm 6{\cdot}5$ % of the atoms % excess. In any one experiment all values of ^{15}N excess of samples were adjusted by multiplying by c/x where c was the chosen atoms % excess of the incubation gas mixture and x was the actual value analysed. This step was necessary because contaminating N_2 in the other gases used in the mixture caused small variations in the percentage of ^{15}N in the mixtures of different composition which were used in an experiment; the step thus put all measurements on a common basis. Gas analyses were prepared from mass-spectra, the relative peak heights of which gave the partial pressures of the components of the mixture.

RESULTS

The relationship between pO_2 and qO_2 for nodules and bacteroids

The effect of nodule size and slicing. The general form of the effects of pO_2 upon nodule respiration is seen in Fig. 1, which illustrates the results obtained with 23-day nodules. When qO_2 was plotted against pO_2 (the qO_2/pO_2 curve) the result was a distinct two-step curve with the first maximum at about 50 % O_2 and the second at about 90 % O_2. This type of curve was repeatedly obtained throughout this work as long as nodules of a single age were used. The plants from which the nodules used in this experiment were taken fell into two well-defined groups: those with few large (3–4 mm. diam.) nodules and those with many small (1·5–3 mm. diam.) ones. These nodules were of the same age to within one day and each plant bore the same

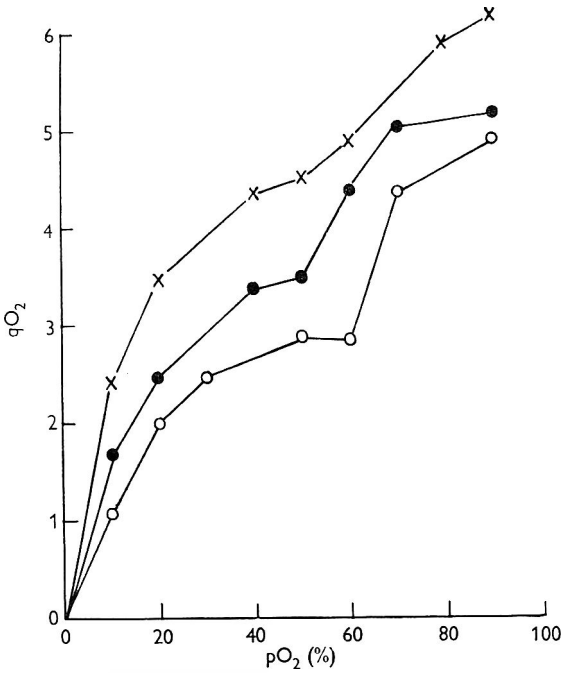

Fig. 1. The effects of nodule size and slicing on qO_2/pO_2 curves for nodules. Respiration measured at 30°. ×, 23-day old nodules: large sliced; ●, 23-day-old nodules: small, whole; ○, 23-day-old nodules: large, whole.

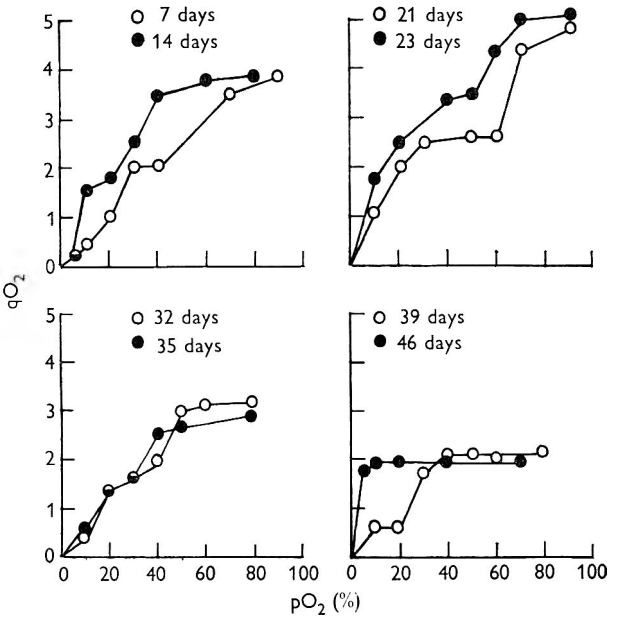

Fig. 2. The effects of nodule age on the qO_2/pO_2 curves for intact nodules, measured at 30°.

weight of nodules. The effects of this difference in size are seen in Fig. 1. The small nodules had a slightly higher qO_2 at each pO_2 value and the maximum occurred at slightly lower pO_2 than was the case for the large nodules. In all subsequent experiments only the large nodules were used because they were more easily handled.

When the large nodules were sliced (about 1 mm. thick) the general form of the qO_2/pO_2 curve was still apparent, but the first maximum was not as marked as with the intact nodules and the qO_2 at each pO_2 value was raised. The magnitude of the first step of the curve was increased by a little more than a third and that of the second step was slightly reduced.

The effects of nodule age on the qO_2/pO_2 curve. The qO_2/pO_2 curves were determined for nodules of different ages. The results are illustrated in Fig. 2. The two-step curve was found at all ages except 46 days. With young nodules (7–14 days) the two

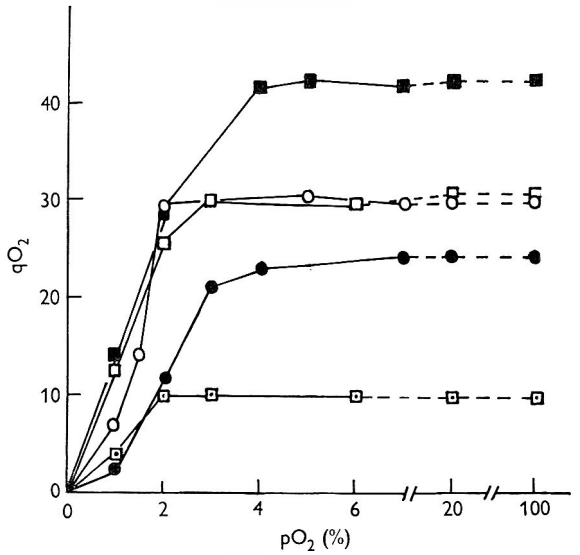

Fig. 3. The qO_2/pO_2 curves for bacteroids isolated from nodules of various ages. Bacteroids from nodules aged: ○, 8 days, succinate; ●, 15 days, succinate; □, 22 days, succinate; ■, 47 days, succinate; ⊡, 22 days, endogenous.

maxima occurred at lower pO_2 values than with actively nitrogen-fixing (21–23 days) nodules. As the nodules aged, both maxima occurred at progressively lower pO_2. At 46 days the first maximum, when present at all, occurred at less than 5 % O_2 and nodule respiration was completely saturated with respect to O_2 at 10 %. From previous work (Bergersen, 1958, 1960*a*) it is known that fixation ceases when the nodules are about 42 days old. Concurrently with this experiment measurements were made of the respiration of isolated bacteroids and the relative proportions of the various nodule fractions were determined.

The effects of nodule age on the qO_2/pO_2 curves for bacteroids. Bacteroids were prepared from nodules aged 8, 15, 22 and 47 days and the respiration measured at a range of pO_2 (Fig. 3). The maximum qO_2 was attained at 2–3 % O_2, and the curves are similar to those given by Burris & Wilson (1939). The qO_2/pO_2 curves are typical of the saturation of an enzyme with increasing concentrations of its substrate and there was no evidence of a two-step curve as seen with intact nodules. The main

effect of nodule age was that which has already been reported, as variations occurred in the maximum qO_2 which were in close agreement with the results of Bergersen (1958). At all ages the endogenous respiration was one-third of the respiration with succinate as substrate and was very stable, the qO_2 of 22-day-old bacteroids being unaltered after they had been standing in buffer at room temperature for 30 hr.

The effects of age on the composition of nodules. The composition of nodules is given in Table 1. For these determinations the nodules were crushed and the dry weights of the various fractions obtained after centrifugal separation. The nature of the fractions was checked microscopically. The composition became fairly stable from 15 days with the bacteroids making up about 25 % of the dry weight and the cell walls and large particles of the host accounting for about 40 %.

Table 1. *The composition of soybean root nodules of various ages determined on a dry-weight basis*

Fraction	Nodule age (days)				
	5	8	15	22	30
	Fraction (%)				
Large particles and cell walls (1)	80	56	43	39	38
Bacteroids (2)	3	19	28	25	25
Small particles (3)	1	2	2	3	3
Soluble (4)	15	23	26	26	26

(1) Sedimented from nodules crushed in buffer at 300 *g* for 4 min.
(2) Sedimented from the supernatant of (1) by 4000 *g* for 6–8 min.
(3) Sedimented from the supernatant of (2) by 6000 *g* for 20 min.
(4) Supernatant from (3).

The effects of pO_2 upon nitrogen fixation ($^{15}N_2$ uptake)

It has already been shown by Burris *et al.* (1955) that pO_2 affects nitrogen fixation by excised soybean nodules, and it was necessary to ascertain that these effects were not due to an increase or a decrease in the time during which N-fixing capacity was retained by the excised nodules. Figure 4 shows that this was not the case, since at 20, 37 and 81 % O_2, fixation was linear with time for at least 90 min. No fixation at all was detected at 5 % O_2; this is in agreement with all the present experiments, in which N_2 fixation could be extrapolated to zero at about 5 % O_2 for intact nodules. In this experiment, exposure to $^{15}N_2$ commenced 55 min. after the first nodule was detached.

Nitrogen fixation increased with increasing pO_2 up to 30°–50 %, above which it was inhibited. With intact nodules, the rise in fixation with increasing pO_2 from 10 to 50 % was greater than the decrease which occurred from 50 to 80 %, but with sliced nodules the inhibition was as great as the stimulation over a similar increment of pO_2 (Table 2; Fig. 5).

The effects of nodule age upon ^{15}N uptake with different pO_2 values. The amount of nitrogen fixed at the optimum pO_2 varied with nodule age, being greatest at 28–32 days (Table 2), and the optimum pO_2 decreased from 50 % at 32 days to 30 % at 40 days.

The relationship between the qO_2/pO_2 curve and $^{15}N_2$ uptake with varying pO_2. The

data in Table 2 clearly show that the pO_2 range of the first maximum of the qO_2/pO_2 curve corresponded with the pO_2 range for maximum nitrogen fixation. Figure 5 illustrates the form of the curves for nodules aged 32 days. This relationship was not found in older nodules (36 and 40 days) when respiration was measured at 30°. In these cases the first maximum of the qO_2/pO_2 curve occurred at a lower pO_2 than at 23° (compare Fig. 2 and Table 2).

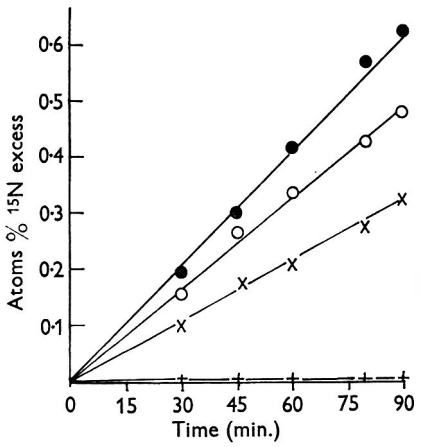

Fig. 4

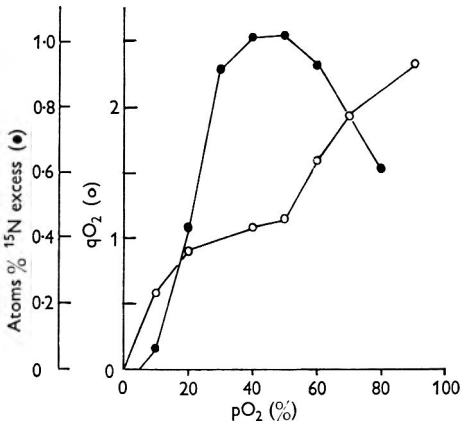

Fig. 5

Fig. 4. The time course of $^{15}N_2$ uptake by 26 day old intact nodules. ●, 37% O_2, ○, 81% O_2, ×, 20% O_2, 10%; +, 5% O_2, all gas mixtures contain 10% $^{15}N_2$ (46 atoms %).

Fig. 5. The relationship between the qO_2/pO_2 curve and the nitrogen fixation/pO_2 curve for intact 32 day-old nodules. Both fixation and respiration measured concurrently at 23°.

The effects of pO_2 on the kinetics of nitrogen fixation. The kinetics of nitrogen fixation by intact nodules were examined by measuring the effect of N_2 concentration upon the nitrogen fixed in 1 hr. with different pO_2 values. The ^{15}N excess of the nodules after 1 hr. was a valid measurement of the velocity (v) at any particular N_2 concentration (s) since fixation was linear with time for at least 90 min. The Dixon (1953) modification of the Lineweaver & Burk (1934) graphical method was then used to determine the Michaelis constant (K_m) and the maximum velocity ($V_{max.}$) for nitrogen fixation in the Michaelis-Menten equation

$$\frac{1}{v} = \frac{1}{V_{max.}} + \frac{K_m}{V_{max.}} \cdot \frac{1}{pN_2}$$

by plotting $1/v$ against $1/s$ at each pO_2, for nodules aged from 25 to 27 days. The oxygen tension in this work as in the respiratory work was taken as the initial value. The N_2 concentration was taken as the final pN_2 measured in a gas sample taken at the end of the incubation. At each pN_2 for any pO_2, duplicate determinations of ^{15}N excess were made on each of two separate samples of nodules.

Statistical methods were necessary in order to exploit the data fully. The following is an outline of the methods of analysis used as a result of examination of the form of the data. The variance of $1/v$ increased with $1/v$ so that a weighted regression procedure was necessary. To determine the weight function the regression of the

difference between (or range of) the sample means of two duplicates on the average of the means was determined in a relationship of the form: range $= b_1(1/v)+b_2(1/v)^2$. This relationship itself was fitted using weights inversely proportional to $(1/\text{range})^2$. The coefficient of the linear term, b_1, was significant while b_2 was not, although there is little doubt of the reality of the curvature. However, these data do not conform to a relationship: range $= b(1/v)^2$, as suggested by Wilkinson (1961). The weights were taken as the inverse of the squares of the range estimated from the regression relation of any average $1/v$. The weighted regression of $1/v$ on $1/\text{pN}_2$ was fitted using standard least squares procedure. The confidence limits of the intercepts on the x axis $(-1/K_m)$ and on the y axis $(1/V_{max})$ were calculated using standard procedures.

Table 2. *The influence of the partial pressure of* O_2 *upon respiration and nitrogen fixation of excised soybean nodules of various ages*

Nodule age (days)	Measurement	Partial pressure of O_2 (%) 5	10	20	30	40	50	60	70	80	90
23	qO_2*	—	1·66	2·48	—	3·41	3·60	4·38	5·07	—	5·10
	^{15}N excess†	—	0·064	0·202	—	0·626	0·653	0·550	0·451	0·428	—
	(atoms %)	—	0·060	0·156	—	0·766	—	0·583	0·467	0·420	—
23 (sliced)	qO_2*	—	2·45	3·50	—	4·36	4·52	4·87	—	5·89	6·23
	^{15}N excess† (atoms %)	—	0·050	0·195	—	0·410	0·423	—	0·311	0·143	0·056
28	qO_2	—	0·40	0·72	—	1·10	1·20	1·41	1·83	—	1·97
	^{15}N excess†	—	0·188	0·649	—	0·949	1·071	1·113	0·882	—	0·769
	(atoms %)	—	0·199	0·661	—	0·975	1·127	—	0·851	—	0·760
32	qO_2	—	0·62	0·81	—	1·04	1·18	1·64	1·94	—	2·31
	^{15}N excess	—	0·067	0·434	0·919	1·105	—	0·926	—	0·613	—
	(atoms %)	—	0·071	0·428	0·921	1·116	1·111	0·930	—	0·609	—
36	qO_2	—	0·75	1·23	1·46	1·70	1·68	2·04	—	2·90	—
	^{15}N excess	—	0·110	0·479	0·920	1·003	—	0·901	—	0·840	0·780
	(atoms %)	—	0·110	0·482	0·926	1·012	—	0·900	—	0·832	0·712
40	qO_2	0·25	0·68	1·37	1·52	1·80	—	2·77	—	2·90	—
	^{15}N excess	—	0·019	0·195	0·598	0·598	0·531	0·403	—	0·399	—
	(atoms %)	—	0·019	0·205	0·629	0·559	0·497	0·494	—	0·380	—

qO_2 = μl./hr./mg. dry wt. nodule. Respiration was measured on 20 nodules in each Warburg vessel; 0·5 ml. M/15 phosphate buffer (pH 7·0), was added to prevent drying out of the tissue; centre wells contained 0·2 ml 20 % KOH.

^{15}N uptake was measured by analysing the portion of the nodules which was soluble in 3N-HCl after 1½ hr. exposure at 23° to gas mixtures enriched in ^{15}N. Figures for duplicate nodule samples are shown.

* The respiration of the 23-day-old nodules was measured at 30°. All other qO_2 values shown were measured at 23°.

† Corrected ^{15}N excess figures; all results are expressed as if the incubation gas contained 55·8 atoms % excess ^{15}N.

The results are given in Table 3. It will be seen that V_{max} increased with pO_2. K_m was only slightly affected by pO_2 at the lower levels but above 40 % increased very sharply. The intercepts on the y axis for 60 and 80 % O_2 were not significantly different but the slope was greater for 80 % O_2. These results meet the criteria for competitive inhibition of nitrogen fixation by oxygen when comparing 60 and 80 % O_2. The intercepts on the y axis for 50 and 60 % O_2 were just significantly different, but the slope was greater for 60 % O_2. Thus, comparing these two partial pressures

of O_2, the results approach competitive inhibition. The Dixon–Lineweaver–Burk plots for these three partial pressures of O_2 are presented in Fig. 6.

Figure 7 shows the change in K_m with pO_2 and the change in nitrogen fixation at a constant pN_2 (10 %). These latter figures were determined from the regression diagrams of $1/v$ on $1/pN_2$ at each pO_2. Figure 7 thus shows the change in the kinetics of the nitrogen-fixation reaction in relation to the earlier experiments, in which the effects of pO_2 upon fixation were determined with pN_2 equal to 10 % (Table 2 and Fig. 5).

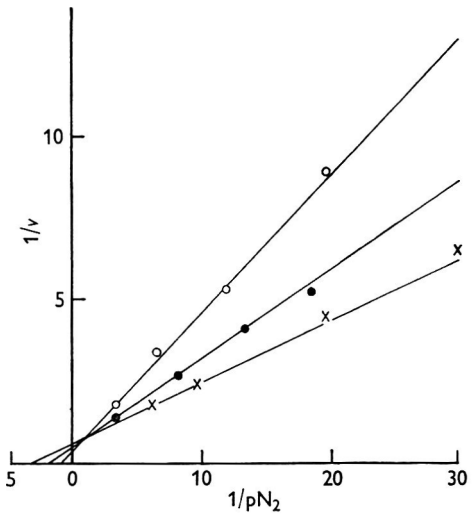

Fig. 6

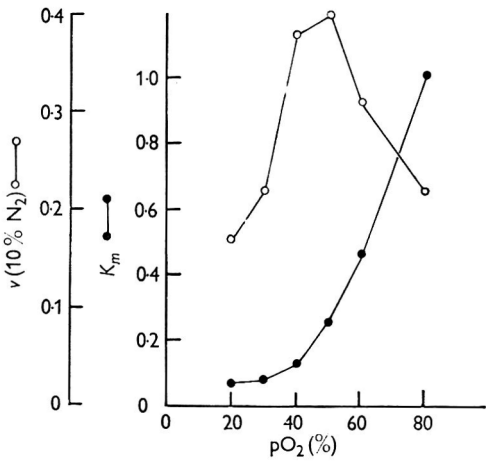

Fig. 7

Fig. 6. The Dixon-Lineweaver-Burk plot for nitrogen fixation by intact 26 day-old nodules at 50, 60 and 80 % O_2. ○—○, 80 % O_2; ●—●, 60 % O_2; ×—×, 50 % O_2.

Fig. 7. The relationship between the change in K_m with pO_2 and the change in fixation (v) with pO_2 when the gas phase contained 10 % $^{15}N_2$.

Table 3. *Data from the Lineweaver–Burk plots of nitrogen fixation at different* pO_2 *values. The intercepts on the x axis gave* $-1/K_m$ *and on the y axis* $1/V_{max.}$

	$1/V_{max.}$		$V_{max.}$		$-1/K_m$		K_m	
Initial pO_2 (%)	Esti-mate	95 % limits	Esti-mate	95 % limits	Esti-mate	95 % limits	Esti-mate	95 % limits
20	3·4157	3·9365 2·8949	0·2927	0·2540 0·3454	13·9361	18·8963 10·3181	0·0718	0·0529 0·0969
30	2·5058	3·1368 1·8748	0·3991	0·3188 0·5334	11·8712	18·2242 7·5261	0·0842	0·0549 0·1329
40	1·1769	1·5145 0·8393	0·8497	0·6603 1·1915	7·8766	12·7561 4·6755	0·1269	0·0784 0·2139
50	0·7245	0·9440 0·5050	1·3802	1·0593 1·9802	3·7775	5·4518 2·4055	0·2647	0·1834 0·4157
60	0·5660	0·7168 0·4152	1·7667	1·3951 2·4085	2·1954	3·0111 1·4992	0·4554	0·3321 0·6670
80	0·4036	0·6247 0·1825	2·4777	1·6008 5·4795	0·9402	1·5798 0·3954	1·0636	0·6330 2·5291

DISCUSSION

There are intriguing effects of oxygen tension both on nodule respiration and on nitrogen fixation. In discussing these results it is convenient to divide this part of the paper into sections, but it should be realized that the results are composed of inter-related observations.

The effects of pO_2 on nodule respiration. In the widest sense the results presented are in agreement with the work of others (e.g. Allison *et al.* 1940; Ebertova, 1959) who have concluded that the oxygen tension in legume root nodules in air is low. This is shown by the 2–3-fold increase in qO_2 when the external pO_2 is increased from air tension (20 %) to 90 %. The additional information which has come from the present work is that this increase in respiration is not simple. The failure of others to observe the two-step nature of the qO_2/pO_2 curve can be attributed largely to the fact that no precautions were taken about nodule age. Nodules of a range of ages would have the first maximum at different pO_2 values and hence the whole curve would tend to be smoothed out. An explanation of the qO_2/pO_2 curve is offered.

Interpretation of the nodule respiration data. From studies of the anatomy and cytology of soybean nodules the bacteroids are seen to be the innermost component since in the central tissue in which they occur they are enclosed within membrane envelopes in the cytoplasm of the host cells (Bergersen & Briggs, 1958). It may also be noted again that the tissues of soybean nodules are uniform in cell age since these nodules have no growing point (Bergersen, 1958). From these considerations and assuming (i) that bacteroids within the nodules have a qO_2 similar to their endogenous qO_2 as measured in the Warburg; and (ii) that root tissue has a similar qO_2 to the plant tissue component of nodules, it is possible to offer an explanation of the unusual pattern of nodule respiration with increasing pO_2. Because the bacteroids are the innermost respiring component it is logical to suggest that their respiration is the last to be saturated with respect to O_2 as the external pO_2 increases; that is to say, the second step of the qO_2/pO_2 curve for the nodules represents the bacteroid respiration increasing to a maximum with increased penetration of O_2. Table 1 shows that for nodules aged 22 days the bacteroids composed 25 % of their dry weight. Figure 3 shows that the maximum respiration of bacteroids isolated from these nodules occurs at 2–3 % O_2. If we now consider the data for nodules aged 21 days (Fig. 2), the bacteroids (22-day sample, Fig. 3) respiring at the maximum endogenous rate would account for 2·5 μl./hr. of O_2 uptake. This is the magnitude of the second step of the qO_2/pO_2 curve (Fig. 2, 21 days).

Turning to the plant tissue component of the nodule respiration, it is seen that this is made up of the respiration of the cortical tissue and the central (bacteroid-containing) cells, the peripheries of which contain mitochondria (unpublished electron microscope observations). It was found that 23-day soybean root segments had a qO_2 of 2·52 at 20 % O_2 and 3·36 in 100 % O_2. These values are close to those of Allison *et al.* (1940), namely, 2·12 and 2·88, respectively. They support the suggestion that the first part of the qO_2/pO_2 curve represents the saturation with O_2 of the plant tissue respiration of the nodule, because 75 % of the nodule dry weight is plant tissue and 75 % of 3·36 (the maximum qO_2 of root tissue) is close to the magnitude of the first maximum of the curve for these nodules (Fig. 2). The suggested explanation is summarized in Fig. 8, which shows how the nodule qO_2/pO_2 curve

could be the sum of the respiration of the plant tissue and of the bacteroids as the respirations of these components are successively saturated with O_2.

Assuming that this interpretation is correct, it is also possible to deduce the internal pO_2 at various external oxygen tensions. These deduced values are shown on the lower scale of Fig. 8.

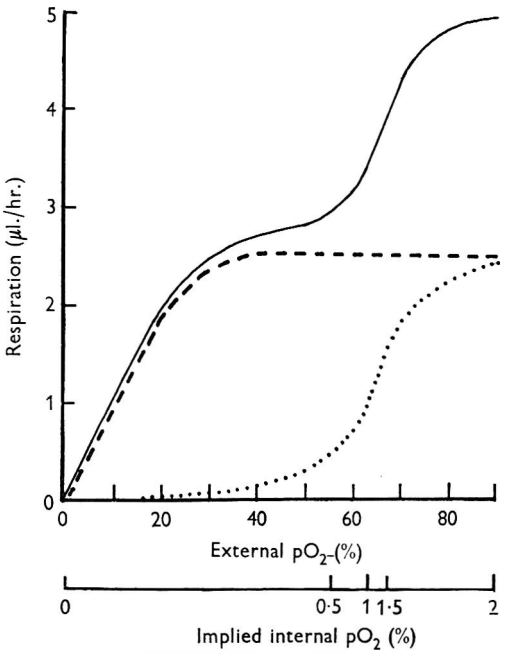

Fig. 8. The explanation of the qO_2/pO_2 curve for nodules. The observed curve is considered to be the sum of the curves for the plant tissue and the bacteroids as these are successively saturated with respect to O_2. ——, Observed respiration 1 mg. nodules aged 21 days; – – –, observed respiration 0·75 mg. root aged 21 days; ·····, calculated respiration 0·25 mg. bacteroids.

This explanation of the nodule respiration data implies that there is an O_2 diffusion barrier between the host respiration and that of the bacteroids. The latter, with their high respiratory rate (about five times higher than that of the root tissue cells in terms of the dry weight) would respire any O_2 passing the outer respiring regions of the nodule as, with increasing external pO_2, these regions approached saturation with respect to O_2. There would be no break in the curve unless there was a further barrier which did not permit the passage of appreciable amounts of O_2 until a certain partial pressure had been exceeded. This diffusion barrier may be the membrane envelopes within which the bacteroids are enclosed (Bergersen & Briggs, 1958).

The effects of pO_2 upon nitrogen fixation. The data of Table 2 fully agree with those of Burris *et al.* (1955) who found similar responses to increased pO_2 with sliced nodules. However, in my work there was considerably less variation in $^{15}N_2$ uptake than was the experience of these authors or of Aprison, Magee & Burris (1954), who found it necessary to use sliced nodules to decrease variation. This decreased variability of nitrogen fixation in the present work is attributable once again to the use

of nodules of a single age in any one experiment. The use of nodules of mixed ages and of successive nodule samples from ageing plants will give rise to errors because the shape of the fixation/pO_2 curve changes with age.

The nature of the stimulation of nitrogen fixation with increased pO_2 up to about 50 % in any one nodule sample remains obscure. The increased respiration of the host tissues in this pO_2 range may provide more substrates to the bacteroids or more acceptors for the fixed nitrogen. There is a significant correspondence between the first maximum of the qO_2/pO_2 curve and the optimum pO_2 for nitrogen fixation (Fig. 5). If the interpretation of the respiratory data which has been offered is correct, it would therefore seem that the penetration of O_2 into the vicinity of the bacteroids results in inhibition of nitrogen fixation. This has been confirmed by the kinetic data.

Parker & Scutt (1960) showed that, within certain limits, O_2 and N_2 competed as terminal hydrogen acceptors in nitrogen fixation by *Azotobacter vinelandii* and thus fixation may be regarded as a form of respiration. That a similar phenomenon might account for the inhibition of nodule nitrogen fixation at high pO_2, described by Burris *et al.* (1955), has been suggested by Bergersen (1960*b*). The kinetic studies reported here show that there was in fact a competitive inhibition of nitrogen fixation by O_2 at high external pO_2. The value for K_m of 0·07 atmosphere N_2 for 20 % O_2 is higher than the 0·025 obtained by Burris *et al.* (1955), probably because they used sliced rather than intact nodules. The Michaelis constant (K_m) also rose sharply in a range at which an internal O_2 diffusion barrier became permeable. The competitive nature of the inhibition is not necessarily due to a direct competition for the active site of the nitrogen-reducing enzyme. Any diversion of reducing power from any part of an electron transport chain could have a competitive effect. Thus, diversion of the bacteroid-reducing power to aerobic respiration could have a competitive effect even if the actual reduction of nitrogen or nitrogen containing intermediate compounds took place at a site remote from the bacteroids but linked with them by an electron transport chain. The results reported in the present work therefore support the hypothesis proposed elsewhere (Bergersen 1960*b*), that one of the main functions of the bacteroids in nodule nitrogen fixation is that they are a source of reducing power which is used for the ultimate reduction of N_2 to NH_3.

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The Production of Sporidesmin and Sporidesmolides by Wild Isolates of *Pithomyces chartarum* in Surface and in Submerged Culture

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SUMMARY

Four wild and two laboratory isolates of *Pithomyces chartarum* were grown under identical conditions, in submerged and in surface culture; yields of organisms and utilization of medium constituents are reported. Sporidesmin was produced in submerged culture and in surface culture by all the isolates examined but these showed differences of up to at least 100-fold in ability to produce the metabolite. Sporidesmolides were not isolated from cultures which did not sporulate.

INTRODUCTION

The production of sporidesmin and sporidesmolides by *Pithomyces chartarum* in surface culture was studied by Done, Mortimer, Taylor & Russell (1961), who showed that sporidesmin appeared in the cultures on the third day of incubation. They were unable to show significant differences in the amount of the metabolite present in the cultures for 4 days thereafter. By contrast the production of sporidesmolides was proportional to the growth of the organism and particularly to the number of conidia produced. It has been postulated that *P. chartarum* is implicated in the aetiology of facial eczema and in this connexion Russell, Synge, Taylor & White (1962) showed that the depsipeptides (Shemyakin, 1960) isolated from pasture where the disease occurred were identical with the sporidesmolides. These results were obtained by using an organism (strain C) isolated as a distinctly high-sporing laboratory sector from a culture obtained from a pasture at Claudelands, Hamilton, New Zealand in March 1958 (Thornton & Ross, 1959). It was therefore of interest to determine whether sporidesmin and sporidesmolides were produced by field isolates and also whether such isolates differed in ability to produce these metabolites. It is known (Morton, 1960) that many fungi produce fewer spores in submerged culture than in surface culture. Thus it is possible to compare the same organism growing on the same medium at the same temperature under conditions

where one culture produces many times the number of conidia produced by the other. These contrasted experimental conditions were used with *P. chartarum* in an attempt to obtain further information on the possible relationships of sporidesmin- and sporidesmolide-production with sporulation.

METHODS

Organisms. Pithomyces chartarum occurs as a common saprophyte on debris in pastures in the north of New Zealand. Many isolates were obtained from pasture debris and from agar plates exposed in pastures where stock were prone to facial eczema. From these field isolates four (S73a, SA10b, SA19a and SA26a(i)) were selected as single spores for detailed study. In addition, two isolates (C, SA26a(ii)) were obtained as single spores from high-sporing sectors of laboratory cultures of the fungus. The isolates differed from one another in small morphological and cultural characters. The following descriptions are based on 21-day cultures on potato glucose agar incubated at 24°. Isolate C (= strain C, Done *et al.* 1961; Commonwealth Mycological Institute, Kew, England, herbarium no. 74473) was obtained from Dr R. H. Thornton, Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand. Ross (1960) stated that isolate C is a high-sporing isolate which rarely sectors in culture with optimum growth at about 27°. Cultures of isolate C were flat, zonate, dark coloured when over 10 days old with some floccose superficial mycelium, pigmented verrucose mycelium; intercalory chlamydospores were common among the mycelium. Conidia were formed scattered in large numbers among the superficial mycelium; they were typically muriform, with 3 transverse septa (rarely 2) and with 2 or more longitudinal septa; these conidia were 17–25 × 9·5–13 μ in size. Isolate S73a was isolated from a sporing colony on debris of *Paspalum dilatatum* (May 1959, Mount Albert, Auckland). Like isolate C optimum growth was obtained between 25–28° but the cultures were light coloured with more superficial floccose mycelium. Dark chlamydospores and dark verrucose mycelium were commonly produced. Conidia were formed in small clusters in among the floccose superficial mycelium; conidia (21–29 × 9·5–13 μ) were muriform with 2 but more usually 3, transverse septa. Isolates SA10b, SA19a and SA26a(i) were collected from agar plates exposed at Mahuta, Dargaville, New Zealand, in late January and February 1960; unlike isolates C and S73a they were unstable in culture and sectors were common. In sporing cultures conidia were formed superficially but scattered and in densely compacted clusters. Isolate SA10b rarely produced conidia during incubation for 14 days. Cultures were almost colourless as dark mycelium and chlamydospores were rarely formed. Maximum growth was observed at 20–28°. Cultures of isolate SA19a were moderately sporing, floccose zonate; conidia were irregular in size varying from 16 to 35 μ in length and 9·5 to 13 μ in width, and with 2–5 (commonly 3) transverse septa. Its growth was limited at 26°. Sectoring was common in cultures of isolate SA26a(i) which grew equally well at 28° as at 24°. Conidia were regular in size varying from 19 to 27 μ long and 9·5 to 13 μ wide, with 3 transverse septa. Like isolate C, isolate SA26a(ii) was selected from a distinct high sporing sector on a culture of isolate SA26a(i) on potato-glucose agar. Isolate SA26a(ii), unlike its parent, produced conidia that were erratic in size (16–30 × 7–14·5 μ) but like its parent, it commonly showed sectoring and it grew equally well at 28° as at 24°.

Cultivation of organisms. Isolates were grown on potato glucose agar which was prepared by covering potatoes (chopped, 200 g.) with water (300 ml.). The mixture was boiled for 30 min., strained through muslin, then glucose (20 g.), agar (Davis 'Bacteriological', Christchurch, New Zealand; 12 g.) and Marmite (0·2 g.) added. The solution (pH 5·5–5·8) was diluted to 1 l. with water. The isolates S73a, SA19a, SA26a(i) and SA26a(ii) were inoculated on to potato glucose agar films on the inside of 15 × 1 cm. test tubes. The cultures were incubated 21 days at 25°, were freeze-dried and then sealed. Freeze-dried cultures were stored at 0°. Isolate SA10b was inoculated on to potato glucose agar slopes that were incubated for 21 days at 25°, then sealed and stored at −40°. A sample of each culture thus prepared was transferred aseptically to potato carrot agar (Done *et al.* 1961). Plates were incubated for 14 days at 25°, treated with sterile 0·05 % (v/v) Lissapol N (Imperial Chemical Industries Ltd., 16, The Terrace, Wellington, New Zealand; 5 ml.), rubbed with a stout platinum loop and the suspension obtained transferred aseptically to a 25 ml. blood bottle. The suspension was diluted with 0·05 % (v/v) Lissapol N to a final concentration of $6 \cdot 10^5$ spores/ml. except in the case of isolate SA10b where no spores were seen in the inoculum. The inocula (0·1 ml.) were added aseptically to the culture vessels. Inocula of isolate C were prepared as described previously.

Surface cultures on potato carrot medium. Thirty-six l. of potato carrot medium (Done *et al.* 1961; containing, g./l.: potato carrot solids, 20–25, sugars 10, nitrogen 0·5; pH 5·0) were prepared and 150 ml. added to each of 240 pint milk bottles. Forty milk bottles were used for each isolate. After inoculation the 240 culture vessels were stacked on a plane slightly inclined from horizontal. The vessels of each isolate were distributed at random throughout the stack which was incubated at 25° ± 1° for 7 days.

Submerged cultures (i) comparative experiments. Potato carrot medium (7·2 l.) was prepared and distributed in 150 ml. amounts into each of 48 Erlenmeyer flasks (capacity 750 ml.); 8 flasks were used for the growth of each isolate. The 48 flasks were distributed at random on the shaker after inoculation. They were shaken in an horizontal plane at 110 rev./min., each flask describing a circle of radius 5 in. Cultures were incubated at 25° ± 1° for 4–7 days.

Submerged cultures (ii) sporidesmin production experiments. Forty-nine Erlenmeyer flasks (capacity 750 ml.) each containing potato carrot medium (150 ml.) were inoculated with isolate C or isolate S73a and the cultures were grown for 4 days under the conditions described in the previous paragraph for comparative experiments.

Harvesting and extraction

Surface cultures. Six vessels from each group of 40 were selected at random; 2 of these were used as inoculum for potato glucose plates. These plates were incubated for 21 days at 24° and then examined for contaminants. Cultures from 4 vessels were filtered and sugar (Hanes, 1929), nitrogen (Keldjahl), pH value and dry-weight determinations made on the separate filtrates. The residual fungus was washed with water, dried in a vacuum oven at 20°/0·5 mm. Hg pressure and weighed. The dry fungal tissue was ground to a powder, thoroughly mixed and spore numbers and sporidesmolide estimations done on weighed samples. The remaining 34 vessels of each isolate were combined and the resulting 6 batches of culture extracted con-

currently in similar equipment. The extraction and estimation of the sporidesmin content of the extracts obtained were carried out as described by Done *et al.* (1961).

Submerged cultures. In the comparative experiments 2 vessels of each group of 8 were used as inocula for potato glucose agar plates. Plates were incubated and examined for contaminants as described above. The contents of the remaining 6 vessels of each group were bulked and filtered. Nitrogen, sugar, pH value and dry-weight determinations were made on the filtrates. The residual mycelium was washed with water, dried in a vacuum oven at 20°/0·5 mm. Hg pressure and weighed. Weighed samples of the dry powdered mycelium were used for estimations of sporidesmolides and spore numbers. In sporidesmin production experiments 4 of the 49 vessels were selected at random and used for estimations of medium constituents, amount of organism and for checking the homogeneity of the isolate. The remaining 45 cultures were combined, extracted and the sporidesmin content of the extract determined iodometrically and by the tissue-culture toxicity method (Done *et al.* 1961).

Sporidesmolides were estimated by a modification (suggested to us by M. E. Carruthers) of the method of Done *et al.* (1961) since it was found that the estimation was expedited by centrifugal separation of the material insoluble in diethyl ether. Chloroform extracts of the residue after centrifugation were satisfactorily filtered by gravity through charcoal. The amino acid composition of sporidesmolides from the different isolates was determined by the method of Russell *et al.* (1962).

RESULTS

The utilization of medium constituents and yields of fungus, spores, sporidesmin and sporidesmolides in a typical set of comparative surface cultures are summarized in Table 1. Five comparative experiments of this sort were carried out; the results obtained in each set were similar. The yields of sporidesmin given in Table 1 were obtained by iodometric assay and tissue-culture toxicity tests of extracts of cultures prepared under similar conditions. Good agreement between the two assay procedures was observed except in the case of isolate SA10b where the figure obtained by the iodometric procedure was 5–10 times greater than that obtained by the tissue-culture toxicity test. The biological potency of an extract of 200 l. of culture of isolate SA10b was therefore determined by the corneal opacity test (kindly carried out for us by Mr P. H. Mortimer; Done *et al.* 1961). The result obtained agreed with that obtained by the tissue-culture toxicity method. Thus this figure is given in Table 1.

In submerged culture growth of all isolates was more rapid than in surface culture. The growth of isolate C during the 7 days after inoculation is shown in Fig. 1 and is typical of the other 5 isolates. After the fourth or fifth day breakdown of fungal tissue was noticed by microscopical examination, decreased weight of mycelium on harvest and an increase in the nitrogen concentration of the spent culture medium. The utilization of medium constituents and the production of spores and sporidesmolides are therefore given for 4-day cultures in Table 2. Three comparative experiments of this sort were done in submerged culture; similar results were obtained in all. In one of these half the cultures (i.e. 4 vessels of each isolate) were harvested 4 days after inoculation and the remainder at 7 days. Spores

Table 1. *Utilization of medium constituents, and yields of organism, spores, sporidesmin and sporidesmolides by six isolates of* Pithomyces chartarum *in surface culture for 7 days at 25°*

Isolate	Final pH value*	mg./ml. Δ dry wt.	Δ sugar	ΔN	Mycelium† dry wt.	mg./l. Spores (× 10^{-5}/ml.)	Sporidesmolides	Sporidesmin‡
C	6·57	14·0	6·5	0·45	6·2	110	110	0·7
S73a	6·25	12·2	4·5	0·31	6·7	lost	150	2·1
SA10b	6·1	9·4	2·9	0·23	5·8	0·0	0·0	0·005
SA19a	6·15	13·6	4·7	0·4	6·5	180	155	1·4
SA26a(i)	6·33	12·6	5·2	0·39	6·6	21	43	0·3
SA26a(ii)	6·12	12·0	4·8	0·33	6·0	140	140	0·6

Δ dry wt., Δ sugar and ΔN refer to the decrease in dry weight, sugar and nitrogen concentration in the medium before inoculation and after harvest. * Of culture filtrate after incubation for 7 days, † Weight of fungus after harvesting and drying to constant weight. ‡ The average of values obtained by iodometric assay and tissue-culture toxicity test except in the case of isolate SA10b where the latter value is given.

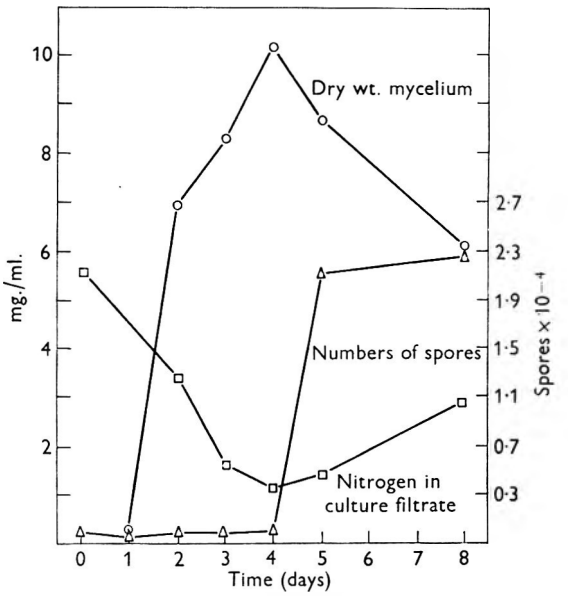

Fig. 1. Growth of *Pithomyces chartarum* in submerged culture. ○—○, Mycelium dry weight; △—△, spore numbers; □—□, nitrogen in culture filtrate.

and sporidesmolides were present only in cultures of isolate C and the quantities present were the same in the 7-day-old culture as in the one 4 days old. In submerged culture isolate S73a often grew abnormally and formed discrete spherical colonies, or a pillar of growth in the centre of the base of the flask. The colonies were often 2–3 cm. in diameter on the third day of incubation; it is likely that such cultures were not completely 'submerged'. This would account for the appearance of conidia in cultures of isolate S73a (Morton, 1960). The occurrence of this abnormal growth was erratic and sometimes difficult to detect in vessels where very

heavy growth had occurred. Thus the figure given in Table 2 for the number of spores produced by isolate S73a in submerged culture is open to doubt.

A strict comparison of the amounts of sporidesmin produced by different isolates in submerged culture was not possible because of the limited capacity of the shaker. The average yield of sporidesmin from 6 batches of cultures of isolate C was 0·3 mg./l. whilst that obtained from 4 batches of isolate S73a was 1·5 mg./l. These results were obtained on extracts of 4-day cultures since preliminary experiments indicated that cultures 7 days after inoculation contained little sporidesmin.

Table 2. *Utilization of medium constituents, and yields of organism, spores and sporidesmolides by six isolates of* Pithomyces chartarum *in submerged culture after 4 days at 25°*

Isolate	Final pH value	mg./ml. Δ wt.	Δ sugar	ΔN	Mycelium dry wt.	Spores ($\times 10^{-5}$/ml.)	Sporidesmolides (mg./l.)
C	6·30	26·7	7·48	0·47	15·1	0·45	19
S73a	6·40	24·7	7·03	0·47	12·2	0·35	0·0
SA 10b	6·10	18·7	6·97	0·46	12·7	0·0	0·0
SA 19a	6·25	17·1	6·65	0·39	9·2	0·0	0·0
SA 26a(i)	6·41	22·7	7·05	0·46	13·9	0·0	0·0
SA 26a(ii)	6·30	22·3	7·05	0·41	10·0	0·0	0·0

Abbreviations have the same meaning as in Table 1.

Table 3. *Comparison of the weight of sporidesmolides isolated from cultures of six isolates of* Pithomyces chartarum *with the amount estimated polarimetrically*

Isolate	Optical rotation*	Sporidesmolides (mg./l.) By gravimetric method	By polarimetric method
C	−205°	110	110
S73a	−196°	158	153
SA 10b	—	0·0	0·0
SA 19a	−199°	138	134
SA 26a(i)	−205°	28	28
SA 26a(ii)	−198°	173	167

* Specific optical rotations were determined in chloroform solution at 20° in sodium light.

The polarimetric determination of sporidesmolides assumes constant composition of the isolated depsipeptides (Russell, 1962). Such an assumption was not necessarily tenable for cultures of different isolates. Thus the sporidesmolides from each isolate were also separated and determined gravimetrically. Some of the properties of the isolated sporidesmolides were determined. The specific optical rotations are given in Table 3. All samples sublimed in a characteristic manner when heated above 200°. Hydrolysates of the 6 samples contained valine, leucine, isoleucine and *N*-methyl-leucine, the relative proportions of the 4 amino acids found in each sample (in the order given) was 2:1:0·2:1.

The effect of sequential subculture on the production of sporidesmin by isolate S73a in surface culture was determined. The subcultures are expressed in Fig. 2 in

terms of $x+n$ where x is a single small (<5) integer and n is the known number of subcultures counting from the freeze-dried culture described in the methods section. Figures were only obtained for every other subculture since between each sporidesmin production subculture the isolate was grown on potato glucose plates to make sure that contaminants were absent. Results obtained for isolate C grown under analogous conditions are given for comparison in Fig. 2.

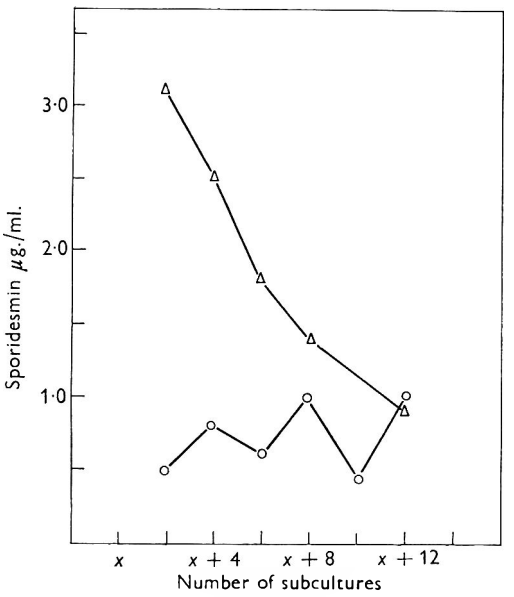

Fig. 2. Production of sporidesmin by *Pithomyces chartarum* isolates S73a and C in sequential subculture. △—△, S73a; ○—○, C; × refers to the parent freeze-dried culture as indicated in the text.

DISCUSSION

The selection of the isolates reported here was largely intuitive, taking into account geographical location, year of isolation and the differing number of conidia produced by the isolates grown on potato glucose agar. In cultures of all the isolates used in the work hyphal anastomoses were commonly seen and with four isolates (S73a, SA19a, SA26a(i), SA26a(ii)) nuclear numbers within the cells were erratic. In addition, in cultures of the three latter isolates sectoring often occurred. Such behaviour has been observed with several species of filamentous fungi, e.g. the growth of isolates of *Penicillium cyclopium* on apple pulp medium (Jinks, 1952). Hence it is likely that in culture *Pithomyces chartarum* exhibits the 'dual phenomenum' demonstrated by Hansen (1938) for a number of Fungi Imperfecti. The results summarized in Fig. 2 are also consistent with this view; presumably they indicate the adaptive behaviour of an heterokaryon (Pontecorvo, 1946) transferred from debris of *Paspalum dilatatum* to sequential subculture on potato carrot medium. Two conclusions emerge. First, the physiological and morphological characters displayed by the isolates are dependent on the cultural conditions and do not justify their classification as strains, races or mutants. Thus they are referred to as isolates in this paper with a code number to indicate their origin. Secondly, in experiments

designed to compare the growth of different isolates or their ability to produce a particular metabolite it is necessary to provide information about the number of sequential subcultures the organism has undergone in the laboratory. Thus we have subjected field isolates to a minimum number of subcultures before preparing freeze-dried parent cultures and these have been used for inocula in all comparative experiments.

The differences observed between growth in submerged and in surface culture resemble those described for other organisms (Morton, 1960). Thus in submerged cultures growth was more rapid with a shorter lag period. Conidia were produced by isolate C but only 0·25 % of the number produced in surface culture were found. In submerged culture production of 1 mg. dry weight fungus required the use of about 0·04 mg. N. A similar value was observed in surface cultures of isolate SA10b. In cultures bearing large numbers of conidia 0·06 mg. N was needed to produce 1 mg. dry-weight fungus. Large numbers of spores are formed in submerged and in surface culture (Done *et al.* 1961) only when the growth rate is decreasing; therefore sporulation in static surface culture whilst accompanied by high N utilization takes place only at a stage when the medium becomes depleted in this constituent. The simplest explanation of these paradoxical results seems to be that sporulation in static surface culture is stimulated by a local accumulation of metabolic products. In well mixed submerged culture breakdown of mycelium begins before such products reach a sufficiently high concentration and thus provides additional nutrient factors that can result in further mycelial growth. There is no experimental evidence for this suggestion; all aspects of the physiology of spore formation remain obscure.

Except for isolate SA10b the amount of sporidesmin recovered from surface cultures of the various isolates was of the same order. The small differences shown in Table 1 are thought to be real because they were observed in replicate experiments. From submerged cultures of isolates C and S73a sporidesmin was recovered in amounts comparable with those obtained from surface cultures of these two isolates. Thus whilst the submerged and surface cultures differed greatly with respect to the number of spores produced, sporidesmin production remained about the same.

In a previous paper (Done *et al.* 1961) attention was directed to the relationship between the number of spores in culture and the amount of sporidesmolides isolated therefrom. The results here given in Tables 1 and 2 amplify and confirm this relationship. No sporidesmolides were isolated from non-sporing cultures. A change from submerged culture to surface culture resulted in the formation of conidia and the isolation of sporidesmolides; but no examples were observed of the formation of one without the other with the possible exception of isolate S73a. This relationship between the numbers of spores and the amount of sporidesmolides isolated suggested that the proportion of the different depsipeptides (Russell, 1962) present might differ from one isolate to another and thus be of value in differentiating between isolates. However no differences were observed in the composition of the sporidesmolide fractions from the isolates investigated.

The authors are greatly indebted to Mr J. Allen whose mechanical expertise provided a shaker of exceptional reliability. Details of the design will be supplied to those interested.

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Some Observations on the Cultivation, Fruiting and Germination of *Fuligo septica*

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SUMMARY

Conditions optimal for the laboratory culture of unpurified *Fuligo septica* plasmodium were examined. The organism was grown in the laboratory for over 2 years, during which time regular sporulation occurred. Investigation of factors which affected sporulation suggested that this was induced by material present in older cultures; light did not precipitate it. Spore germination occurred readily in the laboratory; some experiments on germination were made. *F. septica* plasmodium was purified by a migration technique combined with antibiotic treatment. Two-member cultures were established with two yeasts and a *Penicillium* sp. isolated from plasmodium, and with a baker's yeast. Several other organisms (including Gram-positive and Gram-negative bacteria and yeasts) were not satisfactory as associate organisms in two-member cultures. Satisfactory axenic culture was not obtained, slow growth for a few weeks only being obtained on an autoclaved suspension of baker's yeast.

INTRODUCTION

Much of the work carried out in recent years with members of the Class Myxomycetes has used *Physarum polycephalum*, since this organism proved able to grow abundantly in unpurified laboratory culture (Howard, 1931; Camp, 1936), was easily purified, and subsequently grown in two-member and in axenic culture (Howard, 1931; Cohen, 1939; Hok, 1954; Daniel & Rusch, 1956, 1961). It readily completes its life cycle in the laboratory. Relatively few other myxomycetes have been grown in the laboratory throughout the whole of the life cycle (Alexopoulos, 1960; Gray, 1961), and fewer still have been grown in two-member or in axenic culture (Sobels, 1950; Hok, 1954; Kerr & Sussman, 1958). It was therefore thought desirable to extend the range of organisms studied in this group of slime moulds. Work with the species *Fuligo septica* was begun since its spores are known to germinate readily and since it can produce large amounts of plasmodium under certain conditions, e.g. on heaps of spent tan in tanneries. The present aim was to produce axenic cultures and to investigate their nutritional requirements. However, considerable difficulty was experienced in the pursuit of this object and observations were made on the fruiting habit and spore germination of unpurified material whilst attempts were being made to produce purified material and to establish two-member and axenic cultures.

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METHODS

Source of organism. *Fuligo septica* sclerotium was collected from heaps of spent tan at the tannery of the Kingston Tanning Co., Ltd., Kingston-upon-Thames, Surrey. This sclerotium when placed on moistened filter paper in a humid chamber gave a typical bright yellow reticulate plasmodium.

Maintenance of stock cultures and preparation of inocula for experiments. Several culture media were tried. The best method of culture for the production of plasmodium for maintenance of stock cultures and for experimental purposes was found to be that of Camp (1936) in which pulverized rolled oats were fed to plasmodium supported on moist filter paper in a humid chamber. The culture vessel used was a 6 in. diameter Petri dish in which was placed the inverted half of a 4 in. diameter Petri dish wrapped in filter paper. A shallow layer of tap water was placed in the larger dish, to keep the filter paper moist and the atmosphere inside the larger dish humid, and the whole was autoclaved. It was found that there was less frequent overgrowth by contaminating organisms and better growth of the myxomycete when the oatmeal used was boiled in water, dried overnight (at 60°), ground to a fine powder and autoclaved (121° for 20 min.). Stock cultures of *Fuligo septica* were maintained for more than 2 years in this way, being subcultured at monthly intervals and incubated at 25° in the dark.

Inocula for experiments were cut with a sterile scalpel from the advancing edge of the plasmodial reticulum of these cultures and were standardized as far as possible by taking a measured area (0·25 cm. × 0·25 cm.; 0·5 cm. × 0·5 cm.; or 1·0 cm.^{2}) which was transferred on the filter-paper support.

In recording results growth was estimated by measuring the area of plasmodium formed. This was not an accurate measure of growth since the plasmodial reticulum varied in density, but it gave sufficient information for the present purpose.

Experiments were carried out to determine optimal conditions for the growth of unpurified plasmodium, the effects of substrate, pH value, temperature, aeration, light and humidity being considered. Methods of purifying plasmodial material by migration and antibiotic treatments were investigated. Details of procedures are given in the next section together with results.

Tests for purity of plasmodium. Preliminary tests for purity of plasmodium were carried out by flooding with glucose yeast-extract broth and incubating at 25° for 5 days washed agar plates across which plasmodium had been allowed to migrate. The washed agar was prepared by the method of Cohen, 1939; 2% agar was made up in M/75 phosphate at pH 6·0. The agar was washed before use in running tap water for 24 hr. and in distilled water for 24 hr. This washed agar + phosphate medium will be referred to as Cohen's agar. When these preliminary tests of purification became negative plasmodium was transferred to a nutrient substrate. In this method of testing there was no evidence of false negatives due to inhibition of growth of any contaminants by antibiotics from the plasmodia (Sobels, 1948*b*). Confirmatory tests for pure cultures were then carried out by inoculating agar blocks (about 1 cm.^{2}) bearing plasmodial track from fresh Cohen's agar migration plates to the following media: malt agar, glucose yeast-extract agar, yeast-extract agar, peptone water, nutrient gelatin, thioglycollate broth, Robertson's cooked meat medium, medium for detection of cellulose-decomposing organisms. Aerobic

incubation at 25° of negative tests in all of these media was continued for three weeks, and anaerobic incubation at 25° in the first five media for 3 days.

In all experiments unless otherwise stated plasmodium was incubated at 25° in the dark in a humid atmosphere, incubation being in a closed container with a shallow layer of water in the bottom. Incubation of spores in experiments on germination was at 25° in the dark.

RESULTS

Optimal conditions for growth of unpurified Fuligo septica *plasmodium*

Culture medium. The growth of *Fuligo septica* on a variety of media was examined: oatmeal agar (pH 5, 6), ground oatmeal scattered on Cohen's agar, aqueous extract and ethanolic extract of oatmeal (each sterilized by autoclaving), malt agar, nutrient gelatin, yeast-extract agar, glucose yeast-extract agar, glucose yeast-extract broth, tan-extract agar, Cohen's agar, living yeast suspension (*Saccharomyces cerevisiae*) spread on the surface of oatmeal agar, malt agar or Cohen's agar. Slow growth was obtained on oatmeal agar (pH 5, 6), on aqueous and ethanolic extracts of oatmeal, on malt agar and on tan-extract agar. When particulate nutrient was present on the surface of the agar, as in the case of oatmeal scattered on Cohen's agar and of yeast suspension on oatmeal agar, malt agar or plain agar, the growth was much improved. It was best on Cohen's agar + oatmeal where it was equal to that of controls set up as described for stock cultures. Several of the media used were toxic (nutrient gelatin, yeast-extract agar, glucose yeast-extract agar and glucose yeast-extract broth), the plasmodium being dead within 2 days of inoculation in all cases. Control plasmodium on Cohen's agar survived for at least 14 days.

pH value. Attempt was made to determine the optimal pH value for plasmodial growth by using as culture fluid in place of tap water, in cultures similar to those used for stock cultures, a series of buffer solutions (Sørensen) ranging from pH 3 to 10. Suitable buffers were not found, however, those used in general being toxic at M/15 and having insufficient buffering power at lower concentrations. Determinations of pH value were made, however, over a 5-week period of incubation on the culture fluid of cultures maintained by the method described for stock cultures. The culture fluid, sterile tap water, had an initial pH value of 7·3. As growth of the plasmodium proceeded the pH value of the culture normally decreased from the initial value of pH 7·3 to pH 4·5–6·0 in 2–3 weeks, then becoming relatively constant (Table 1). In such cultures growth was good at all stages and in general the plasmodium presented a normal appearance, i.e. it was bright yellow in colour, formed a characteristic reticulum over the filter paper support and exhibited rapid streaming of protoplasm. When the pH value did not decrease in the usual way (e.g. culture 2 in Table 1 where the pH value rose to above pH 8) growth was poor and the plasmodium abnormal in appearance until the pH eventually decreased.

Temperature. To examine optimal growth temperature cultures maintained as described for stock cultures were incubated at 25°, 30° and 37° (at 25° and 30° for 6 months). Incubation was also carried out for a few weeks at room temperature (18°–20°). The relative amounts of growth and the appearance of plasmodia were recorded. Table 2 shows typical results. Normal growth occurred, and continued for the 6-month period of the experiment, at 25° and 30°, being more rapid at 30°.

Table 1. *Changes in pH value of developing* Fuligo septica *cultures grown on ground oatmeal on a filter-paper support at 25°*

Culture	Days: 1	6	12	18	23	26	34	Growth	Appearance
	pH value of culture fluid								
1	7·6	6·5	6·7	4·6	4·6	4·5	4·6	Good throughout	Pale yellow becoming normal at 18 days
2	8·4	8·5	8·2	7·6	7·3	6·3	5·6	Poor initially, improving at about 23 days	Pale yellow, plasmodium densely massed; becoming normal at end of experiment
3	7·6	7·8	5·4	5·2	6·5	4·9	5·0	Good throughout	Normal bright yellow plasmodium showing rapid streaming of protoplasm
4	7·6	7·2	7·3	6·2	6·8	5·9	5·9	Good throughout	
5	7·8	6·5	5·3	5·1	5·7	4·7	6·8*	Excellent. More rapid than in cultures 3 and 4	
6	7·1	5·6	4·8	4·8	5·4	4·7	5·2		

* Had fruited 5 days previously.

At 37° no growth occurred and the inoculum died within 1 or 2 weeks. At room temperature (18°–20°) growth was normal but slower than at the higher temperatures.

Anaerobic cultivation. Aerobic and anaerobic (95% (v/v) hydrogen + 5% (v/v) carbon dioxide in a McIntosh and Fildes' jar) incubations of plasmodium were carried out on malt agar and on Cohen's agar. *Fuligo septica* behaved as an obligate aerobe under the conditions used; no growth or migration occurred during anaerobic incubation, and the plasmodium died.

Table 2. *Growth of* Fuligo septica *at various temperatures*

The cultures were grown on ground oatmeal on a filter-paper support.

Temp.	Culture	Growth and appearance at: One week — Plasmodial area (cm.²)	One week — Appearance	Two weeks — Plasmodial area (cm.²)	Two weeks — Appearance
25°	(i)	8	Normal	18	Normal
	(ii)	12	Normal	14	Normal
30°	(i)	25	Normal	100	Normal
	(ii)	12	Normal	41	Normal
37°	(i)	Dead	—	—	—
	(ii)	1	Contracted mass	Dead	—

Light. The effect of light on the plasmodium was observed by using culture methods as for temperature experiments. Incubation was carried out in daylight, including exposure for 2 hr. to direct afternoon sunlight, and in the dark. The plasmodium on being placed in light almost immediately developed 'swellings' which appeared to be formed by rapid streaming of the protoplasm through lesions in the plasmodial strands. This phenomenon has been observed previously in several myxomycetes (Baranetzki, 1876; Gray, 1938). The plasmodium was negatively

phototactic, and after incubation for 4–5 days in light lost its pigmentation, becoming cream in colour. Initially growth was retarded in the light, but after 2 weeks the plasmodium appeared to return to normal apart from pigmentation.

Humidity. Plasmodial growth in humid (nearly saturated) and non-humid atmosphere was compared. Better growth was obtained in humid than in non-humid atmosphere.

Purification of plasmodium

Purification of *Fuligo septica* was attempted by several methods. The migration method of Cohen (1939) was unsuccessful with migration of *F. septica* plasmodium on Cohen's agar, even at the maximum migration rate of 0·5 cm./hr. achieved at 30°. (Cohen related successful purification to speed of migration.) The present findings were contrary to those of Cohen who found that one strain of *F. septica* when migrating at only 1·5 cm./day was readily purified by his method. The difference between Cohen's results and the present ones may have been due to differences in the accompanying microflora. Unsuccessful also were migration of plasmodium on other supports (silica gel, cellophan laid over Cohen's agar, glass, moistened filter paper), enrichment with *Saccharomyces cerevisiae* followed by migration (Cohen, 1939), treatment of plasmodium with ultraviolet radiation, and heat treatment of spores.

The method finally adopted was a combination of a migration technique with treatment by antibiotics similar to that used by Sobels (1948*a*) and Hok (1950). Penicillin + streptomycin incorporated in Cohen's agar each at 2500 units/ml. were used. These high concentrations of antibiotic were necessary since one of the contaminating organisms was relatively resistant; the plasmodium was unaffected by these antibiotics. Migration over Cohen's agar + antibiotics was carried out for 2 days, followed by 1 day on Cohen's agar and a further 2 days on the antibiotic agar. This treatment removed bacterial contaminants in 90 % of the tests, but yeast and mould contaminants persisted for longer. Further migration on Cohen's agar for as long as 18 days was necessary to complete the purification. An attempt was made to decrease this long migration period by using antifungal agents but those tested (griseofulvin, viridin, copper sulphate, cycloheximide, *o*-chlorophenol, *p*-chlorophenol, *β*-phenylethyl alcohol) were more toxic to the myxomycete than to the contaminating yeasts and moulds.

An alternative method of purification in which tubes were used for migration in place of Petri dishes was found to be convenient. The tubes were 40 cm. long, 1 cm. internal diameter, with each end (about 3 cm.) bent up at an angle of 45°, and contained a shallow layer of Cohen's agar. The direction of movement of the plasmodium could be controlled when necessary by the negative phototactic response of the plasmodium when the tube was illuminated at one end. By using this combination of migration technique and antibiotic treatment 85 % of the attempted purifications were successful. The method was tedious and the resulting plasmodial fragment usually only about 1 mm.^{2}, but no better method was found.

Two-member cultures

Attempts were made to establish *Fuligo septica* plasmodium in two-member culture with (*a*) organisms isolated from the plasmodium, (*b*) various Gram-positive and Gram-negative bacteria from other sources.

(*a*) Purified plasmodium was inoculated to malt agar (10 ml. slopes in 6 in. × 1 in. tubes) together with one isolate from the plasmodium. Eight Gram-negative rods, five yeasts, and one *Penicillium* sp. were tested in this way as associate organisms. One of the Gram-negative rods and three of the yeasts were also inoculated with plasmodium on to Cohen's agar. When growth of the plasmodium occurred transfers were made through several subcultures to confirm establishment of true two-member culture.

Of the fourteen organisms tested only three were successfully established in two-member culture with the plasmodium. These were two yeasts (one white- and the other black-pigmented) and a *Penicillium* sp. Of these three organisms, the white-pigmented yeast gave growth comparable to that of unpurified cultures of plasmodium; the other yeast and the *Penicillium* sp. gave slightly less good growth. In one experiment a small amount of growth was obtained with one of the Gram-negative rods and with two other yeasts. This, however, was not repeatable. Malt agar was more satisfactory for two-member cultures than plain agar; better growth was obtained and the associate organisms did not need to be added at intervals since they grew on the malt agar. The white-pigmented yeast seemed to be the organism of choice for routine two-member cultures. The plasmodium was maintained on malt agar in association with this organism for 3 months without any deterioration of the myxomycete strain becoming apparent.

(*b*) Purified plasmodium was inoculated onto washed agar on which were streaked washed suspensions of various Gram-negative bacteria, Gram-positive bacteria and yeasts, previously grown in shaken glucose yeast-extract broth cultures at 30° for 24 hr.: *Pseudomonas fluorescens*, *Escherichia coli*, *Erwinia carotovorum*, *Serratia marcescens*, *Corynebacterium* sp., *Bacillus cereus*, *Micrococcus aurantiacus*, *Micrococcus flavus*, *Saccharomyces cerevisiae*, *Torula rosea*. Incubation was as in (*a*). Of these ten organisms not isolated from plasmodium the *Corynebacterium* sp. permitted poor growth of plasmodium which deteriorated after a few days; *Escherichia coli* gave slow growth continuing for 2 weeks. *Saccharomyces cerevisiae* gave continuing growth of plasmodium on subculture; this was slower, however, than with the yeast isolated from the plasmodium.

Axenic cultures

The following preparations were tested as nutrients for pure cultures of *Fuligo septica*: sterile ground oatmeal; aqueous extract of oatmeal; ethanolic extract of oatmeal; oatmeal agar; autoclaved baker's yeast suspension in distilled water; yeast autolysates (one prepared from baker's yeast, dried, and incubated in distilled water at 37° for 4 hr. and at room temperature overnight; the other from brewer's yeast, ground, incubated in distilled water at 50° for 24 hr. and sterilized by autoclaving or by filtration); sand-ground brewer's yeast (sterilized by filtration); ethanol + ether-dried yeast; aqueous extract of baker's yeast; ethanolic extract of yeast; yeast disintegrated in a Mickle disintegrator (centrifuged at high speed to 'sterilize'); malt agar; Difco yeast-extract casein agar. The sterilization of these preparations was by autoclaving unless otherwise stated. In addition, purified plasmodium was placed on a cellophan membrane overlying a culture of living yeast (originally isolated from plasmodium) on malt agar.

Only one preparation of those tested gave any growth of plasmodium, namely,

the autoclaved baker's yeast suspension in distilled water. The growth was, however, very slow as compared with that of the control and the plasmodium began to deteriorate after 3 weeks. At no times was the plasmodium normal in appearance, being pale yellow instead of a bright deeper yellow. A similar autoclaved yeast preparation incorporated in the agar did not support growth of the plasmodium.

The yeast autolysates and the Difco yeast-extract casein agar were toxic to the plasmodium (cf. results with unpurified cultures; also observations on *Physarum polycephalum* reported by Hok, 1950).

Sporulation of Fuligo septica *in laboratory culture*

The following observations were made with unpurified plasmodium on the effect on sporulation of the age of the culture and of various environmental conditions.

Age of culture. The sporulation of two groups of *Fuligo septica* cultures was observed over a period of 2 years. Both groups of cultures were set up as described for stock cultures and were fed with the oatmeal preparation at weekly intervals. One group was subcultured every 4 weeks and the second group was maintained without subculture until sporulation occurred, sclerotium was formed or the plasmodium died. Humid conditions were maintained by adding sterile tap water when necessary.

Subculture of plasmodium to fresh medium prevented sporulation; of 171 cultures observed only 7 (4%) sporulated within 28 days. On the contrary the cultures maintained under exactly similar conditions but without subculture showed a relatively high percentage of sporulation throughout the entire 2-year period; of 171 cultures observed 82 (48%) sporulated, the remaining cultures dying or forming sclerotia. The length of the vegetative stage differed greatly from culture to culture and tended to be longer and more variable at the end of the 2-year laboratory cultivation than at the beginning.

In a group of unsubcultured cultures similar to the above addition of oatmeal was stopped after a period of feeding. This starvation of previously well-fed cultures did not promote sporulation.

Effect of other nutrients. The following nutrients were used in place of ground oatmeal: aqueous oatmeal extract, ethanolic oatmeal extract, malt agar, and oak bark and valonia (the dried acorn-cups of *Quercus aegilops*), both obtained from the tannery from which *Fuligo septica* was obtained. The plasmodium did not sporulate when these materials were tested as alternatives to oatmeal.

Physical nature of solid substrate. This was varied, filter paper, cotton wool and glass surfaces being used. No effect on sporulation of the plasmodium was observed.

Light. Cultures (unsubcultured) maintained as in the experiments on the effect of age of culture were exposed to daylight for 16–18 hr. per day. Light did not induce fruiting under our conditions.

Temperature. Cultures (subcultured and unsubcultured) maintained as in the experiments on effect of age of culture were incubated at 30° for 15 months after which incubation of this line of cultures was continued at 25°. Subculture to fresh medium prevented sporulation at 30° as it did at 25°. At 30° a lower frequency of sporulation (33%) in unsubcultured plasmodia occurred than at 25°, although on transfer of this line of cultures from 30° to 25° sporulation was apparently stimulated (82%). However, the number of cultures (44) used in this experiment was too small for any firm conclusion to be reached about this apparent stimulation of sporulation.

Desiccation. Cultures maintained as in the experiments on effect of age on culture were allowed to desiccate slowly at 25°. This did not induce sporulation.

Attempts to induce sporulation

The sporulation plasmodia used in these experiments were produced in cultures maintained as described in the experiments on the effect of age of culture. Attempts were made to demonstrate the existence in sporulating plasmodium of a substance inducing sporulation: (i) sporulating plasmodium (detectable at least 24 hr. before completion of the process) was transferred to a wet sterile cellophan membrane which separated the sporulating plasmodium from a vegetative plasmodium; (ii) sporulating plasmodium was transferred directly to vegetative plasmodium; (iii) freshly formed aethalium was transferred to vegetative culture; (iv) vegetative plasmodium was transferred to a culture which had just sporulated. All these attempts to show the existence of any inducing substance or substances in sporulating plasmodium, aethalium or culture medium were unsuccessful.

Attempts to interrupt the sporulation process

Attempts to interrupt sporulation were made by transferring sporulating plasmodium from cultures similar to those used in the previous section, to similar fresh culture medium, to a vegetative plasmodium in similar culture, to Cohen's agar and to malt agar, and by adding to sporulating plasmodium fresh nutrient in the form of powdered oatmeal or a suspension of a yeast isolated from plasmodium.

Transfer of sporulating plasmodium to a vegetative plasmodium, to Cohen's agar or to malt agar interrupted the sporulation process, the plasmodium returning to its normal vegetative state. Sporulating plasmodium transferred to similar fresh oatmeal medium, however, continued sporulation to completion. It is possible since only a few samples of plasmodium were used in the latter experiment (owing to the difficulty of detecting early stages of fruiting), that this difference in observations was due to the length of time after onset of sporulation at which the transfers were made. An irreversible stage in the sporulation process may have been reached in the latter case. The addition of fresh nutrient to sporulating plasmodia had no effect on the sporulation process.

Spore germination

A few experiments on spore germination were carried out with unpurified spore material produced in laboratory stock cultures of *Fuligo septica* as described in the previous section. Germination in distilled water of two batches of spores, one batch used immediately after formation and the other after storage for 6 months in a dry state at room temperature, was observed. The spores germinated by one of the methods described by Gilbert(1928*b*) in which the swarm cell escapes through a wedge-shaped aperture in the spore wall. This aperture was readily observable on microscopic observation. When determination was made of % germination 300 spores were counted at random throughout the preparation and the number of these which had germinated recorded. *F. septica* spores showed no dormancy period in distilled water; newly formed spores germinated as well as those 6 months old. A high percentage germination (60–70 %) occurred within a few hours.

Newly formed spores were also inoculated to stock culture medium; they germinated readily on the moist filter-paper support and in the presence of ground oatmeal developed into normal plasmodium. The young plasmodium ingested large numbers of the remaining spores, which gave it a dark brown appearance.

Since Elliot (1949) showed improved germination of some myxomycete spores after treatment with bile salts this was tested for *Fuligo septica*. Spores (4 months old) were shaken for a few minutes in a solution (10 g./l.) of bile salts, washed three times in sterile distilled water, resuspended in distilled water and incubated. Since the % germination without this treatment was usually high, and plasmodium readily obtained, treatment with bile salts showed no advantage.

Observation on the effect of cold and heat shock on spores (6 months old) was made by exposing them in distilled water suspension to the following temperatures: −20° for 30 min., 2° for 30 min., 60° for 5 min., 60° for 10 min., 60° for 20 min., 60° for 30 min. The suspensions were then incubated in distilled water. Exposure to low temperatures (Table 3) before germination decreased the % germination; −20° had a greater effect than 2°. Treatment at 60° resulted in failure to germinate except in spores exposed for only 5 min., where less than 1 % germination occurred after 2 weeks.

Table 3. *Germination at* 25° *of* Fuligo septica *spores after exposure in aqueous suspension to different temperatures*

Exposure temperature	Exposure time (min.)	Germination after 24 hr. incubation (%)
20°	30	22
2°	30	44
60°	5	0*
60°	10	0
60°	20	0
60°	30	0
None	None	69

* < 1 % germination after 14 days incubation.

The effect of spore concentration was investigated. An arbitrary, fairly concentrated, suspension of spores (7 months old) containing 51,000 spores/mm.^{3} as determined by a haemacytometer count, was prepared in distilled water and diluted 1/2, 1/3, 1/4, 1/5, 1/6, 1/8 and 1/16; 0·02 ml. samples of each dilution were set up in a van Tieghem cell and the % germination recorded at intervals. The more dilute suspensions of spores showed earlier and higher % germination than the more concentrated suspensions (Table 4).

DISCUSSION

Fuligo septica, a myxomycete which has not been much used in work with the slime moulds, can be readily grown in unpurified culture in the laboratory, and will complete its life cycle, fruiting and germination, under such conditions. It is therefore a suitable organism for laboratory study. Environmental conditions which favour the growth of *F. septica* are similar to those found by other workers

Table 4. *Germination at 25° of* Fuligo septica *spores in aqueous suspensions of different concentrations*

The initial suspension contained 51,000 spores/mm.^{3} as determined by a haemacytometer count.

Dilution of original spore suspension	Germination (%) at			
	2 hr.	3·5 hr.	6 hr.	24 hr.
Undiluted	0	< 1	< 1	14
1/2	0	0	< 1	18
1/3	0	0	< 1	19
1/4	0	< 1	< 1	7
1/5	0	0	< 1	6
1/6	0	0	< 1	—
1/8	< 1	< 1	< 1	17
1/16	5	6	12	48

to be suitable for other myxomycete species. For example Hok (1950) found that growth of *Physarum polycephalum* was good between pH 4·0 and 7·0 and between 18° and 25°. *Badhamia* sp. similarly tolerated a wide pH range, pH 4·0–7·5 (Jahn, 1932). *B. utricularis* (Sobels, 1950) grew well at 20°–24°, was slightly inhibited at 30° and died at 35°. In the present work *F. septica* grew well between pH 4·5 and 7·5 and over a temperature range of 18°–30°. Like *P. polycephalum* (Moore, 1935; Allen & Price, 1950) *F. septica* was found to be aerobic. In contrast to the findings of Hofmeister (1867) and Stahl (1884) who reported positive phototaxis of *F. septica* in weak light, in the present work *F. septica* was negatively phototactic. *B. utricularis* was shown by Sobels (1950) to be negatively phototactic. As observed by Gray (1938) the yellow pigmentation of *F. septica* was lost in the light. Growth was initially retarded in the light but exposure to direct sunlight was not lethal (cf. Gray, 1938).

The growth of *Fuligo septica* was better when nutrient was available in particulate form than when soluble nutrients only were present. A similar observation was made by Hok (1950) for *Physarum polycephalum*. *P. polycephalum* has since, however, been grown successfully in pure culture on a non-particulate medium (Daniel & Rusch, 1956, 1961), thus showing that this organism has not the specific requirement for particulate nutrient which has from time to time been postulated for this group of organisms.

Many of the earlier workers who claimed to have established two-member or axenic cultures appear to have used insufficiently rigorous and comprehensive tests for purity and their claims must be accepted with some reserve (e.g. Vouk, 1913; Skupienski, 1928; Howard, 1931; Cohen, 1939). Of these workers, some found that two-member cultures could readily be achieved with several bacterial, yeast or mould species (e.g. Vouk, 1913; Skupienski, 1928; Cohen, 1941; Hok, 1950), whilst others found such cultures more difficult to establish (e.g. Cohen, 1939; Sobels, 1950). In the present work *Fuligo septica* grew well in two-member culture only with two yeasts and a *Penicillium* sp. which had all been isolated from plasmodium, and with a laboratory strain of *Saccharomyces cerevisiae*. The myxomycete showed little or no continuing growth with a variety of Gram-positive and Gram-negative bacteria, some isolated from plasmodium and some from other sources. It is possible that particle size and the capacity of the plasmodium to ingest such

material may have some bearing on the nature of organisms suitable for two-member cultures. Gilbert (1928*a*) observed that swarm cells and small plasmodia of *Dictydiaethalium plumbeum* would ingest only smaller fungus spores. Kidder, Dewey & Fuller (1954) suggested that size of certain food molecules was important for some protozoal species. Axenic culture of *F. septica*, as found by Sobels (1950), proved difficult in our work, a small amount of growth being obtained with only one preparation from autoclaved baker's yeast.

Although sporulation of *Fuligo septica* occurred regularly in the laboratory, the presence of an inducing substance in the cultures could not be demonstrated, and the factors causative in the sporulation process were not determined. As observed by Seifriz & Russell (1936) with *Physarum polycephalum*, however, a definite rhythm of sporulation occurred, subculture within a certain period always averting sporulation. Seifriz & Russell suggested that the presence of 'toxic' or stimulatory substances in old cultures is the factor which most probably precipitates sporulation. The present observations with *F. septica* are in accordance with this interpretation. The interruption of the sporulation process which occurred on subculture is explicable by removal of the sporulating plasmodium from the influence of such substances. The possible effect of size of plasmodium as a pre-conditioning factor is also worthy of investigation (cf. Hok, 1950). In contrast to the findings of Gray (1938) with *F. septica* and *P. polycephalum* and of Hok (1950) with *P. polycephalum*, light was not found to be necessary for sporulation of *F. septica*, nor was it a precipitating factor. Depletion of nutrient did not lead to sporulation as it did with *P. polycephalum* (Camp, 1937).

A high percentage of spore germination was obtained with *Fuligo septica* in distilled water within 24 hr., as found by Gilbert (1929) and Smart (1937) and contrary to Cook & Holt's finding (1928) with this species. Dilute suspensions of *F. septica* spores showed a markedly higher percentage of germination than more concentrated suspensions, in contrast to the findings of Smart (1937) and Wilson & Cadman (1928) with other myxomycete species. Powell (1957) found that the germination of spores of *Bacillus subtilis* was less complete and slower in concentrated suspensions than in dilute suspensions. Thus the spores themselves may produce substances inhibitory to germination, as has been found for some fungal species (Allen, 1955; Krishnan, Bajaj & Daml, 1954).

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Amino Acid Interrelationships in Cysteine Toxicity in *Neurospora crassa*

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SUMMARY

L-Cysteine became toxic to the growth of *Neurospora crassa* (wild, Em 5297*a*), in the range 1·0–2·0 mM in the culture medium. The specificity of cysteine toxicity was shown by absence of toxicity with other sulphydryl compounds (β-mercaptoethanol, thioglycollic acid, reduced glutathione) and with cysteine metabolites, L-cysteic acid and taurine, under similar conditions. The toxicity of L-cysteine was completely overcome by supplements of S-methyl-L-cysteine and to a marked extent by DL-methionine and DL-homocysteine; partial counteraction of cysteine toxicity was observed with L-serine, DL-tryptophan, DL-alanine, DL-valine, DL-homoserine or DL-threonine. DL-Methionine and S-methyl-L-cysteine counteracted the inhibitory effect of L-cysteine in two *N. crassa* mutants, namely, methionineless mutant 38706 and cystathionineless mutant 9666.

INTRODUCTION

The existence of complex inter-relationships between amino acids in the growth and nutrition of micro-organisms and animals is well known. In several instances such antagonisms involve amino acids which possess structural similarities or are connected by interlinked metabolic pathways. The toxicity of one amino acid can be annulled by simultaneous supplementation with another, as in the case of the leucine–isoleucine antagonism in rats (Harper, Benton & Elvehjem, 1955). There are many examples of this kind, but it would appear that these interactions are due to various effects of amino acids on metabolism, some of which are explicable on the basis of mechanisms other than those involving simple structural antagonism. In *Neurospora crassa*, a reciprocal antagonism between threonine and methionine was shown by Doudney & Wagner (1952, 1953) where the toxicity of one compound was counteracted by the other; it was suggested that threonine interfered with the utilization of homocysteine for the synthesis of methionine. The toxicity of homocysteine for *N. crassa* was overcome by threonine or choline but not by betaine, glycine, serine or methionine (Doudney & Wagner, 1955). During our earlier work on the effects of sulphur amino acids on molybdenum toxicity in *N. crassa* (Sivarama Sastry, Ramaiah & Sarma, 1958), preliminary experiments showed that high concentrations of cysteine were toxic to growth and that methionine could annul this toxicity. These findings indicated that cysteine toxicity was quite different from homocysteine toxicity. Cysteine toxicity has been studied in greater detail and in the present paper it was found that S-methyl-L-cysteine and DL-methionine were good antagonists of L-cysteine and that other amino acids, including DL-homocysteine itself, were partially effective in this respect.

METHODS

Organisms. The following *Neurospora crassa* cultures (obtained through the courtesy of Dr H. K. Mitchell, Division of Biology, California Institute of Technology, Pasadena, California, U.S.A.) were used: *N. crassa*, wild strain Em 5297*a*; *N. crassa*, methionineless mutant 38706; *N. crassa*, cystathionineless mutant 9666.

Media. The basal medium used routinely for growing the wild strain was as in earlier studies (Sivarama Sastry, Ramaiah & Sarma, 1958) and had the following composition (g.): glucose, 20; KH_2PO_4, 3; NH_4NO_3, 2; $(NH_4)_2C_4H_4O_6$, 1; $MgSO_4.7H_2O$, 0·5; NaCl, 0·1; trace elements (μg.): Zn, 200; Mn, 200; Cu, 80; Fe, 20; Mo, 20; biotin 5 μg.; all in 1 l. glass-distilled water.

The methionineless and cystathionineless mutants were grown on this basal medium + DL-methionine 50 μg./ml. Under the conditions of growth used, this concentration of DL-methionine was the minimal concentration for optimal growth.

All chemicals used were of analytical grade.

Amino acids and other compounds. The amino acids used in these experiments were: S-methyl-L-cysteine (Mann Chemicals; DL-homocysteine (Hoffmann La Roche); DL-homoserine (California Foundation for Biochemical Research); DL-alanine, DL-glutamic acid, DL-valine (Nutritional Biochemical Corporation, U.S.A.) all others were Merck products.

All the sulphydryl compounds used were added to the required concentrations in the medium as solutions sterilized by Seitz filtration. Other amino acids were incorporated directly in the media which were sterilized by autoclaving at 120° for 10 min.

Culture technique and estimation of growth. In all experiments the organisms were grown on 10 ml. basal medium (adjusted to pH 4·8–5·0) containing all the supplements as described below, in 50 ml. Pyrex conical flasks for 72 hr. at 30° ± 1°. At the end of incubation the mycelia were harvested, thoroughly washed, dried overnight at 60° and weighed. All experiments were run in duplicate and repeated at least four times.

RESULTS

Specificity of L-*cysteine toxicity*

L-Cysteine in the medium at concentrations in the range 1·0–2·0 mM. was toxic to the growth of *Neurospora crassa* Em 5297*a*, growth being nearly 80% inhibited at 2·0 mM. To see whether this was a specific effect or a non-specific one due to an unphysiological concentration of a sulphydryl compound, β-mercaptoethanol, thioglycollic acid and reduced glutathione were tested; they were completely non-toxic up to 2·0 mM. L-Cysteic acid and taurine were also non-inhibitory under similar conditions; thus the toxicity of cysteine was not due to accumulation of these possible products of its metabolism. It would appear that L-cysteine is much more toxic to the growth of these strains of *Neurospora crassa* than other sulphydryl compounds and its own catabolic end products.

Sulphur amino acid antagonism in cysteine toxicity

In view of the above observed specificity of cysteine toxicity for *Neurospora crassa*, the possibility of its antagonism to other sulphur amino acids was explored. In this work, the toxicity of cysteine was studied at 0·5, 1·0, 1·5 and 2·0 mM and

the annulment of growth inhibition by DL-homocysteine, DL-methionine and S-methyl-L-cysteine was tested at molar ratios of 1:0·5, 1:1 and 1:2. The results are given in Table 1.

Table 1. *Effect of some sulphur amino acids on cysteine toxicity in* Neurospora crassa *(wild) Em 5297a*

N. crassa grown on 10 ml. basal medium (pH 4·8–5·0) in 50 ml. conical flasks for 72 *hr. at* 30 ± 1°; with supplements as shown.

Supplements to basal medium*	Cysteine: amino acid (molar ratio)	Concentration of cysteine (mM)† 0·5	1·0	1·5	2·0
		Growth (% control)			
None	—	75·0	47·0	34·0	19·0
DL-Methionine	1:0·5	80·0	60·0	56·3	48·8
	1:1·0	87·5	72·5	67·5	58·8
	1:2·0	90·0	75·0	70·0	68·8
S-Methyl-L-cysteine	1:1·0	95·5	88·5	87·1	88·5
	1:2·0	98·0	93·0	92·0	93·0
DL-Homocysteine	1:2·0	—	75·0	60·5	60·5

* In all cases, L-cysteine was added to give the ratios indicated.
† Values on the basis of growth reached on basal medium alone (mycelial dry weight, 35–40 mg.).

The growth values recorded in Table 1 represent the maximal reversals obtained with levels of the amino acids found optimal by preliminary trials. The data show that the three amino acids tested counteract the growth inhibition caused by cysteine to a very marked extent throughout its inhibitory range. One of the noteworthy features brought out by these results is the superiority of S-methyl-L-cysteine over methionine and homocysteine. Moreover, judged by the completeness of reversal at the higher level of S-methyl-L-cysteine, and by the constancy of growth reached at various molar ratios of S-methyl-L-cysteine, it would appear that there is a distinct competitive antagonism between these two amino acids. The reversal by homocysteine reveals an interesting antagonism among sulphur amino acids since homocysteine itself at higher levels becomes toxic.

Influence of amino acids on cysteine toxicity

It was of interest to examine whether other amino acids could also counteract cysteine toxicity in *Neurospora crassa*. Table 2 records the maximal reversals obtained with the amino acids which were found effective in this regard. These data show that cysteine toxicity can be partially counteracted by other amino acids as well and that serine and tryptophan are somewhat more effective than others. It is important to note that the effects of methionine, serine and glycine on cysteine toxicity are quite different from corresponding phenomena in homocysteine toxicity in Neurospora (Doudney & Wagner, 1955).

Threonine was a good antagonist of homocysteine but not of cysteine. The reverse appeared to hold good with methionine and to a lesser degree with glycine and serine. The data in Tables 1 and 2 taken together suggest, therefore, that the mechanisms by which homocysteine and cysteine become toxic to Neurospora are not identical. Further support for such a conclusion was provided by the absence

of any effect with choline at a cysteine:choline ratio of even 1:3. Other compounds tested and found unable to annul cysteine toxicity were: pyridoxine, *p*-aminobenzoic acid, betaine, folic acid, vitamin B_{12}, nicotinic acid.

Table 2. *Effect of some amino acids on cysteine toxicity in* Neurospora crassa *wild type Em 5297a*

Experimental details as in Table 1.

Supplements to basal medium*	Cysteine: amino acid (molar ratio)	Concentration of cysteine (mM)† 1·0	1·5	2·0
		Growth (% control)		
None	—	46·1	35·5	20·0
Glycine	1:8	74·3	51·3	43·0
L-Serine	1:3	76·3	65·8	51·3
DL-Tryptophan	1:2	63·2	65·0	63·2
DL-Alanine	1:2	74·4	58·1	40·0
DL-Valine	1:2	70·0	42·0	32·0
DL-Homoserine	1:2	74·0	58·4	47·7
DL-Threonine	1:2	70·0	62·5	45·0

* In all cases, L-cysteine was also included to give the ratios indicated.
† Values on the basis of growth reached on basal medium alone (mycelial dry weight, 35–40 mg.).

Sulphur amino acid antagonisms in Neurospora *mutants*

Since the superiority of methionine and S-methyl-L-cysteine over other amino acids in annulling the toxicity of cysteine might be indicative of a specific effect of cysteine on methionine biosynthesis, the sulphur amino acid interrelationships were also studied in two mutants of *Neurospora crassa*, the methionineless mutant 38706 and the cystathionineless mutant 9666 when both were grown on a basal medium containing the least concentration of methionine which gave optimal growth, as mentioned earlier. The results are presented in Table 3. The results, which represent the maximal degree of antagonism obtained, show the presence of sulphur amino acid antagonisms of a nature similar to those seen with the wild strain. However, cysteine toxicity may not involve only an interference with methionine biosynthesis. With the cystathionineless mutant 9666 both S-methyl-L-cysteine and methionine (particularly the latter) were less efficient in counteracting cysteine toxicity than with the wild strain. Nevertheless, the patterns of growth again indicate the competitive antagonism between S-methyl-L-cysteine and cysteine.

DISCUSSION

The present work brings out some interesting features of amino acid inter-relationships in Neurospora. While general amino acid antagonisms are known, there is no record of any specific amino acid inter-relationships in cysteine toxicity in micro-organisms. The results recorded here, considered with the earlier work of Doudney & Wagner (1955) about homocysteine toxicity in Neurospora, show that among several sulphydryl compounds, only cysteine and homocysteine exerted toxic effects at comparable concentrations. However, the two toxieities appear to be different, as indicated by the patterns of antagonism obtained with compounds such as choline, methionine and threonine. Since the amino acids that counteract

cysteine toxicity are those known to be incapable of overcoming homocysteine toxicity, the data suggest that cysteine exerts its toxic effects independently and not by giving rise to abnormal intracellular concentrations of homocysteine, a possibility that would normally be suggested by the known ability of *N. crassa* to perform such a conversion (Horowitz, 1947). Moreover, since cysteic acid and taurine were non-toxic at comparable concentrations, the possibility that the cysteine toxicity was due to such catabolic products appears unlikely.

Table 3. *Influence of methionine and* S-*methyl-*L-*cysteine on cysteine toxicity in* Neurospora crassa *mutants 38706 and 9666*

Experimental details as in Table 1, except that the basal medium also contained DL-methionine at 50 μg./ml.

Supplements to basal medium*	Cysteine: amino acid (molar ratio)	Concentration of cysteine (mM)† 1·0	1·5	2·0
		Growth (% control)		
Mutant 38706				
None	—	64·4	46·6	35·7
DL-Methionine‡	1:2	71·4	66·3	56·4
S-Methyl-L-cysteine	1:2	93·0	92·0	96·4
Mutant 9666				
None	—	46·4	35·7	19·0
DL-Methionine‡	1:2	62·1	48·0	26·2
S-Methyl-L-cysteine	1:2	76·2	76·2	78·0

* In all cases, L-cysteine was included to give the ratios indicated.

† Values on the basis of growth reached on basal medium + methionine alone (50 μg./ml.). Mycelial weight 28–30 mg. in both cases.

‡ The methionine value represents total amount inclusive of that present in basal medium.

The results in Tables 1 and 3 show that in the examined strains of *Neurospora crassa*, S-methyl-L-cysteine was a powerful and competitive antagonist of L-cysteine. In view of the reported occurrence of S-methyl-L-cysteine as a normal metabolite in the wild and methionineless strains of *N. crassa* and the capacity of such strains to utilize this compound as a sole sulphur source (Ragland & Liverman, 1956), the antagonism between cysteine and S-methyl-L-cysteine might be important in the metabolism of sulphur amino acids in Neurospora. Several suggestions have been made in recent years that S-methyl-L-cysteine may itself be incorporated into proteins in Neurospora (Ragland & Liverman, 1956) and that it might be involved in the formation of methionine by transfer of its thiomethyl group to a four-carbon compound e.g. homoserine (Wiebers & Garner, 1960) by an alternative pathway not involving cystathionine. Since cysteine itself is a powerful inhibitor of the cystathionine-cleaving enzyme from rat liver (Matsuo & Greenberg, 1959) it is possible that the annulment of cysteine toxicity by S-methyl-L-cysteine in *N. crassa* may indicate such utilization of S-methyl-L-cysteine for methionine formation under the stress imposed by cysteine toxicity. That homoserine itself did not completely annul cysteine toxicity (Table 2) suggests that synthesis of homoserine may not be totally inhibited and that hence it would be available for the formation of methionine by interaction with S-methyl-L-cysteine by the alternate pathway.

The counteraction of cysteine toxicity by methionine would appear to be significant, and characteristic of *N. crassa*, since in several pathogenic fungi it has been found incapable of antagonizing the toxic effect of cysteine (Königsbauer, 1951). In *Escherichia coli* the reverse phenomenon of a specific cysteine counteraction of methionine toxicity was reported by Cimino (1954). The lack of complete counteraction of cysteine toxicity in Neurospora by methionine may be due to the fact that there may be some difference between its effects as exogenous and endogenous methionine (Wiebers & Garner, 1960). The data about the counteraction of cysteine toxicity by amino acids and the toxicity picture in the mutants here examined (Tables 2, 3) show that cysteine toxicity is a complex phenomenon. It is difficult to envisage a direct antagonism between these amino acids and cysteine, though interaction involving antagonism at the level of absorption of amino acids (O'Barr, Levin & Reynolds, 1958) has to be considered. However, other explanations are also likely; in this context, the tryptophan *v.* cysteine interaction noted in the present work is interesting since an antagonism between cystine and tryptophan was observed in the rat by Tyner, Lewis & Eckstein (1950) and in the larvae of the rice moth *Corcyra cephalonica* St. (Radhakrishna Murthy, Sivarama Sastry & Sarma, 1957). Another feature is that the amino acids other than tryptophan found significantly effective in the present work are those which have been implicated in methionine formation in *E. coli* or *N. crassa* (Gibson & Woods, 1960; Kalan & Ceithaml, 1954; Teas, Horowitz & Fling, 1948).

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The Requirement for Acetate of a Streptomycin-resistant Strain of *Staphylococcus aureus*

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SUMMARY

A streptomycin-resistant strain of *Staphylococcus aureus*, which requires haemin for aerobic growth, grew either aerobically or anaerobically in the absence of haemin provided the medium was supplemented with acetate or pyruvate; growth with these organic acids was increased by uracil and purines. The parent drug-sensitive strain grew aerobically without haemin but when grown anaerobically required either uracil or acetate or pyruvate. With both strains mevalonate replaced acetate and was about ten times more active.

The products of glucose fermentation by both strains showed no gross difference, lactate being predominant (about 85 % of the glucose carbon); only small amounts of acetate were detected. Under aerobic conditions suspensions of the parent strain oxidized glucose to acetate which accumulated. The mutant strain oxidized glucose to acetate only when previously grown with haemin or when haemin was added to the suspension of organisms. When the organism was grown with acetate in place of haemin, lactate was the predominant product. The ability of the mutant to form sufficient acetate from glucose for biosynthetic purposes is apparently dependent on a functional electron transport chain involving haemoproteins. A nicotinamide-adenine dinucleotide-linked lactate dehydrogenase and a pyruvate oxidizing system are present in extracts of both organisms. The activity of these enzymes in the mutant strain was similar whether the organisms were grown on haemin or acetate.

INTRODUCTION

Jensen & Thofern (1953) described a streptomycin-resistant strain of *Staphylococcus aureus* (Var 511) which, unlike the parent sensitive strain, required haemin for growth. Later work showed that the mutant grew without haemin in a medium containing acetate or pyruvate; purines and uracil increased growth under these conditions (Lascelles, 1956). The mutant did not form haem compounds when grown on acetate in place of haemin since organisms grown in this way neither respired nor reduced nitrate; these activities were present only in organisms grown on haemin (Jensen & Thofern, 1953; Lascelles, 1956). In the present work the requirement of the mutant and parent strains for acetate has been examined more thoroughly. In addition, analyses have been made of the end products of glucose metabolism by suspensions of the parent and mutant strains to see whether these provided evidence for differing pathways of glucose breakdown which might account for the requirement of the mutant for preformed acetate when grown without haemin.

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METHODS

Organisms. The two strains of *Staphylococcus aureus* used were obtained from Dr J. Jensen. The parent organism (SG511A) was streptomycin-sensitive and grew without added haemin while the mutant strain (SG511 Var) was streptomycin-resistant and required haemin (Jensen & Thofern, 1953). Stock cultures were maintained on chocolate blood agar as previously described (Lascelles, 1956).

Medium. The basal medium used throughout contained per l.: Difco casamino acids (vitamin-free) 3 g.; L-cysteine hydrochloride, 100 mg.; DL-tryptophan, 50 mg.; $Na_2HPO_4.12H_2O$, 7 g.; K_2HPO_4, 5·5 g.; KH_2PO_4, 500 mg.; NaCl, 2 g.; $MgSO_4.7H_2O$, 100 mg.; ferric citrate, 1 mg.; nicotinic acid, 1 mg.; thiamine hydrochloride, 1 mg.; biotin, 1 μg.; adjusted to pH 7·4. Glucose (1 % final conc.) was added after autoclaving. Supplements to this medium are indicated in the text.

Growth tests. Aerobic cultures were in 18 mm. tubes containing 5 ml. medium incubated in air without shaking. Anaerobic cultures were grown in completely filled glass-stoppered bottles containing 32 ml. medium. The inoculum was prepared by suspending organisms from a chocolate blood agar slope in water to a concentration equivalent to about 0·4 mg. dry wt./ml.; dilutions of this suspension were added to the medium to give an initial concentration density equiv. about 0·001 mg. dry wt./ml. Incubation was at 37°. Amounts of growth and concentrations of suspensions were measured with an EEL colorimeter (Evans Electroselenium Ltd., Halstead, Essex), an instrument reading of 10 being equivalent to 0·42 mg. dry wt./ml.

Preparation of suspensions. Cultures were grown in 250 ml. flasks containing 125 ml. medium inoculated with organisms from the chocolate blood agar slopes to give an initial concentration equiv. about 0·01 mg. dry wt./ml. Incubation was in air (without shaking) for 16 hr. at 37°. Organisms were harvested by centrifugation and washed in one half the original culture volume of 0·02 M-potassium phosphate buffer (pH 7·4). They were finally suspended in 0·04 M-potassium phosphate buffer (pH 7·4) to a concentration equiv. 2–4 mg. dry wt./ml.

Manometry. Conventional Warburg techniques were used. Details of the vessel contents and atmosphere are given in the text. Incubation was at 37°.

Analytical methods. After incubation, suspensions were removed from the manometer cups, the organisms removed by centrifugation and samples of the supernatant fluid analysed for lactate and acetate. Lactate was determined by the method of Barker & Summerson (1941) and acetate as described by Rose (1955). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin (Armour Laboratories Ltd., Hampden Park, Eastbourne, Sussex) as standard.

Assays of enzymic activity. Cell-free extracts were made by shaking 10 ml. suspension (equiv. 10 mg. dry wt./ml. in 0·04 M-potassium phosphate buffer, pH 7·4) with 10 g. ballotini (grade 12) beads for 5 min. in a Mickle disintegrator (Mickle, 1948). After centrifugation at 20,000 *g*, for 10 min. at 0°, the clear supernatant fluid was assayed for lactate dehydrogenase and pyruvate oxidizing activity. Lactate dehydrogenase was measured by following the rate of oxidation of reduced nicotinamide-adenine dinucleotide ($NADH_2$) in the presence of pyruvate at

340 mμ. Each cuvette (1 cm. light path) contained in a final volume of 3 ml.: potassium phosphate buffer pH 7·0, 300 μmoles; sodium pyruvate, 20 μmoles; extract, 0·05–0·2 mg. protein; $NADH_2$ 0·3 μmole (except in the blank). The reaction was started by addition of pyruvate. Units of activity are expressed as μmoles of $NADH_2$ oxidized/min. Pyruvate oxidizing activity was measured by observing the rate of reduction of 2:6-dichlorophenol-indophenol in the presence of pyruvate at 600 mμ. Each cuvette (1 cm. light path) contained in a final volume of 3 ml.: potassium phosphate buffer (pH 7·0), 300 μmoles; $MgCl_2$, 10 μmoles; thiamine pyrophosphate, 0·1 μmole; 2:6-dichlorophenol-indophenol, 0·2 μmole; extract 0·5–2 mg. protein; sodium pyruvate, 40 μmoles (except in the blank). The reaction was started by addition of the substrate. Units of activity are expressed as μmoles of dye reduced/min. The spectrophotometric assays were carried out with an Optica recording spectrophotometer, Model CF4DR (Optica Ltd., Gateshead, Co. Durham).

Special chemicals. Stock solutions of haemin (1 mM) were dissolved in 0·02 N-NaOH in 50% (v/v) ethanol. It was added to media after autoclaving. Sodium DL-mevalonate was prepared by neutralizing the lactone (British Drug Houses, Ltd., Poole, Dorset). Details of other materials were as previously described (Lascelles, 1956, 1960).

RESULTS

Growth of parent and mutant strains

Aerobiosis and anaerobiosis. The parent strain grew aerobically on the unsupplemented basal medium containing glucose, amino acids and B-group vitamins; but anaerobically the addition of purines and uracil or of acetate or pyruvate was

Table 1. *Requirements for aerobic and anaerobic growth of parent and mutant strains of* Staphylococcus aureus

Cultures were grown for 27 hr. in the basal medium supplemented where shown with: PU (a mixture of adenine, xanthine and uracil, 0·1 mM each); sodium acetate or pyruvate, 10 mM.; or haemin, 0·001 mM. Details of the procedures for aerobic and anaerobic incubation are given in Methods. Growth is expressed as optical density readings on the EEL colorimeter.

Additions to basal medium	Growth			
	Parent strain		Mutant strain	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Nil	22	1·5	1·5	0
PU	21	19	0	0
Acetate	20	14	25	17
PU + acetate	21	18	32	22
Pyruvate	21	19	31	22
PU + pyruvate	21	19	30	27
Haemin	17	0	21	0

necessary (Table 1). Of the nucleic acid derivatives tested, uracil was essential as found previously for *Staphylococcus* (Richardson, 1936); the purines merely improved anaerobic growth. Anaerobic growth of the parent strain with acetate was increased by the addition of nucleic acid derivatives.

Haemin promoted growth of the mutant strain only under aerobic conditions (Table 1). The oxygen tension necessary to support full growth under these conditions could not have been high since static cultures in 18 mm. diameter tubes containing 10 ml. medium grew just as well as those in vigorously shaken flasks. Growth of the mutant in the absence of haemin occurred either aerobically or anaerobically when the basal medium was supplemented with acetate or pyruvate (Table 1). The further addition of a mixture of adenine, xanthine and uracil improved the yield but, unlike the parent organism, nucleic acid derivatives alone did not support anaerobic growth.

Replacement of acetate by mevalonic acid. The requirement of some lactobacilli for acetate is replaced by mevalonic acid, a key intermediate in the biosynthesis of isoprenoid derivatives such as sterols (Skeggs *et al.* 1956; Popjak & Cornforth, 1960). This compound also replaced acetate for growth of the mutant strain of Staphylococcus used here (Table 2). Mevalonate was 10 times more active than acetate though the lag period was longer than in cultures growing with acetate. Addition of mevalonate to the basal medium also promoted anaerobic growth of the parent strain.

Table 2. *Activity of mevalonate for growth of the mutant strain* Staphylococcus aureus (*SG* 511 *Var*)

The basal medium was supplemented with adenine, xanthine and uracil (0·1 mM. each). Cultures were incubated aerobically as described in Methods and growth is recorded as EEL colorimeter readings.

Additions to medium (mM.)	Growth	
	28 hr.	52 hr.
Nil	0	0·5
Sodium acetate 1	1	3
Sodium acetate 3	10	42
Sodium mevalonate 0·1	2	14
Sodium mevalonate 0·3	3	37
Haemin 0·001	20	37

Anaerobic metabolism of glucose by suspensions of organisms

The obligatory requirement of the mutant for acetate (or pyruvate) for growth without haemin, whereas the parent strain grew anaerobically without added acetate in the presence of nucleic acid derivatives, suggested that the end products of glucose fermentation by the two strains might differ. No evidence for any major difference was, however, found. Lactic acid was the main end-product formed by incubation of suspensions of both parent and mutant organisms anaerobically in phosphate buffer (Table 3, Expt. 1). Small amounts of CO_2 were also formed but only trace amounts of volatile acids were detected after steam distillation of the fermentation mixtures. Acetoin was not detectable by the test of Barritt (1936). Suspensions of the mutant harvested from cultures containing either haemin or acetate and nucleic acid derivatives behaved similarly (Table 3). Suspensions of both parent and mutant organisms fermented pyruvate to lactic and acetic acids and CO_2 in equimolar amounts, as found previously with other strains of *Staphylococcus aureus* (Krebs, 1937). The mutant strain behaved similarly whether grown with haemin or acetate.

The fermentation of glucose was also studied with organisms suspended in the basal medium instead of phosphate buffer. In these experiments acetate was estimated by the sensitive enzymic method of Rose (1955). With both parent and mutant organisms, irrespective of the medium from which they were harvested, lactate was again the major end-product, amounting to about 1·5 μmole/μmole glucose (Table 3, Expt. 2). The amounts of acetate formed by suspensions of both organisms were of the same order, 0·1–0·15 μmole/μmole glucose. The amount of acetate formed by the parent strain or by the mutant grown with haemin was however always more than that formed by the mutant grown without haemin; but the analyses did not provide any obvious clue to account for the ability of the parent to grow anaerobically in the absence of added acetate.

Table 3. *Fermentation of glucose by suspensions of parent and mutant strains of* Staphylococcus aureus

The organisms were grown on the basal medium supplemented as shown with 10 mM. sodium acetate and 0·1 mM. each of adenine, xanthine and uracil (Ac-PU) or with 0·001 mM. haemin (H). In Expt. 1, each double side-armed manometer cup contained: organisms (equiv. 2·5 mg. dry wt./ml.) 1·0 ml.; potassium phosphate buffer (pH 7·4) 250 μmoles; glucose (when present) 10 μmoles added from one side arm at zero time; H_2O to 2·5 ml.

In Expt. 2, double-strength basal medium (1·25 ml.) was present in place of the phosphate buffer.

Incubation was at 37° in N_2 for 1 hr. in Expt. 1, for 3 hr. in Expt. 2. To estimate CO_2, 0·1 ml. 4 N-H_2SO_4 was added from the other side arm at the end of the reaction to liberate bound CO_2. Lactate and acetate were estimated with suspensions incubated in the same way, except that H_2SO_4 was not added at the end of the reaction.

Expt.	Strain	Growth medium	Glucose (10 μmoles)	Product (μmoles) CO_2	Lactate	Acetate*	Glucose C recovered (%)†
1	Parent	Ac-PU	−	1·7	0	—	
			+	3·2	17·0	—	93
	Mutant	Ac-PU	−	0·7	0	—	
			+	2·7	16·6	—	93
		H	−	1·7	0	—	
			+	3·4	17·0	—	94
2	Parent	Ac-PU	−	0·6	0·2	0·6	
			+	3·3	13·8	2·1	89
		Basal	−	0·6	0·2	0·7	
			+	2·9	14·8	1·6	89
	Mutant	Ac-PU	−	0·4	0·2	0·5	
			+	5·4	15·7	0·6	100
		H	−	0·4	0·2	0·7	
			+	5·6	13·9	1·6	99

* Acetate was not estimated in Expt. 1.

† Glucose (C) accounted for after subtraction of endogenous values.

Aerobic metabolism of glucose by suspensions of organisms

The oxidation of glucose was examined with suspensions of the parent and mutant strains incubated aerobically in the basal growth medium (Table 4). Acetate was the major end-product (1·3 μmole/μmole glucose) formed by the parent organism when harvested from the basal medium with or without acetate. The mutant strain

formed a similar amount of acetate only when previously grown on haemin, or when haemin was added to the manometer cups. Mutant organisms harvested from the acetate+nucleic acid medium formed only small amounts of acetate, lactate being the major end product; such suspensions showed only slight oxygen consumption. The ability of the mutant strain to grow without acetate aerobically in the presence of haemin was therefore correlated with its ability to oxidize glucose to acetate under these conditions.

Table 4. *Aerobic metabolism of glucose by suspensions of the parent and mutant strains of* Staphylococcus aureus

Organisms were grown on the basal medium supplemented with acetate, purines and uracil or with haemin (see Table 3). Each manometer vessel contained: organisms (equiv. 2·5 mg. dry wt./ml.), 1·0 ml. double-strength basal medium, 1·25 ml.; glucose (when present) 10 μmoles, added from the side arm at zero time; H_2O to 2·5 ml. The centre wells contained 0·2 ml. 20 % NaOH. Incubation was at 37° in air for 3 hr.

Strain	Growth medium	Glucose 10 μmoles)	O_2 uptake (μmoles)	Products (μmoles) Acetate	Lactate
Parent	Ac-PU	−	2·5	1·0	0·1
		+	52·5	12·5	0·5
	Basal	−	5	1·65	0·1
		+	62	12·75	0·3
Mutant	Ac-PU	−	1·5	0·6	0·1
		+	2·6	1·2	16·0
	H	−	7·5	1·35	0·1
		+	56	14·25	0·2

Table 5. *Lactic dehydrogenase and pyruvate oxidase activities in extracts of parent and mutant strains of* Staphylococcus aureus

Extracts were prepared as described in Methods from organisms grown in the basal medium supplemented as described in Table 3. The enzyme activities were assayed as described in Methods and are expressed as units/mg. protein. At least 3 different extracts from each group were assayed and the values shown are the mean with the range in brackets.

Strain	Growth medium	Lactate dehydrogenase	Pyruvate oxidase
Parent	Basal	6·4 (5·2–7·7)	0·11 (0·07–0·13)
	Ac-PU	6·0 (5·0–6·5)	Not determined
Mutant	H	5·6 (4·4–6·8)	0·17*
	Ac-PU	5·6 (5·0–6·0)	0·11 (0·08–0·14)

* One estimation only.

Enzymic activity of cell-free extracts

The requirement of the mutant strain for acetate might have been due to an active lactate dehydrogenase coupled with a low pyruvate oxidizing system. In such a situation pyruvate formed from glucose would be rapidly reduced to lactate and might not be available for acetate formation via pyruvate oxidase. However, no evidence for this was found by assay of these enzymes in cell-free extracts (Table 5). Extracts of the mutant and parent strains had a highly active NAD-linked lactate dehydrogenase (specific for the L(+)isomer) and both types of

extract exhibited a pyruvate oxidase system. There was no significant difference in the level of these enzymes in the parent and mutant strains nor did the presence of haemin or of acetate in the growth medium influence the enzymic activity found in the extracts.

DISCUSSION

Nutritional studies have provided abundant evidence that acetate is an essential metabolite for micro-organisms. It is required by some lactobacilli in media lacking lipoic acid which is part of the pyruvate oxidase complex (Reed, 1957). Other lactobacilli require either acetate or mevalonate (Wagner & Folkers, 1961). In these organisms the main function of acetate appears to be as a precursor of mevalonate, a key intermediate in the biosynthesis of isoprenoid derivatives (Popjak & Cornforth, 1960). The precise structure of the bacterial isoprenoids is unknown but they are probably present in the non-saponifiable fraction of the lipids. The activity of mevalonate in replacing acetate for growth of the strains of Staphylococcus studied in this work suggests that the major function of acetate in these organisms is also for the formation of mevalonate.

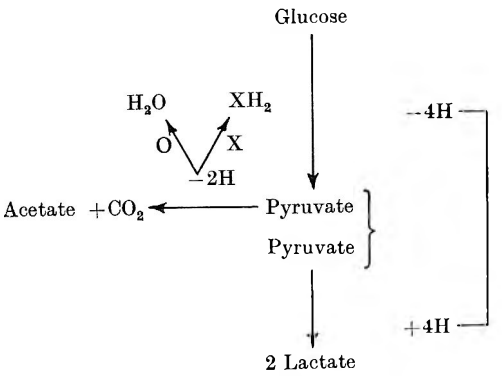

Fig. 1. Scheme for glucose metabolism in Staphylococcus.

Growth experiments with the mutant strain of Staphylococcus suggest that it can form sufficient acetate for its biosynthetic needs only when an electron transport chain involving haemoproteins is functioning; either oxygen or nitrate can act as ultimate hydrogen acceptor under these circumstances (Lascelles, 1956). This interpretation of the growth experiments was confirmed by analysis of the end products of glucose metabolism. Acetate is the major end-product formed by haemin-grown organisms incubated aerobically but with those grown without haemin, lactate is the predominant end-product under both aerobic and anaerobic conditions.

The ability of the parent strain to grow anaerobically without added acetate provided uracil and purines are present suggests that it can make sufficient acetate by anaerobic mechanisms to satisfy its requirements for biosynthetic reactions while the mutant cannot. Evidence for a gross difference in fermentation pathways in the two organisms was not shown by the analyses; but the amount of acetate accumulated by the parent, although slight, was consistently higher than that formed by the mutant.

Since lactate is the main end-product of glucose fermentation by both strains reduction of pyruvate by a NAD-linked lactate dehydrogenase is apparently the main outlet for substrate hydrogen (see Fig. 1). For conversion of pyruvate, derived from glucose, to acetate an additional hydrogen acceptor system is required (X in Fig. 1) and this may be lacking in the mutant strain. Under aerobic conditions, provided there is a complete electron transport chain including haemoproteins, the problem of an outlet for substrate hydrogen is solved by the use of molecular oxygen.

The ability of added pyruvate to replace acetate for anaerobic growth of these staphylococci is readily explained by the pyruvate dismutation system which converts pyruvate to acetate, lactate and CO_2 (Krebs, 1937). Pyruvate was previously shown to be needed for anaerobic growth of other strains of Staphylococcus (Richardson, 1936). The present experiments throw no light on the question of why the parent strain requires for growth *either* nucleic acid derivatives *or* acetate. The whole problem of the requirement of Staphylococcus for nucleic acid derivatives for anaerobic growth remains unsolved.

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Particle Counts and some Chemical Properties of Murray Valley Encephalitis Virus

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SUMMARY

Murray Valley encephalitis virus was grown in baby mouse brain and purified. In five out of six experiments, the number of virus particles per chick egg LD50 was 90 ± 50. Fifteen preparations of purified virus were pooled and the pool examined chemically. The nucleic acid content was 7·8 %, with bases present in the proportions: adenylic acid, 25·5; guanylic acid, 27·5; cytidylic acid, 21·5; uridylic acid, 25·5. No thymidylic acid was found. The virus preparation contained 11 % lipid; phospholipid and total cholestorol were present to 0·8 % and 1·0 %, respectively, of the virus preparation.

INTRODUCTION

The arthropod-borne viruses are divided into groups on serological grounds; the two most studied are groups A and B. The group A virus, Eastern equine encephalomyelitis, was purified by Taylor, Sharp, Beard & Beard (1943) from extracts of infected chick embryos and found to contain protein, ribonucleic acid, lipid and a small fraction of carbohydrate; the nucleic acid was about 4·4 % of the preparations studied. There was a high lipid content (54 %) of which phospholipid was a major component (35 % of the whole virus); cholesterol and neutral fat were also present.

During a study of methods of purification of group B arthropod-borne viruses from infective baby mouse brains (Ada, Anderson & Abbot, 1961), 15 purified preparations of Murray Valley encephalitis (MVE) virus became available and were pooled. The results of electron-microscope examination of individual preparations and of chemical analyses of the pool are reported here.

METHODS

Purification of virus. Infected baby mouse brains were extracted with phosphate buffered saline and the extract treated with protamine sulphate. This suspension was clarified by brief low-speed centrifugation and the virus sedimented from the clear solution by high-speed centrifugation. The sedimented virus was resuspended, adsorbed on to, and then eluted from, a column of hydroxyl apatite. In the final step the virus in the eluate was sedimented by high speed centrifugation and resuspended in a small portion of the overlying fluid. Thus from an original volume of 90 ml. (60 brains), the virus was obtained in a final volume of about 0·7 ml. In those batches

where it could be estimated, the recovery of haemagglutinin was about 50 %. In contrast, the recovery of infectivity averaged only 15 %; the major loss occurred during the last centrifugation step (Ada *et al.* 1961).

Electron microscopy

Preparation of sample. The virus purified as above was obtained as a suspension in buffered saline (potassium phosphate, 0·25 M; sodium bicarbonate, 0·025 M; cystine, 0·85 mM; sodium chloride, 0·15 M; pH 7·7). Immediately after preparation 0·05 ml. of the virus preparation was applied to a column (4·0 cm. × 0·3 cm.) of Sephadex G75 (a cross-linked dextran gel, water regain 7·7 g./g. dry gel; from Pharmacia, Sweden) which had previously been equilibrated with ammonium carbonate (10 g./l.) solution; separate drops of effluent were collected. The first 6 drops of effluent (about 0·35 ml.) contained 90 % of the ultraviolet (u.v.) absorbing material (260 mμ) applied to the column; of this amount drops 3 and 4 (0·13 ml.) contained 65% of the u.v. absorbing material; these were together used for spraying. The gel filtration was carried out at 0–4°, took about 10 min. and yielded a sample of virus in a solution of a volatile salt.

Spraying of virus. The apparatus designed for spraying the infective virus preparation consisted of a desiccator (about 30 cm. high and 25 cm. diameter). The side arm of the spray gun (Backus & Williams, 1950) passed through a stopper and was connected by pressure tubing to a cylinder of medical-grade compressed air. The gauge valve of this cylinder was pre-set to give a pressure of 10 lb./in.^{2} on opening the cylinder valve. Glass tubing fitted with a stop-cock also passed through the stopper and was connected by pressure tubing to a water pump and a mercury manometer. Several carbon-coated electron microscope grids were mounted on the floor of the desiccator directly beneath the spray gun aperture. The distance between gun aperture and grids was usually 12 cm. but this did not appear to be critical. Immediately before final assembly a small beaker containing 10 ml. of a formaldehyde (400 g./l.) solution was taped to the inside wall of the desiccator.

The filtered virus (0·05 ml.) was mixed with an equal volume of a solution containing latex particles (Dow polystyrene latex, run no. L.S. 0·040 A, particle diameter 0·088$\mu \pm$ 0·008 S.D.) and bovine plasma albumin (BPA Armour Laboratories, Cohn fraction V) so that the final 0·1 ml. of solution contained $1{\cdot}5 \times 10^{10}$ latex particles and 0·17 mg. BPA. A sample (about 2μl.) of this solution was loaded by a finely drawn Pasteur pipette into the tip of the gun and the stoppers slipped over the side arm of the gun. This assembly was then placed in the neck of the desiccator and the tubing connexions completed. The desiccator was evacuated to about 40 mm. Hg, the stop-cock closed and the pumping line disconnected. The cylinder valve was then opened for about 2 sec. which was sufficient time for the entire sample to be sprayed. The cylinder valve was closed after 2 sec. and a clamp tightened on the pressure line. The apparatus was left undisturbed for 16 hr., by which time about half the formalin had evaporated. The apparatus was then dismantled, the gun was disinfected in boiling water and the grids removed for examination. Previous experience showed that even after 4 sec. spraying time there was still a pressure of 25 mm. Hg in the desiccator. The apparatus was tested before each spraying by carrying out the procedure with an empty gun.

Examination of droplets

Particle counts. The grids were examined in an electron microscope (Siemens type E4) without shadowing. Pictures of complete droplets were taken at magnifications of × 7000 and photographically enlarged (× 3·5) for counting particles. At least 4000 virus particles/sample were counted. The average ratio of numbers of virus particles to latex particles was 10:1.

Particle size. The electron microscope was calibrated by using Dow Polystyrene Latex, run no. 580G. The particles in this had been found (Farrant & Hodge, quoted by Gerould, 1950) to have a diameter of 256 ± 2·5 mμ; Dow latex, run no. IS040A was found to have particles of diameter 88 mμ. The virus particle size was estimated by using a microscope graticule (0·1 mm. divisions) to measure particles on prints which had been photographically enlarged (× 5).

Titration of virus infectivity. The standard method of titration was to inoculate 0·05 ml. volumes of decreasing ten-fold dilutions of virus either on the chorio-allantoic membrane of 12-day-old chick embryos (6 embryos/dilution), or into the peritoneal cavity of 5–8-day mice, with 12 mice/dilution. The diluent for virus was 10% (v/v) unheated Seitz-filtered normal rabbit serum diluted in physiological saline. Infectivity end-points were determined as 50% lethal endpoints at 4 days in chick embryos; the mice were observed during 12 days.

Chemical analysis. To the remaining purified virus was added 1/3 volume of trichloroacetic acid (400 g./l.) and the suspension allowed to stand at 0–4° for 5–10 min. It was then centrifuged (2000 ***g*** for 10 min.), and the sediment resuspended in a small volume of distilled water. The suspension at this stage contained no infective virus; it was stored at −20°. The frozen suspensions from 15 different preparations were pooled and freeze-dried *in vacuo*. The dried virus was transferred to a weighing bottle and dried further over P_2O_5 in a vacuum desiccator; it weighed 12·9 mg.

Extraction and estimation of lipid. Dried virus was extracted thrice at room temperature with a mixture of chloroform + ethanol (2 + 1 by vol.). The extract was evaporated to dryness under nitrogen and the residue extracted with diethyl ether. The ethereal solution was removed by decantation and the insoluble material added to the main virus residue. After evaporation of the samples to dryness under nitrogen, the ether-soluble material was taken up in a known volume of chloroform from which samples were taken for phosphorus, cholesterol and sugar determinations.

Phosphorus was estimated as described previously (Collins, 1959). Cholesterol was determined by the micromethod of Caraway & Fanger (1955). (We are grateful to Dr J. MacMillan for the use of his Beckman Spinco ultramicro colorimeter.) Samples of the lipid (45μg.) were digested with 0·5 ml. acid (0·25 N-HCl; 16 hr.; 100°) and the digest evaporated to dryness in a desiccator containing caustic soda. The content of reducing sugar was then estimated by the technique of Park & Johnson (1949). Galactose (3μg.) added to a sample of virus lipid before digestion was recovered in this test.

Extraction and analysis of nucleic acid. The nucleic acid was isolated from the defatted virus by three extractions (30 min., 100°) with NaCl (100 g./l.) solution. A sample of the extract was kept for phosphorus estimation; 2 vol. ethanol were added to the remainder (0°; 16 hr.). The precipitated nucleic acid was washed

with 66 (v.) ethanol in water, 95 (v.) ethanol in water and then with diethyl ether.

The nucleic acid content of the 10 % NaCl extract was estimated in two ways: (i) by direct determination of the phosphorus content, assuming this to be all nucleic acid phosphorus; (ii) by determination of the specific absorption at 260 mμ. The formula $Ep = 30{\cdot}98\ E/cl$, where Ep = atomic extinction coefficient at 260 mμ with respect to phosphorus, E = optical density, c = concentration of phosphorus in g./litre and l = thickness of the absorbing layer (Chargaff & Zamenhof, 1948) was used to determine spectrophotometrically the nucleic acid content. A value of 9200 for Ep was assumed (Ada & Perry, 1956). A factor of 10 was assumed for the conversion of the phosphorus value to nucleic acid.

The viral nucleic acid was hydrolysed (M-HCl, 100°, 60 min.) the digest chromatographed and the purine and pyrimidine derivatives detected and estimated. Details of these procedures were given previously (Ada & Perry, 1956).

The virus residue remaining after extraction with hot NaCl solution was washed thrice with distilled water, digested (M-HCl, 100°, 60 min.) and the digest dialysed (24 hr.; 0°) against 3 vol. distilled water. (We are indebted to Dr A. Szenberg for suggesting this procedure.) The content of purine and pyrimidine derivatives in the diffusate was estimated by determining the absorption at 260, 280 and 300 mμ. These values were compared with those given by artificial mixtures of protein and nucleic acid.

RESULTS

The number of particles/infective dose. In five experiments the ratio virus particles/chick embryo infective dose was 90 ± 50 (range 32–160). It may be noted that in these experiments the concentration of virus in the Sephadex column effluent was 25 % as judged by u.v. absorption (260 mμ) and 51 ± 26 % as judged by infectivity titrations of the concentration of virus in deposit 2. In a sixth experiment the infectivity titre of the virus decreased from $6 + 10^{10}$ in deposit 2 to 4×10^8 in the Sephadex sample. In this experiment the ratio particle count/chick embryo infective dose was unduly high at 980. On one occasion the stability of a Sephadex effluent was tested. When kept at 20° for 30 min., the infectivity titre decreased from 6×10^9 to 1×10^9. The corresponding ratios for particle count/chick embryo infective dose were 74 and 290. In three experiments, Sephadex samples were titrated in eggs and in mice. The mouse titre was equal to the egg titre in one experiment, was three times higher in the second experiment and 2·5 times higher in the third experiment.

Virus particle size. Counts were made of the number of virus particles having diameters of 25, 27·5, 30, 32·5 or 35 mμ. Of 378 particles examined: 223 had diameter 25 mμ, 100 diameter 27·5 mμ, 28 diameter 30 mμ, 6 diameter 32·5 mμ, 11 diameter 35 mμ.

Chemical analysis of the virus preparation. The virus obtained from 15 preparations when pooled and dried weighed 12·9 mg. After extraction of lipid, the residual weight was 11·5 mg. and the extracted lipid weighed 1·4 mg. Thus the lipid content was 10·9 % of the total weight. The extracted lipid was estimated from phosphorus analysis to contain 0·10 mg. phospholipid which corresponds to 0·8 % of the virus preparation. Cholesterol was present to the extent of 1·0 % of the virus preparation. The content of reducing substance in an acid digest of virus lipid (45 μg.) was

equivalent to 2 μg. galactose; if assumed to be galactose and this assumed to derive from cerebroside, then the cerebroside content of the virus lipid would amount to a maximum value of 2·2 % of the total virus.

Nucleic acid was estimated in two ways which gave similar results. As estimated by u.v. absorption the preparation contained 7·8 % nucleic acid of which 92 % (920 μg.) was present in the extract made with 10 g./l. sodium chloride solution. The sodium chloride extract contained 2·93 μM phosphorus; this would correspond to about 910 μg. nucleic acid. Paper chromatography of an acid digest of the nucleic acid in the sodium chloride extract showed four components, corresponding in R_F values to adenine, guanine, cytidylic acid and uridylic acid; no component corresponding in R_F value to thymidylic acid was observed. The components were present in the following proportions: adenylic acid, 25·5; guanylic acid, 27·5; cytidylic acid, 21·5 and uridylic acid, 25·5.

DISCUSSION

When the infectivity of purified Murray Valley encephalitis virus (deposit 2) was titrated in chick embryos, the average infective dose contained about 100 virus particles. There was some evidence that mouse infectivity titration would have yielded a lower figure perhaps 40 virus particles/mouse infective dose. Previous work had indicated that there was a greater decrease in infectivity than in haemagglutinin titre or in u.v. absorption during the last stage of purification; the relative change was about four-fold. Thus, perhaps there were 10 virus particles/mouse infective dose in the original brain extract. This recalls the figure of 10 virus particles/chick embryo infective dose for many strains of influenza virus (Donald & Isaacs, 1954). The distribution curve of virus particle size was very skew. One possibility is that the undamaged particles had a narrow distribution of size and were about 25 mμ diameter. Particles with a greater diameter may be damaged and flattened.

No confidence can be placed on the results of chemical analysis of a virus preparation unless the purity of the preparation has been demonstrated; this requirement has here been met only partially. The main evidence of purity was the fact that the electron microscope picture of deposit 2 (Ada *et al.* 1961) showed little but virus particles. Because of the inadequacy of this evidence and the fact that the results were from a single chemical analysis, the values obtained must be regarded as preliminary. However, if the results are taken at their face value, three comments are worth making. Earlier work showed that phospholipase A destroyed viral infectivity (Anderson & Ada, 1961); this was believed to mean that intact phospholipid was necessary for the viability of this virus. The present findings show that the virus particles contained only a small amount of lipid, particularly of phospholipid, in an amount which might all be carried on the viral surface. Phospholipid, cholesterol and cerebroside account for a maximum of 38 % of the viral lipid. If it be assumed that lipid components (particularly phospholipid phosphorus) have not been lost from the virus during isolation, it seems possible that the remaining lipid is a triglyceride. The relative proportions of lipid, phospholipid and cholesterol in the virus preparation (10:0·7:0·9) are different from those in 7-day mouse brain (10:7·1:1·2; Folch-Pi, 1955). In contrast the ratio of phospholipid:cholesterol: triglyceride in influenza virus is similar to that found in the chick embryo chorio-

allantoic membrane (Frommhagen, Freeman & Knight, 1958). It would be of interest to know the composition of Murray Valley encephalitis virus derived from other hosts. With this idea in mind, virus grown on chick embryo chorioallantoic membrane was treated with phospholipase A and found to be fully inactivated (Anderson & Ada, unpublished).

The failure to find thymidylic acid indicated that the viral nucleic acid was largely if not entirely RNA. This agrees with the earlier finding (Anderson & Ada, 1959) that pancreatic ribonuclease destroyed the infectivity of Murray Valley encephalitis virus nucleic acid. Lack of material prevented an attempt to identify the sugar component of this nucleic acid. As might be expected, the base ratio found for the viral nucleic acid differed from that of mammalian cell RNA. It is not yet known whether the Murray Valley encephalitis virus contains carbohydrate other than that associated with the nucleic acid or lipid fractions.

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Decarboxylase and other Reactions of some Gram-negative Rods

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SUMMARY

Tests for decarboxylation of amino acids, oxidation of gluconate, growth in potassium cyanide broth, utilization of malonate and deamination of phenylalanine were applied to 269 strains of aerobic Gram-negative rods, other than members of the Enterobacteriaceae. The results indicated that some of these tests might be of value in the identification of organisms outside this family. The decarboxylase and KCN tests permitted separation of *Pasteurella septica* from *P. pestis* and *P. pseudotuberculosis*. Phenylalanine deaminase was detected in only one of the strains tested. Possible mis-classification of *Pseudomonas maltophilia* and of the glanders bacillus was suggested.

INTRODUCTION

Haynes (1951) used the oxidation of gluconate to ketogluconate as a means of characterizing *Pseudomonas aeruginosa*; a test based on this reaction has proved valuable within the Enterobacteriaceae (see Cowan, 1955; Shaw & Clarke, 1955). Tests for amino acid decarboxylase activity, utilization of malonate, deamination of phenylalanine and ability to grow in the presence of potassium cyanide are of value in characterizing members of this family, and it has been suggested (Sneath & Cowan, 1958) that genera such as *Pasteurella* and *Vibrio* should be included in surveys of the Enterobacteriaceae. These tests have now been applied to a selection of aerobic Gram-negative rods (other than enterobacteria); the results are reported here.

METHODS

Strains. A total of 269 strains maintained in the National Collection of Type Cultures was used.

Biochemical tests used were as follows.

Amino acid decarboxylase activity. By Møller's (1955) method, with incubation of the cultures for 4 days. Falkow's (1958) method was not used since, at least among the Enterobacteriaceae, the results do not always agree with Møller's method; Ewing (1960) regarded the latter as the standard reference method for use in taxonomy.

Gluconate oxidation. By the method of Shaw & Clarke (1955); cultures were tested for the presence of reducing substances after incubation for 2 and 7 days.

Growth in the presence of KCN. By the method described by Rogers & Taylor (1961), a modification of that of Møller (1954); incubation was for 2 days at 37° or 3 days at 30° or 22°.

Malonate utilization and phenylalanine deamination. By the combined medium of Shaw & Clarke (1955); cultures were examined after incubation for 1 and 7 days.

For the detection of phenylalanine deaminase in the Proteus and Providence groups, Ewing, Davis & Reavis (1957) found this combined medium to be less satisfactory than phenylalanine agar.

Cultures were incubated at 37° with the following exceptions: *Chromobacterium amethystinum*, *Pasteurella pestis*, *Pseudomonas chlororaphis*, *P. diminuta*, *P. fluorescens*, *P. graveolens*, *P. mucidolens*, *P. syncyanea*, *Vibrio metchnikovii*, *V. percolans* and *V. proteus* at 30°; *Chromobacterium lividum* at 22°.

RESULTS

The results are shown in Table 1. Because of their exacting nutritional requirements, strains of the genus *Haemophilus* were not included in this work. Huet (1959), however, reported the presence of lysine decarboxylase in some strains of *H. influenzae*. The two strains of *Bordetella pertussis* tested were in phase IV (Leslie & Gardner, 1931) and grew well on simple media. Media were not supplemented to ensure the growth of the more fastidious organisms. In a few instances a negative result may have been due to failure of the organism to grow under the conditions of the test; e.g., the three strains of *Moraxella lacunata* failed to grow in the basal medium used for the KCN test.

Decarboxylases

Attempt to distinguish the reactions whereby arginine is metabolized (Møller, 1955) were not made. A positive reaction is here referred to, for convenience, as indicating the presence of arginase; for our purpose it was immaterial whether an arginase, a decarboxylase or a dihydrolase system was involved. Arginase activity was pronounced in *Aeromonas* spp., *Chromobacterium violaceum*, *Loefflerella* spp. and most strains of *Pseudomonas*. Strains of *Brucella* spp. (except *B. ovis*) showed arginase activity after incubation for 6–10 days but are here recorded as negative.

Ornithine decarboxylase activity was found in most strains of *Pasteurella septica* and in some strains of *P. haemolytica* var. *haemolytica* but not in *P. haemolytica* var. *ureae*. Lysine decarboxylase activity was detected in *Pseudomonas maltophilia* and in the genus *Vibrio* where it was usually associated with ornithine decarboxylase; this is in agreement with the results of Ewing, Davis & Edwards (1960) and Hugh & Ryschenkow (1961*a*). With strains of *Alcaligenes* difficulty was encountered in reading the reactions, due to alkalinization of the controls; this effect was not found with other organisms which did not produce acid from glucose (e.g. *Bordetella bronchiseptica*). Ewing *et al.* (1960), who studied only 5 strains of *A. faecalis*, detected weak arginase activity in one of them.

Gluconate oxidation

The results of this test are liable to different interpretations by different workers. Shaw & Clarke (1955) stated 'a positive test showed a yellowish-brown precipitate of cuprous oxide and there was a characteristic smell of decaying cabbage'. Haynes (1951) after heating and rapid cooling of his reaction mixtures set them aside overnight and then compared the amount of precipitate with standards prepared from calcium ketogluconate. A worker familiar with the qualitative examination of urine for 'sugar' would record a green precipitate (indicating about 0·5% glucose) as +, and a yellowish one (about 1%) as ++. In the present work a

Table 1. *The reactions of some Gram-negative bacilli*

Organism	No. of strains tested	Number of positive reactions: Decarboxylases: Arginine	Lysine	Ornithine	Gluconate oxidation: At 2 days	At 7 days	Growth in KCN	Malonate utilization: At 1 day	At 7 days	Phenylalanine deaminase: At 1 day
Achromobacter anitratus	11	.	.	.	1w	1	9	.	2	.
A. equuli	7	.	.	.	.	.	1	.	.	.
Actinobacillus lignieresi	6	.	.	.	.	.	.	.	.	.
Aeromonas spp.	7	7	.	.	7	5	7	.	1w	.
Alcaligenes denitrificans	1	.	.	.	.	.	1	.	.	.
A. faecalis	5	1w	.	.	.	1	4	.	1w	1
A. viscosus	1	.	.	.	.	.	1	.	.	.
Bordetella bronchiseptica	11	.	.	.	.	.	11	.	.	.
B. parapertussis	3	.	.	.	.	.	.	.	.	.
B. pertussis (phase IV)	2	.	.	.	.	.	.	.	.	.
Brucella abortus	13	.	.	.	.	.	.	.	.	.
B. melitensis	7	.	.	.	.	.	.	.	.	.
B. neotomae	4	.	.	.	.	.	.	.	.	.
B. ovis	1	.	.	.	.	.	.	.	.	.
B. suis	7	.	.	.	.	4	.	.	.	.
Chromobacterium amethystinum	1	.	.	.	.	.	.	.	.	.
C. lividum	3	1	.	.	.	.	2v	.	.	.
C. typhi-flavum	1	.	.	.	.	1	1	.	1	.
C. violaceum	14	14	.	.	.	.	14	.	.	.
Flavobacterium meningosepticum	1	.	.	.	.	.	.	.	.	.
Loefflerella mallei	9	9	.	.	.	.	5	.	.	.
L. pseudomallei	9	9	.	.	.	.	9	.	1	.
Lophomonas alcaligenes	1	.	.	.	.	.	.	.	1	.
Moraxella bovis	3	.	.	.	.	.	.	.	.	.
M. lacunata	3	.	.	.	.	.	.	.	.	.
M. liquefaciens	1	.	.	.	.	.	.	.	.	.
M. lwoffii	3	.	.	.	.	.	.	.	.	.
Pasteurella haemolytica	4	.	.	2	.	.	3v	.	.	.
P. haemolytica var. *ureae*	4	.	.	.	.	.	.	.	.	.
P. pestis	10	.	.	.	.	.	.	.	.	.
P. pseudotuberculosis	20	.	.	.	.	.	.	.	.	.
P. septica	26	.	.	23	.	.	24v	.	.	.
Pseudomonas aeruginosa	14	14	.	.	14	14	14	3	14	.
P. chlororaphis	1	1	.	.	1	1	.	.	1	.
P. diminuta	1	.	.	.	.	.	.	.	.	.
P. fluorescens	4	4	.	.	4	4	2	.	3	.
P. graveolens	1	1	.	.	.	.	.	.	.	.
P. maltophilia	3	.	3	.	.	.	3	.	3	.
P. mucidolens	1	.	.	.	.	.	1	.	.	.
P. ovalis	2	2	.	.	1	1	.	1	2	.
P. polycolor	1	1	.	.	1	1	1	.	1	.
P. syncyanea	1	1	.	.	.	.	.	.	.	.
P. spp. (achromogenic)	5	5	.	.	.	.	3v	1	4	.
Vibrio alcaligenes	3	.	.	.	.	.	.	.	.	.
V. choleraeasiaticae	14	.	14	14	.	.	4	.	.	.
V. el Tor	9	.	9	9	3	2	7	.	.	.
V. metchnikovii	1	.	1	.	.	1	.	.	.	.
V. percolans	1	1w	.	.	.	.	.	.	.	.
V. proteus	2	.	2	.	.	.	.	.	.	.
Vibrio spp.	6	.	6	6	4	2	5	.	.	.

v = variable results. w = weak reaction. . = negative.

strain of *Klebsiella aerogenes* was used as a positive control organism; hence the results of positive tests are strong reactions, indicated by a yellow to dark brown precipitate after heating. Traces of green precipitate may have been due to excess phosphate and were ignored. Some tests which were recorded as negative showed, after standing overnight at room temperature, a small precipitate of cuprous oxide; this was most evident with *Loefflerella pseudomallei* but was also noted with the strain of *Lophomonas alcaligenes* and with some strains of *Achromobacter anitratus*.

Strong gluconate-oxidizing ability was shown by *Aeromonas* and most *Pseudomonas* strains. Strains of *P. aeruginosa* gave similar results when tested after 2 and 7 days, but two of the aeromonads were positive at 2 and negative at 7 days;

presumably the ketogluconate had undergone further metabolism. (Since opinions on the taxonomy of the genus *Aeromonas* are conflicting: see Eddy, 1960; Ewing, Hugh & Johnson, 1961, attempts to distinguish species within this genus have not been made). Occasional strains of *Achromobacter anitratus*, *Alcaligenes* and *Vibrio* spp. showed gluconate oxidation, as did the single available strain of *Chromobacterium typhi-flavum*. This latter organism has not yet been allotted to a suitable taxon; other strains received under this designation have all been re-characterized as *Enterobacter cloacae*. It is not a chromobacterium as defined by Sneath (1960), who reported the gluconate reaction of *C. lividum* as often positive and that of *C. violaceum* as doubtful or negative. Neither of these *Chromobacterium* species showed gluconate oxidation in our study, nor did *C. amethystinum* which Sneath (1960) regarded as a gelatinous psychrophilic chromobacterium; our strain will now grow at 37°. Three of 4 American strains and 1 of 3 Danish strains of *Brucella suis* appeared to be gluconate-positive, after incubation for 7 days. All strains which appeared to oxidize gluconate were tested in control medium without gluconate to show the absence of reducing substances from any capsular or slime material produced. False positives were not observed.

Growth in the presence of KCN

With the exception of *Moraxella lacunata*, *Brucella ovis* and a few other strains of *Brucella* spp., all the organisms tested grew in the basal medium without added cyanide. Strains of *Pasteurella septica* generally grew in the presence of KCN but some gave variable results in different tests. Mollaret (1961) reported the variability of *P. septica* in this test. Hugh & Ryschenkow (1961*a*) noted subspecies differences with the KCN test in their study of 152 strains of *Vibrio comma* (*V. choleraeasiaticae*). Our results indicated the El Tor variety to be less sensitive to the presence of cyanide. Four of five strains of *Alcaligenes faecalis* grew in KCN broth; Doxiadis, Pavlatou & Chryssostomidou (1960) reported 33 strains of 'Bacillus faecalis alcaligenes' to be KCN-negative. The KCN-sensitive strain (NCTC 1347) was considered by Galarneault & Leifson (1956) to be one of *Lophomonas alcaligenes*. Fey (1959) reported that 8 of 9 strains of *A. faecalis*, 3 strains of 'B. viscosum equi' and 9 strains of *Bordetella bronchiseptica* did not grow in KCN broth: in our hands, all strains of *B. bronchiseptica* and 1 of 7 strains of *Achromobacter equuli* (*Actinobacillus equuli* in *Bergey's Manual*, 1957) grew in the presence of KCN.

Utilization of malonate

Use of malonate after incubation for 24 hr. was shown only by some strains of *Pseudomonas*. After incubation for 7 days, strong positive reactions were given by many *Pseudomonas* spp., two strains of *Achromobacter anitratus* and the single strains of *Chromobacterium typhi-flavum* and *Lophomonas alcaligenes*; a few strains of other organisms gave weak reactions.

Many strains of *Achromobacter anitratus* caused some alkalinization of the medium but by comparison with a known positive (*Klebsiella aerogenes*) no difficulty was encountered in reading the results. Pigment production at the surface of the medium by pigmented pseudomonads caused some difficulty in determining the use of malonate; this was overcome by using an indicator-free medium and determining the alkalinity after incubation. The colour of *Chromobacterium violaceum* tended

to interfere and this was overcome in the same way. Our results confirmed Sneath (1960) who reported that chromobacteria rarely used malonate.

Mollaret (1961) reported 3 strains of *Pasteurella pseudotuberculosis* of serotype IV as malonate-positive in 48 hr. at 28° or 4–7 days at 18°; 301 strains of other serotypes were malonate-negative as were *P. pestis* and *P. septica*. These incubation temperatures were not used by us for *P. pseudotuberculosis* and use of malonate was not found in this genus.

Phenylalanine deaminase

The presence of phenylalanine deaminase is no longer considered to be a specific character of the genus *Proteus* although its occurrence in other genera is uncommon. Shaw & Clarke (1955) noted that weak reactions were obtained occasionally with strains of *Klebsiella aerogenes*, *Escherichia coli* and some 'paracolons'. The occurrence of weak reactions for phenylpyruvic acid in a minority of strains of *Aeromonas* spp. was noted by Eddy (1960), Ewing *et al.* (1961) and Lysenko (1961).

The single strain of *Alcaligenes faecalis* (NCTC 415) which gave a positive reaction after incubation for 24 hr. was that designated as 'type' by Winslow, Kligler & Rothberg (1919); phenylpyruvic acid was not detected after incubation for 48 hr. Phenylalanine deaminase activity was not found in any other genus tested in our work, after 1 or 7 days of incubation. The absence of phenylalanine deaminase activity has been reported for *Chromobacterium* (Sneath, 1960), *Pasteurella* (Henriksen & Jyssum, 1961; Mollaret, 1961), *Pseudomonas* (Lysenkc, 1961) and *Vibrio choleraeasiaticae* (Hugh & Ryschenkow, 1961*a*).

DISCUSSION

The *Achromobacter–Pseudomonas* group, at present poorly defined, is under investigation by many workers. The strains of *Achromobacter anitratus* studied by us were oxidase-negative non-motile Gram-negative rods or coccobacilli which oxidized glucose but did not reduce nitrate. Buttiaux (1961) separated 'B. anitratum' from *Achromobacter*; by using the arginine hydrolysis test of Sherris, Shoesmith, Parker & Breckon (1959) he found that 7 of 14 strains of 'B. anitratum' hydrolysed arginine but none of 11 strains of *Achromobacter*. Thornley (1960) devised a test for the anaerobic breakdown of arginine which permitted separation of *Achromobacter* and *Pseudomonas* strains. In our study, arginase activity was characteristic of the genus *Pseudomonas*, the exceptions being *P. diminuta*, *P. maltophilia* and *P. mucidolens*. Thornley (1960) noted that *P. diminuta* did not break down arginine in her test and suggested that the organism should be regarded as belonging to some other genus than *Pseudomonas*; Rhodes (1958) thought that *P. diminuta* might be more closely related to *Acetobacter* or *Vibrio*. The absence of lysine decarboxylase from the pseudomonads makes it necessary to consider whether *P. maltophilia* (Hugh & Ryschenkow, 1961*b*) should be included in this genus; Thibault (1961) would exclude it for this reason. The achromogenic pseudomonads tested here were not a homogeneous group in their reactions although all showed arginase activity, but in one strain it was weak and delayed. Buttiaux (1961) found arginine to be metabolized by only 11 of 16 non-pigmented strains of *Pseudomonas*.

Disagreement exists about the taxonomy of the genus *Pseudomonas*. Gaby (1955) considered *P. chlororaphis* and *P. putida* to be indistinguishable from *P. aeruginosa*

on the basis of biochemical and growth characters, whereas *P. fluorescens*, *P. fragi*, *P. graveolens*, *P. mildenbergii*, *P. mucidolens*, *P. ovalis* and *P. putrefaciens* differed primarily from *Ps. aeruginosa* in their optimum growth temperature; *P. mildenbergii* was further found to be indistinguishable from *P. ovalis* and *P. fluorescens* from *P. putrefaciens*. Sneath (1960) considered *P. syncyanea* to be closely related to *P. aeruginosa*, whereas Haynes (1961) reported that a strain (ATCC 9979, NCTC 9943) received as *P. syncyanea* corresponded to the erroneous description of *P. mildenbergii* in *Bergey's Manual* (1957) and was really *P. convexa*. The identity of the phytopathogen *P. polycolor* with *P. aeruginosa* was shown by Elrod & Braun (1942) and Hoff & Drake (1960). Rhodes (1959) considered that further subdivisions of soil- and water-inhabiting fluorescent pseudomonads were not justified beyond *P. fluorescens*. She was of the opinion that *P. convexa*, *P. mildenbergii* and 7 other species agreed well with her definition of *P. fluorescens* and that 9 other species, including *P. ovalis* and *P. syncyanea*, showed only minor differences from it. Lysenko (1961) considered that Rhodes' definition of *P. fluorescens* was too broad and that insufficient account had been taken of other species in the genus. Our results, on the basis of only four tests, cannot be expected to clarify the position, but the single strain of *P. polycolor* reacted in an identical manner with that of strains of *P. aeruginosa*, and some similarities between *P. chlororaphis*, *P. fluorescens* and *P. ovalis* were found.

The finding of ornithine decarboxylase activity in *Pasteurella septica* and the ability of this organism to grow in the presence of KCN are two further criteria for distinguishing it from *Pasteurella pestis* and *P. pseudotuberculosis*. From an analysis of characters with the aid of an electronic computer, Talbot & Sneath (1960) suggested that *Pasteurella septica* was distantly related to these two species, which were closely related to each other.

Leifson (1960) regarded *Vibrio percolans* as a *Lophomonas*; in our study it showed weak arginase activity unlike the type strain (NCTC 9991) of *Lophomonas alcaligenes* (Galarneault & Leifson, 1956). We have studied only the one strain of *L. alcaligenes* although Galarneault & Leifson regarded our strain NCTC 1347 of *Alcaligenes faecalis* as a strain of *L. alcaligenes*. We did not follow these authors in regarding *Vibrio alcaligenes* as synonymous with *L. alcaligenes*.

The presence of arginase activity in *Loefflerella pseudomallei* is in accordance with current opinions on its classification—as *Pseudomonas pseudomallei* in *Bergey's Manual* (1957). *Loefflerella mallei* also showed arginase activity; this organism is listed in *Bergey's Manual* as *Actinobacillus mallei*. Cowan & Steel (1961) noted that *L. mallei* oxidized glucose whereas *A. lignieresi* fermented it, a fundamental difference in their opinion. The reactions given by organisms now listed in *Bergey's Manual* (1957) as species of *Actinobacillus* (e.g. *A. equuli*, *A. lignieresi* and *A. mallei*) serve to indicate that this genus is taking over the function once fulfilled by 'Bacterium' as a convenient place for poorly studied Gram-negative rods.

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Note added in proof

With the exception of *Pseudomonas polycolor*, we did not study any *Pseudomonas* strains from plant sources. Dr B. P. Eddy of the Low Temperature Research Station, Cambridge, has informed us that some of the plant pathogenic pseudomonads he investigated were arginine dihydrolase negative; Dr A. M. Paton of the University of Aberdeen, in a personal communication, has stated that a large proportion of the pseudomonads found as saprophytes or pathogens on plants do not produce 2-ketogluconate from gluconate.

Davis & Park (1962) have reported that the generic name *Lophomonas* is invalid and they have proposed the new generic name *Comamonas* to replace it. The type strain *C. percolans* is maintained as NCTC 1937 and in our paper is called *Vibrio percolans*.

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The Metabolism of Iron-, Zinc- and Manganese-Deficient *Nocardia opaca*

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SUMMARY

The metabolism of iron-, zinc- and manganese-deficient cells of *N. opaca* was studied. Zinc-deficient organism showed inability to utilize reserve carbohydrate as compared with organism grown under normal conditions. Manganese deficiency caused a much decreased DNA synthesis. Iron deficient organism lacked catalase and the oxygen uptake with certain substrates (pyruvate, phenylacetate, *n*-dodecane) was markedly affected.

INTRODUCTION

In a previous paper (Webley, 1960) it was shown that iron-, zinc- and manganese were required for optimum growth of *Nocardia opaca* and that deficiency of manganese caused marked morphological changes in the organism, irrespective of the substrate on which it was grown. In the present work a study of organisms deficient in these trace elements revealed several differences in their chemical composition and metabolism as compared with organisms grown on complete medium.

METHODS

Organism. Nocardia opaca strain T 16 described previously (Webley, 1954) was used.

Production of metal-deficient organisms. Details about the treatment of glassware, and the composition and preparation of media for individual deficiencies were given in a previous paper (Webley, 1960). After growth no special precautions in the harvesting of the organisms were necessary.

Manometry. Carried out by the conventional Warburg techniques at 25°.

Fractionation of the cell material. The Mickle disintegrator (Mickle, 1948) was used to break organisms, in suspensions containing 10–20 mg. dry wt. organism/ml., with ballotini beads (no. 12) for 15–20 min. The Vibrogen cell mill (E. Bühler, Tübingen, Germany) was used when large volumes (100 ml.) of suspension were to be broken. After removal of the glass beads the broken preparations were centrifuged at 35,000*g* in a refrigerated centrifuge. The debris was washed twice with distilled water at 35,000*g* and proved, on examination with the electron microscope, to be principally cell-wall material with some amorphous material. The cell-free extracts and debris were used directly or were freeze-dried, as required.

Carbohydrate determinations. Usually descending chromatograms were run in butan-1-ol + acetic acid + water (4 + 1 + 5, by vol.) on Whatman no. 1 paper with aniline phthalate or benzidine + trichloroacetic acid (Bacon & Edelman, 1951) as a spray for revealing sugars. The anthrone method of Fairbairn (1953) was used for

the estimation of total carbohydrate. The results were calculated in terms of glucose although paper chromatography showed that other sugars (particularly arabinose) were present in the hydrolysates of whole organisms. Therefore the results can only be regarded as comparative. Estimations on the paper chromatograms were done as described by Duff & Eastwood (1950).

Lipid determinations. The method of Reichert (1944) was used; the organisms were treated with boiling methanol and then extracted with diethyl ether (after removal of methanol). An apparatus was adapted for extracting smaller amounts of material (equiv. 50 mg. dry wt.) than required in the original method; two extractions were made.

Nucleic acid determinations. The freeze-dried cell contents were separated into fractions containing RNA and DNA as described by Elson, Gustafson & Chargaff (1954). The sediment containing the DNA fraction was hydrolysed with 12 N-perchloric acid (Marshak & Vogel, 1951). The solution containing nucleotides derived from RNA was evaporated to dryness *in vacuo* at 20°, taken up in N-hydrochloric acid and hydrolysed at 100° for 1 hr. (Markham & Smith, 1951). The purine and pyrimidine bases obtained from DNA were chromatographed in two dimensions on Whatman no. 1 paper: (*a*) upwards in isopropanol + hydrochloric acid + water (Wyatt, 1951); (*b*) downwards in *n*-butanol + ammonia + water (MacNutt, 1952). The mixture of purine bases and pyrimidine nucleotides from the RNA was chromatographed upwards in isopropanol + hydrochloric acid + water. Bases and nucleotides were detected under u.v. radiation, eluted with 0·1 N-HCl and estimated spectrophotometrically, using extinction coefficients quoted by Markham & Smith (1951) and Wyatt (1951).

RESULTS

Chemical analysis of metal-deficient organisms

From Table 1 it can be seen that, whereas the total nitrogen contents of the complete and Mn deficient organisms were very similar, the Zn- and Fe-deficient cells had, respectively, lower and higher total nitrogen contents. The explanation of

Table 1. *Chemical analysis of* Nocardia opaca *grown in complete medium or in Fe-, Mn- or Zn-deficient medium*

N. opaca grown in 200 ml. complete medium or in Fe-, Mn- or Zn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 7 days and washed 3 times with double-distilled water and made up to known volume. A sample of the washed suspension was used for determination of total nitrogen, carbohydrate and lipid content. The figures given as % of the dry wt. of the cell suspension.

	Total nitrogen	Carbohydrate (as glucose)	Lipid
	mg./100 mg. dry wt. organism		
Complete	7·97	13·4	13·9
−Fe	11·90	4·5	—
−Mn	7·65	11·5	13·1
−Zn	5·17	39·3	10·60

these differences is more clearly understood on examination of the carbohydrate figures. These show that the Zn-deficient organisms had a much higher carbohydrate content than the complete and Mn-deficient organisms while the Fe-

deficient organisms were much lower in carbohydrate. Similarly, when grown on other substrates (sucrose, glycerol, phenylacetate, acetate), the Zn-deficient organisms always had the highest carbohydrate content. Irrespective of growth conditions, when organisms were hydrolysed for 4 hr. at 100°, paper chromatography showed glucose and arabinose in all organisms. The glucose spot was much more intense in the hydrolysate of the Zn-deficient organisms than in the others. The lipid content of the Zn-deficient organisms was slightly less than that of the complete or Mn-deficient organisms. The equipment used did not permit the production of sufficient Fe-deficient organisms for their lipid content to be determined.

Table 2. *Carbohydrate content of* Nocardia opaca *during growth on Zn-deficient medium*

N. opaca grown in 200 ml. complete medium, or Zn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Samples 5–10 ml. periodically removed aseptically and centrifuged. Organisms washed twice with distilled water and suspended in 2·0 ml. water. Carbohydrate analysis (anthrone reaction) on samples of suspension and supernatant fluid of original sample.

	Complete-medium organisms		Zn-deficient organisms	
Days	Residual glucose in medium (mg./ml.)	Carbohydrate in organism (mg./100 mg. dry wt.)	Residual glucose in medium (mg./ml.)	Carbohydrate in organism (mg./100 mg. dry wt.)
4	14·7	9·8	12·5	15·2
5	4·6	15·6	12·25	31·5
6	1·15	34·9	9·0	35·7
7	0·28	5·5	6·0	24·8
8	0 31	—	4·05	32·5
9	Nil	6·6	2·0	26·4
11	Nil	8·3	Nil	12·0

Effect of zinc deficiency on carbohydrate content of organisms

In view of the greater carbohydrate content of Zn-deficient organisms grown on all substrates tested, an experiment was set up to follow the carbohydrate content of the organisms during growth under these conditions. For this two 1 l. culture flasks (Jobling Cat. no. 1410) each containing 200 ml. of medium, one complete and the other Zn-deficient, were set up. At intervals 5–10 ml. samples were removed. The organisms were centrifuged, well washed and finally suspended in 2 ml. distilled water. A sample was removed from each suspension for determination of dry weight and for carbohydrate content (Table 2). In the complete-medium-grown organisms the carbohydrate content decreased rapidly after reaching its maximum, whereas in the Zn-deficient organisms it did not. The decrease in the carbohydrate content of the complete-medium organisms took place when the glucose had been almost exhausted. It appears that reserve carbohydrates are affected by zinc deficiency.

The polysaccharide material in the complete and Zn-deficient organisms was extracted by the procedure of Palmstierna (1956). The moisture content of a sample of wet organism was estimated and the necessary amounts of KOH and water added to give 300 g./l. solution of KOH. This was heated at 100° for 3 hr. After mixing with water, the solution was poured into ethanol and the precipitated crude glycogen dialysed in aqueous solution. The polysaccharide was recovered by pouring the

dialysed solution, after evaporation *in vacuo*, into ethanol. The crude polysaccharide was purified by reprecipitation from water with glacial acetic acid. The yield was 24 % of the dry weight of Zn-deficient organisms and 14 % of the dry weight of complete-medium grown organisms. Both specimens gave glucose, together with small amounts of mannose and arabinose, after hydrolysis and chromatography in the usual way. Paper chromatographic analyses of hydrolysates of the cell fractions were also carried out (Table 3). All the arabinose and most of the glucose was associated with the cell debris.

Table 3. *Paper chromatographic analysis of sugars in cell debris and cell-free extract from complete and Zn-deficient* Nocardia opaca

N. opaca grown in 200 ml. complete medium or Zn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 6 days, washed 3 times with double-distilled water. Suspensions (equiv. 10–20 mg. dry wt. ml.) disintegrated and centrifuged at 35,000 *g*. Cell debris and cell-free extract freeze-dried. Freeze-dried fractions hydrolysed with N-H_2SO_4 at 100° for 5 hr. in sealed tubes and analysed for sugars after chromatographic separation as described in Methods. The results are the average of two determinations.

	Organisms grown on			
	Complete-medium		Zn-deficient medium	
	Cell debris	Cell-free extract	Cell-debris	Cell-free extract
	Sugar (mg./100 mg. dry wt. preparation)			
Glucose	4·0 (8·2)*	7·6 (5·2)	21·7 (14·4)	5·7 (5·0)
Arabinose	12·6 (10·1)	Nil (Nil)	12·3 (10·5)	Nil (Nil)
Mannose	Trace (2·4)	3·4 (4·1)	Trace (2·6)	1·6 (4·1)
Ribose	Nil (Nil)	0·6 (2·0)	Nil (Nil)	2·7 (3·8)

* The results of a second experiment with a different batch of organisms are given in parentheses.

Nature of the carbohydrate in the cell-free extract. The crude polysaccharide from Zn-deficient organisms was fractionated by the Cetavlon procedure of German, Jones & Nadarajah (1961). From the major fractions at pH 7·5 and 9·5. Cetavlon was removed with 2 N acetic acid and the recovered glycogen reprecipitated from aqueous solution with glacial acetic acid. Both samples gave infrared absorption peaks at 930, 845 and 758 mμ, as obtained for glycogen by the above authors. Hydrolysis with N-H_2SO_4 at 100° for 5 hr. and subsequent paper chromatography showed that glucose was the main constituent, accompanied by a small amount of ribose (from nucleic acid), and mannose, in the pH 7·5 and 9·5 fractions, respectively.

The fractions were combined for optical rotation determination, the mixture had $[\alpha]_D + 165°$ (conc. 5 g./l. of aqueous solution). The glycogen isolated gave a brown colour with iodine, having a maximum u.v. absorption at 360 mμ, E360 1·07 (an appropriate value due to light scattering). In these experiments we used a 8g./l. aqueous solution of glycogen (1·56 ml.)+ 1·60 ml. saturated ammonium sulphate+ 0·08 ml. potassium trio-idide solution (1·25 g./l. I in 2·5 g./l. KL), the conditions are comparable to those used by Schlamowitz (1951).

Effect of trace element deficiency on RNA and DNA content of Nocardia opaca

Webley (1960) showed that Mn deficiency caused marked morphological effects during the growth of *Nocardia opaca*. The organisms did not fragment in the usual way but continued to grow in the mycelial phase. As the cultures became older, bulbous swellings frequently appeared on the filaments. Amino acid analysis (DeKock & Morrison, 1958) of cell debris from complete, Zn- and Mn-deficient organisms showed the following: aspartic acid, glutamic acid, serine, α-alanine, valine, leucine, glucosamine, diaminopimelic acid, muramic acid. Muramic acid has been associated only with bacterial cell walls (Strange, 1956; Work, 1957). The acid was present in the cell debris from complete, Zn- and Mn-deficient organisms in the ratio 1·4:1·3:1·0, respectively.

Table 4. *RNA compositions of freeze-dried cell-free extracts from* Nocardia opaca *grown in complete medium or Zn- or Mn-deficient medium*

N. opaca grown in 200 ml. complete medium or Zn- or Mn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 6 days and washed 3 times with double-distilled water. Cell suspensions (10–20 mg./dry wt. ml.) disintegrated and centrifuged at 35,000 *g*. Cell-free extract freeze-dried. Purine bases and pyrimidine nucleotides obtained from the RNA fraction and estimated as described in Methods.

	Organisms from media		
	Complete	Zn-deficient	Mn-deficient
	Base proportions (moles %)		
Guanine (G)	33·0*	32·1	30·1
Adenine (A)	23·0	23·8	22·2
Cystòsine (C)	23·8	24·6	25·4
Uracil (U)	20·2	19·6	22·2
Purines/pyrimidines	1·27	1·26	1·10
G+U/A+C	1·14	1·07	1·10

* Values are the means of at least two chromatographic analyses of hydrolysis products.

Table 5. *DNA composition of freeze-dried cell-free extract from* Nocardia opaca *grown in complete medium or in Zn-deficient medium*

N. opaca grown in 200 ml. complete medium or in Zn- or Mn-deficient medium with 20 g. glucose/l. on a shaker at 25°. Organisms harvested after 6 days and washed 3 times with double-distilled water. Suspensions (equiv. 10–20 mg./ml. dry wt.) disintegrated and centrifuged at 35,000 *g*. Cell-free extract freeze-dried. Purine and pyrimidine bases obtained from DNA fraction as described in Methods.

	Organisms from media		
	Complete	Zn-deficient	Mn-deficient
	Base proportions (moles %)		
Guanine (G)	33·5*	36·4	35·0
Adenine (A)	19·7	19·5	20·6
Cytosine (C)	30·4	26·7	35·0
Thymine (T)	16·4	17·5	9·3
Purines/pyrimidines	1·14	1·26	1·26
A+T/G+C	0·56	0·59	0·43

* Values the means of two chromatographic analyses of hydrolysis products.

Hydrolysis of the cell debris with perchloric acid gave only traces of purine and pyrimidine bases on paper chromatograms (see Table 3), no ribose was contained in this fraction.

The relative proportions of the bases in the RNA and DNA fractions from freeze-dried cell-free extract are shown in Tables 4 and 5. The composition of the RNA was similar for the differently grown organisms. The DNA was similar in the complete and Zn-deficient organisms but the recovery of pyrimidines appears to be slightly low in both cases. The amounts of bases in the Mn-deficient organisms were so low that only approximate values could be obtained. To confirm the deficiency of thymine relative to adenine in the DNA, large-scale fractionation and isolation would be necessary. The nucleic acid contents of the cell-free extracts are given in Table 6. The absolute amounts of DNA and the ratio of DNA to RNA were lower in the Mn-deficient organisms.

Table 6. *Nucleic acid content of cell-free extracts from* Nocardia opaca *grown in complete medium or Zn- or Mn-deficient medium*

Values calculated from analysis of the amounts of bases and nucleotides obtained by hydrolysis of DNA and RNA fractions.

	Organisms grown in media		
	Complete	Zn-deficient	Mn-deficient
	mg./100 mg. freeze-dried preparation		
DNA	3·41 (2·97)	1·96	1·43 (1·09)
RNA	3·46 (1·98)	1·97	2·45 (4·75)
DNA/RNA	0·99 (1·50)	0·99	0·58 (0·23)

The results for a second batch of organisms given in parentheses. The second Mn-deficient batch was harvested after 10 days, the others after 6 days.

Warburg experiments with trace element deficient organisms of Nocardia opaca

Catalase activity. Table 7 shows that Fe-deficient organisms lacked catalase activity. A similar result was reported for *Aerobacter aerogenes* by Waring & Werkman (1944).

Oxidation of added substrates. Table 8 shows that the oxidation of glucose by Fe-deficient organisms was not nearly so much affected as was that of other substrates. A similar result was obtained by Waring & Werkman (1944) with iron-deficient *Aerobacter aerogenes*. The marked effect on pyruvic oxidation suggested that this acid might accumulate in iron-deficient cultures. Iron-deficient growth experiments were set up with glucose, gluconate, sucrose or glycerol as substrates. After growth for 7 days a strong reaction for pyruvic acid was obtained with the Rothera test when applied to the supernatant fluid after removal of organisms by centrifugation. Similar tests on the supernatant fluids from complete, Zn- and Mn-deficient cultures gave negative results. Further confirmation of the presence of pyruvic acid in Fe-deficient cultures was obtained as follows. 2:4-Dinitrophenylhydrazine in 2 N-HCl was added to a suitable sample of the supernatant fluid from an Fe-deficient culture in which glucose was the substrate. The precipitated hydrazone, when examined by infrared analysis, was identical with the hydrazone prepared from pure pyruvic acid. No similar pyruvic acid derivative was obtained

from corresponding supernatant fluids of organisms grown on complete medium, Zn- or Mn-deficient media.

The oxygen uptakes of Zn- or Mn-deficient organisms in the presence of the substrates used by complete and Fe-deficient organisms (Table 8) were generally inferior to those of complete organisms. But the results were too variable to draw conclusion about effects on specific substrates. Addition of the respective trace elements to the Warburg vessels in a form and concentration similar to those used for the complete medium in the growth experiments did not affect the oxygen uptake.

Table 7. *Catalase activity of trace element-deficient* Nocardia opaca

Organisms grown in complete medium or in Fe-, Mn- or Zn-deficient medium containing 20 g. glucose/l. Harvested washed, and suspended in double-distilled water (equiv. 10 mg. dry wt./ml.). In main compartment of Warburg vessel 0·1 ml. suspension +0·5 ml. M/15 buffer (pH 7·2) and distilled water to give 2·5 ml. In side arm 0·2 ml. H_2O_2 (3 g./l.). H_2O_2 tipped after 15 min. equilibrium time.

Medium	O_2 output in 3 min. (μl.)
Complete	57·0
Fe-deficient	2·1
Mn-deficient	59·0
Zn-deficient	54·0

Table 8. *Oxidation of various substrates by Fe-deficient* Nocardia opaca

N. opaca grown in complete medium or in Fe-deficient medium containing 20 g. glucose/l. After 7 days cells harvested and washed twice and suspended in double-distilled water to contain equiv. 10–12 mg. dry wt. organism/ml. In Warburg vessels 1·0 ml. suspension +0·5 ml. M/15 buffer (pH 7·2) +0·5 ml. M/50 substrate and distilled water to give 2·5 ml.

	Organisms grown on	
	Complete medium	Fe-deficient medium
	Q_{O_2}-Endogenous*	
Glucose	26·9	18·1
Gluconate	14·3	6·1
Butyrate	34·4	4·5
Phenylacetate	10·9	3·2
Pyruvate	11·5	1·1
n-Dodecane	26·3	Nil

* Endogenous Q_{O_2} was 9·12 and 5·3 for complete medium grown organisms and Fe-deficient medium grown organisms, respectively.

DISCUSSION

The results recorded in Table 2 suggest that Zn-deficient *Nocardia opaca* is unable to utilize its reserve polysaccharide as rapidly as organisms grown in the complete medium. Stein & Fischer (1960) showed that crystalline α-amylase from *Bacillus subtilis* is a zinc protein complex. Perhaps the polysaccharide splitting enzyme in *N. opaca* is also dependent on zinc.

The effect of manganese deficiency on DNA synthesis by *Nocardia opaca* is

interesting in the light of previous work (Webley, 1960) on the morphology of this organism when grown in Mn-deficient medium. It is well established that in normal growth the DNA content increases before cell division (Belozersky & Spirin, 1960). Jeener & Jeener (1952) showed that the deoxyribonucleoside-dependent *Thermobacterium acidophilis* R 26, when cultured in the absence of DNA, produced elongated cells which did not divide. In the light of these observations it is reasonable to suggest that when *N. opaca* is cultured under conditions of Mn deficiency the production of filamentous forms is caused by failure of normal DNA synthesis.

The presence of pyruvic acid in Fe-deficient cultures suggests that the pyruvic oxidase of the organism may be iron dependent. O'Kane (1954) showed that the pyruvate oxidase of *Clostridium butyricum* required ferrous ion.

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