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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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, spelt according to the *Shorter Oxford English Dictionary*.

(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 as results come to hand. It is better to wait until a comprehensive paper can be written.

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this is necessary, and not to recapitulation. Typescripts should also carry four key words for index purposes.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.) as well as attempting to convey their meaning to their readers.

(6) Editors do not alter typescripts except to increase clarity and conciseness. If an editorial alteration changes an author's meaning one implication is that it was expressed ambiguously. When an editor can grasp the meaning of a sentence unequivocally it may be assumed that anyone can.

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A manuscript should be submitted in double-spaced typing with wide margins, and on paper suitable for ink corrections. The paper must be written in English and should, in general, be divided into (*a*) Summary; (*b*) Introduction; (*c*) Methods; (*d*) Results; (*e*) Discussion (if any) and general conclusions; (*f*) Acknowledgements; (*g*) References.

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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). Where more than one paper by the same author(s) has appeared in one year the references should be distinguished in the text and

the bibliography by the letters *a*, *b*, etc., following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

References at the end of the paper should be given in alphabetical order according to the name of the first author of each publication, and should include the title of the paper as well as both initial and final page numbers. Titles of journals, books, reports and monographs should be set out in full and not abbreviated. References to books should include year of publication, title, edition, town of publication and publisher, in that order. When the reference refers to a particular page or chapter in a book, this should be given after the edition.

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Chemical Formulae. These should be written as far as possible on one line. The chemical nomenclature adopted is that followed by the Chemical Society (*Journal of the Chemical Society* 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 *Journal of the Chemical Society* 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 its correct sense, i.e. g./100 g. of solution; otherwise '%(v/v)' and '%(w/v)' must be used when the figure is larger than 1 %.

Proprietary Substances and Materials. At first mention, the correct designation and the manufacturer's address should be given in the text.

Chemical Nomenclature. Follow the 1969 revision of *Policy of the Journal and Instructions to Authors*, The Biochemical Society, 7 Warwick Court, London, W.C.1.

Nomenclature of Enzymes. The system published in *Report of the Commission of Enzymes of the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s*., is used.

Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in the *Journal* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The correct name of the organisms, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in brackets when the name is first mentioned. Names of bacteria must conform with the Bacteriological Code and the opinions issued by the International Committee on Bacteriological Nomenclature. Names of algae and fungi must conform with the International Rules of Botanical Nomenclature. Names of protozoa must conform with the International Code of Zoological Nomenclature. Bacteriological Code, Iowa State College Press, Ames, Iowa, U.S.A. (1958); Botanical Code, International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952); Zoological Code, International Trust for Zoological Nomenclature, London (1961). One or two small changes have been made to these rules at later International Congresses.

The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

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

Latin Names. The species name is in italics (underlined once in typescript) and is used in full at first mention in each paragraph, but in subsequent mention with the name of the genus abbreviated, single letter abbreviations being used where they are not ambiguous. The genus name is in italic when the whole genus is referred to. When used trivially, genus names are in Roman (not underlined). Anglicized versions are not underlined and are used without capitals. Strain names or numbers are in small capitals (underlined twice in the typescript).

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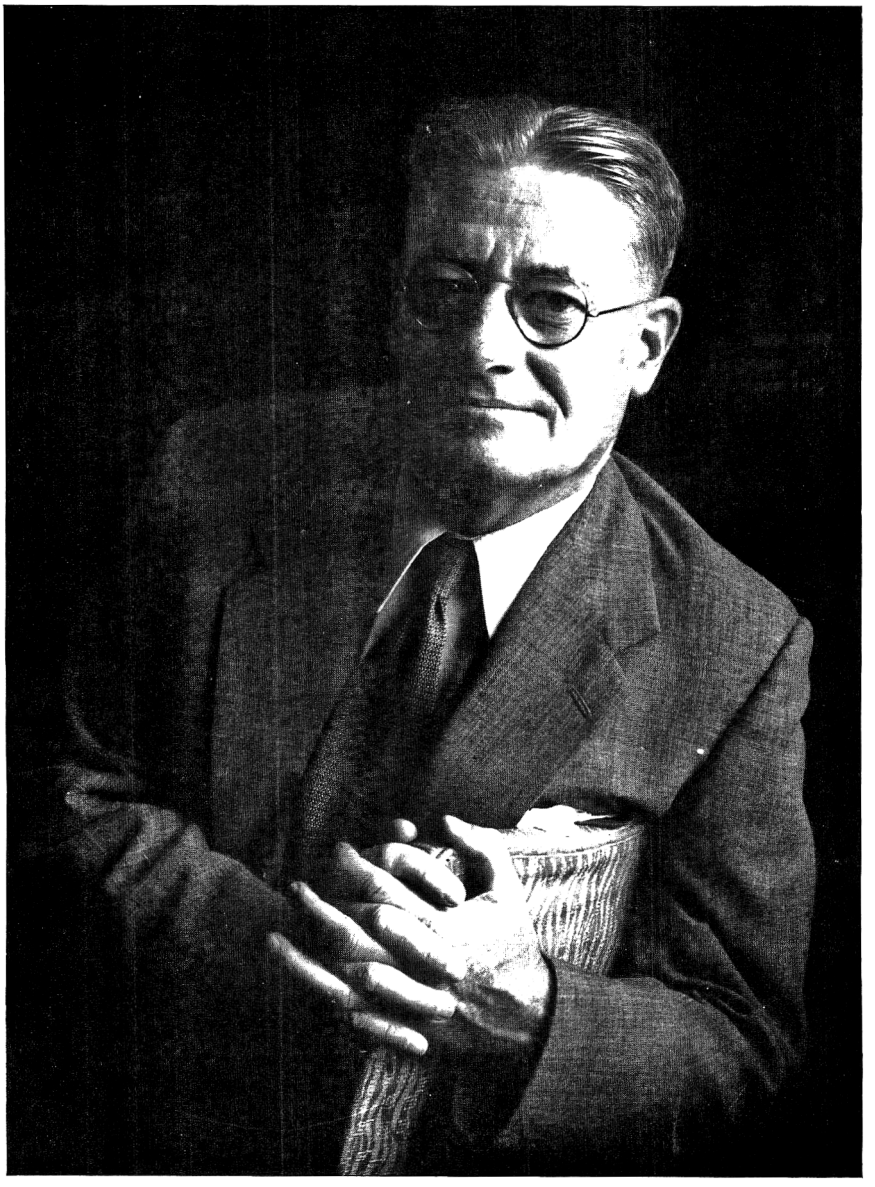

1958 *Photo by Dr William H. Feldman, The Mayo Clinic*

LORD FLOREY, O.M. M.B. PH.D. F.R.S.
1898–1968
Honorary member of the Society for General Microbiology

(*Facing p.* 289)

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In Memoriam, H. W. Florey: an Episode

With the sudden death of Lord Florey at the age of 69 on 21 February 1968 the Society lost a very distinguished original member. Among the lay public he is chiefly known for the work which led to the demonstration of the therapeutic powers of penicillin, 12 years after its discovery by Fleming. The 'golden age of antibiotics' thus initiated is reflected by the fact that whereas isolated papers on microbial antagonism had appeared scattered through the literature during the previous 50 years, no less than 15 % of the papers appearing in our journal during the first 5 years of its existence (1947–52) dealt wholly or largely with antibiotics.

Florey's achievements were, of course, great in other fields: his other scientific work, for example; or the training in experimental methods applied to broad pathological problems which so many young workers received in his department and disseminated all over the world; or his fruitful presidency of the Royal Society (1960–65); or the key part he played in the founding of the Australian National University and in particular the John Curtin School of Medical Research. These and other achievements have been chronicled elsewhere (e.g. E. P. Abraham, 1970) and it may be appropriate in a journal such as ours to pay respect to his memory by singling out his microbiological work, and in particular to attempt to describe in more intimate detail the work on penicillin carried out in his department at the beginning of World War II.

A carefully checked scientific account of this early work is to be found in chapter 15 of the monograph on antibiotics written by Florey and colleagues (Florey *et al.* 1949) and in some little-known but informative papers in volume 6 of the *Journal of the History of Medicine*, 1951. Naturally, no hint was permitted in any publication from Florey's department of the crucial part he played in encouraging and inspiring his colleagues, in directing the project so effectively and unobtrusively, or in the clear-cut elegance of his own laboratory work.

Florey's interest in naturally-occurring antibacterial substances goes back to the 1920s. In a joint paper with N. E. Goldsworthy (1930), a variety of bacteria were shown to produce diffusible substances which inhibited the growth of other bacteria, and the comment was made that the phenomenon was well known. The paper was concerned with the occurrence of lysozyme in the secretions and mucosa of the intestinal tract of various animals, and Florey continued to work on lysozyme, first with Dr Marjory Stephenson at Cambridge—very briefly—then with Mrs S. T. Harrison at Sheffield. When he moved to the Sir William Dunn School of Pathology at Oxford as professor in 1935 the work was pursued; lysozyme was purified by E. A. H. Roberts, and its substrate was characterized and mode of action investigated by E. B. Chain and L. A. Epstein.

Florey and Chain decided that it would be of interest to examine some of the other naturally-occurring antibacterial substances which had been described, including those formed by *Pseudomonas pyocyanea* (*aeruginosa*), *Bacillus brevis* and *Penicillium notatum* which, it so happened, was among the Dunn School cultures. A few preliminary experiments with the penicillium were made by Chain and Epstein in 1938/39 with results

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described by the latter as 'not impressive' (Falk, 1944), but in October 1939 more sustained work began when Florey engaged a full-time junior research worker, N. G. Heatley, to assist him personally. Heatley's first day's work, on 2 October 1939, was to drill holes in Petri dishes for the well type of assay described by Florey and Goldsworthy, which had the great merit that the solutions to be assayed did not need to be sterile. Heatley remained permanently at the Dunn School; his forte was improvization, a quality normally of little value to a research worker, though it proved useful under the wartime conditions of restriction and shortage.

In November 1939 Florey applied for and obtained from the Rockefeller Foundation a grant towards the investigation that he and Chain had planned.

During the winter of 1939/40 several aspects of the penicillin problem were explored simultaneously. The cylinder-plate diffusion assay method, developed from Florey and Goldsworthy's well-plate, provided a simple means of assay with which bacterial contamination of the test solution did not, within limits, interfere. Until an arbitrary standard was adopted in March 1941, potency was expressed simply in terms of the diameter of the zone of inhibition, though in certain experiments internal standards were set up and calibration curves were made. Methods of propagating the fungus were tested, and it was grown on stationary shallow layers of the modified Czapek-Dox medium (containing glucose, sodium nitrate and other salts) used by Clutterbuck, Lovell & Raistrick (1932). Correlations were sought between penicillin content of the medium and its pH and colour, and the colour, thickness, degree of corrugation and age of the mycelial mat. The characteristics of the penicillin-rich transpired droplets on the non-wetted upper surface of the mycelium were also recorded. The effect of different kinds of inocula and of variations in growth conditions such as temperature, age, surface/volume ratio, initial pH of medium and accessibility of air and carbon dioxide were semi-quantitatively examined, and various additions to the medium were tested, such as glutamine, glycerol, Marmite, peptone, malt extract, yeast extract, extra phosphate buffer, thioglycollic acid and meat extracts. Sabouraud's medium and one containing a peptic digest of horse or cow muscle, and the partial replacement of sodium by ammonium, or of glucose by sucrose or lactate, were also tried. No marked increase in penicillin yield followed from any of this or later work at Oxford; the one positive finding to emerge was the acceleration in rate of growth and penicillin production when a boiled extract of brewer's yeast was added (at the suggestion of Dr P. Fildes), such an extract being subsequently used as a routine.

It was found early in this phase of the work that, provided full aseptic precautions were taken, the medium harvested from under the mycelium could be replaced by fresh sterile medium and that about the same penicillin titre would be reached in two-thirds of the time needed by an *ab initio* culture. As many as 12 such serial replacements were done and much time was saved, both in the shortened incubation period and in the shorter time needed for sterilizing, cleaning and setting up of fresh cultures. The replacement technique was, however, abandoned about 2 years later when the frequency of contamination by a stubborn sporing air organism, despite regular use of a steam disinfectant-spray, made it unprofitable. None of the simplified media tried for replacement gave as good a yield as the Czapek-Dox + yeast-extract medium, though the concentration of the yeast extract could be decreased from 10 % (v/v) to 5 or even 2·5 % without appreciably lowering the yield.

Meanwhile, experiments on extraction and purification were being carried out,

mainly by Chain and by E. P. Abraham, who had joined the Dunn School in early January 1940. The crude culture fluid was concentrated by vacuum distillation and later by freeze-drying, and classical separation procedures were tried, with limited success. A good method which was used commercially for several years was derived from the observations of Clutterbuck *et al.* (1932) that penicillin was completely removed from a watery solution at pH 2 when shaken three times with ether, but was only partly removed at pH 7·2. No activity was recovered if the ether were evaporated alone, but if evaporated over water, the residual water contained a considerable proportion of the original activity. Thus penicillin passed more freely into ether at acid than at neutral pH. It is curious that what now seems a fairly obvious deduction—that penicillin should be back-extractable from ether into neutral buffer—had not been made by Clutterbuck *et al.* (1932) and had only been conceived with difficulty at Oxford; indeed, one member of the group considered the deduction unsound.

The resulting solvent transfer extraction process, in which penicillin was extracted from the cooled acidified crude culture fluid by shaking with ether, and then taken from the separated ether phase back into water at neutral pH, was not only simple but could be applied directly to unconcentrated culture fluid. It effectively freed penicillin from proteins, carbohydrates and salts, and enabled extracts to be simultaneously concentrated. There were, however, two minor difficulties: the first, loss of activity at low pH, could be minimized by cooling the solutions; the second was the formation of troublesome emulsions in the primary extraction, and this could be overcome by centrifuging. Other solvents could be used in place of ether, such as isopropyl ether, chloroform and amyl acetate; the latter was soon used exclusively as it was less volatile, less inflammable, and far less of it was lost in solution in the spent culture fluid. Another minor improvement was to use phosphoric acid, which buffered at pH 2 to 3, unlike the hydrochloric acid used originally.

The production of concentrates by solvent transfer from the end of March 1940 opened the way to experiments *in vivo* by Florey and M. A. Jennings. At about the same time, A. D. Gardner, with the help of J. Orr-Ewing, began a long series of bacteriological studies, confirming and extending Fleming's observations, using the increasingly purified material. He also gave advice and assistance in the selection of strains of the pathogens used later in the mouse-protection experiments. Gardner made the interesting observation that penicillin in sublethal doses profoundly affected bacterial morphology; cocci were grossly swollen and rods were elongated, sometimes up to ten times their normal length (Gardner, 1940).

The brown powder obtained by lyophil-drying the concentrate completely inhibited the growth of staphylococci at a concentration of less than 1 mg./l. This spectacular potency was taken to suggest near-purity of the material, but later work showed it to be less than 2 % pure.

The first parenteral administration to an animal was made on 19 March 1940, when Chain asked J. M. Barnes, who was at that time working on another project with mice, to give each of two mice an intraperitoneal injection of 40 mg. of a preparation in 1 ml. water; no ill effects were recorded (Stewart, 1965). This particular preparation was not made by solvent transfer, though practically all subsequent material was. During the next 6 weeks Florey made various pharmacological experiments. The preparation's low toxicity was confirmed by the tolerance of mice to a single intravenous injection of 10 mg., and of young rats to repeated subcutaneous doses. Intra-

venous injections into an anaesthetized cat had no effect on blood pressure or respiration. Human leucocytes remained actively motile in a 1 mg./ml. solution for at least 3 hr, and tissue cultures were unaffected. The activity of the material against staphylococci was not greatly dependent on inoculum size. Penicillin could be detected in the blood after subcutaneous injection and was rapidly excreted in the urine.

Would this highly active and apparently almost non-toxic antibacterial be effective *in vivo*? Florey started the first mouse protection experiment to examine this question on 25 May, a Saturday—an indication of the urgency he felt, since normally no one would dream of starting such an experiment at a week-end.

Eight mice were given a dose of 110 million mouse-virulent streptococci intraperitoneally. One hour later two were given a single subcutaneous dose of 10 mg. of penicillin and two were given four doses of 5 mg. at approximately 2 hr intervals, and a fifth similar dose after a further 4 hr.

All four control mice which had received no penicillin died between 13 and 16½ hr after infection, when the treated mice were all alive and well. The treated mice receiving the smaller dose died after 2 and 6 days, the other two surviving without sign of sickness. The unit for penicillin had not been adopted at that time, but the 25 mg. received by the surviving mice probably contained no more than a total of 200 units.

It is typical of Florey's drive that within 26 hr of the *beginning* of this experiment—long before it was completed—plans had been laid for increased production of penicillin by all means possible. The first requirement was vessels for growing the fungus in stationary culture on layers of medium 1 to 1½ cm. deep. The conical flasks available were wasteful of both incubator and autoclave space, and a variety of alternative containers were tried—bottles, trays, pie dishes, bed-pans, plain or varnished tins, etc. These were used for several months but it became clear that special culture vessels would have to be obtained, though in wartime Britain this presented difficulties. The obvious material for their construction, glass, was ruled out because the delivery time quoted was 6 months, and the cost of the mould, £500, was prohibitive. Ceramic slipware was then considered. It happened that Florey knew a consultant physician in Stoke-on-Trent, Dr J. P. Stock, to whom he wrote sending sketches of the kind of vessel required and asking which firms, if any, might be persuaded to produce them. Stock passed the sketches to James MacIntyre & Co., Ltd. of Burslem and informed Florey by telegram. The following day Heatley went to Stoke, but his train was delayed and he was only able to visit the firm on the next day, 31 October. He found that they had already made three (unfired) prototypes which the modeller was able to trim with the knife to a finally selected design. Three finished vessels arrived in Oxford on 18 November, and the first main batch of 174 were ready, as promised, on 23 December. They were fetched by van that day, washed, charged and sterilized the next, and half of them were inoculated on Christmas Day 1940. These rectangular containers stacked well in autoclave and incubator. Each held 1 l. of medium in a layer 1·7 cm. deep, and hundreds of them were eventually obtained. They effectively solved the culture vessel problem.

The harvesting operation was next partly mechanized by a special trolley on which the rectangular vessels could be tilted so that their contents drained towards the spout. The medium was sucked out through a replaceable sterilized tube held in a 'changing pistol' which simultaneously delivered an excess of sterile filtered air into the vessel to take the place of the fluid removed. The vessels were then tilted backwards and 1 l. quantities of replacement medium were added from bottles or tins.

Extraction of the penicillin rapidly became a limiting factor in production. Initially, containers of culture fluid and solvent had been cooled in ice. With increase in scale they (and the operator) were cooled in the cold room. Discomfort led, after several unsuccessful attempts, to a workable but intricate extraction apparatus which could be used at room temperature.

The crude culture fluid, cooled in a worm surrounded by ice, was acidified with phosphoric acid immediately before being broken up into uniform droplets which fell slowly through a column of amyl acetate, giving up their penicillin to it, and passing to waste. The solvent slowly moved in a counter-current direction, penicillin-rich solvent being collected from the top of the column while fresh solvent was fed in at the bottom. Emulsion formation did not occur, but a gummy material collected at the bottom of the column and had to be removed periodically. Six such columns were mounted, with a warning bell, indicator lights, etc., on a stand made from a bookcase discarded from the Bodleian Library.

Its capacity was 12 l. of fluid/hr, and it ran from January 1941 till it was superseded in the spring of 1942 by a much easier and more reliable system devised by Florey in which the cooled acidified culture fluid was deliberately emulsified with amyl acetate by means of a hand-operated disc-paddle, and the emulsion was then broken by passing it through a Sharples centrifuge. This principle was extended by A. G. Sanders, who designed, and with J. Kent built, two successive plants. The penicillin-rich amyl acetate leaving the Sharples centrifuge was collected and emulsified with a smaller volume of water to which sufficient sodium or barium hydroxide was added to keep it at a neutral pH. The emulsion settled spontaneously, and the watery concentrate was put through another cycle with chloroform instead of amyl acetate. The final relatively small volume of mahogany-coloured watery penicillin concentrate was then worked up by hand. These entirely satisfactory plants, the first in the main building and the second, larger, version in what had been the post-mortem room in the Animal House of the Sir William Dunn School of Pathology, were remarkable for their motley components—milk churns, a milk cooler, a domestic bath, a bronze letter-box, cisterns, aquarium pumps and various taps and valves. The larger of the two processed about 160 l. of crude culture fluid in 3 hr, after which the gummy deposit in the centrifuge bowl had to be removed. It is remarkable that so much of the penicillin survived contact with the heavy metals in these plants. (When rectangular unvarnished tins were used as culture vessels excellent growth of the fungus was obtained repeatedly, but with each re-use the yield of penicillin fell to virtually nil after the fifth or sixth re-use.)

One item which fortunately was obtainable in quantity even in the most difficult war years was the 1 gal., thick, narrow-mouthed glass bottle closed with a stout metal screw-cap, as used for soft drink concentrates, etc. It was adopted as the standard container for the original extraction apparatus and for all harvesting operations, and modifications enabled it to be used for special purposes. Thus, for filtration, the metal cap was adapted to hold a Buchner funnel, suction being applied through a side tube. A similar bottle containing the fluid to be filtered was fitted with a bung carrying a wide tube. When inverted over the Buchner funnel the level of liquid in the latter automatically kept level with the open end of the wide tube till filtration was complete; since the bottles were the same size, the receiver did not overflow. Some of the bottles had a small hole drilled near the bottom and closed by a screw-operated cork pad which enabled them to be used as separating funnels; they were held inverted in a special stand, the lower layer being run out through a glass tap or some other controlling device

mounted in a bung, while air was admitted through the small hole. A chain of operations, e.g. filtration followed by solvent extraction, could often be carried out without the pouring of material from one container to another, as would be done with conventional equipment.

Up to the end of 1940 the penicillin work had engaged the full time of one research worker and the part time, to varying degrees, of six others. No full-time technical help had been available, but at this time (early 1940) two trained technicians, G. A. Glister and S. A. Cresswell, were engaged for the culture and extraction work. Glister remained at the Dunn School for about 5 years, and when he left became Works Chemist of the Ministry of Supply penicillin factory at Speke. With their help a steady but still minute supply of penicillin became available, which enabled Florey to carry out more extensive mouse-protection experiments with different dosage schedules and up to 25 control and 25 treated mice. At least four more experiments were done with the same streptococcus, three with a staphylococcus, and two each with *Streptococcus pneumoniae* and *Clostridium septicum*; a good deal was learned from these experiments (Chain *et al.* 1940).

The mouse experiments were so promising that it was imperative to extend the trials to man. Since, in Florey's words, 'a man is 3000 times the size of a mouse', this required production greater by more than three orders of magnitude—the point at which, under normal conditions, the help of a commercial firm would be sought. But conditions were far from normal, with bombing by the Germans at its height and all firms more than fully engaged with Government contracts. After exploring the situation, Florey realized that there was nothing for it but to try to make penicillin on the required scale in the laboratory. Clearly, more technical help would be needed, but trained technicians could not be obtained. As an experiment two girls, both partly trained in nursing, were taken on temporarily in December 1940—the first female technical help ever to be employed in the Dunn School. They were Ruth Callow (sister of Chain's assistant, D. Callow) and Claire Inayat Khan. So successful was this innovation that more girls, Betty Cooke, Peggy Gardner, Megan Lankester and Pat McKegney, none of them trained technicians, were at different times taken on. These first 'penicillin girls', ably trained and organized by Glister, played an invaluable part in the production of penicillin for the first clinical trials. It is untrue that the entire resources of the Dunn School were given over to penicillin work. Apart from the technical assistance just mentioned, together with some from J. Kent, E. Vincent and D. Callow, the maximum research effort at any one time probably did not exceed the equivalent of four full-time research workers, and was most of the time less than this. Heatley noted in his diary on 31 December 1940 that the scale of production had been increased nearly 1000-fold during the year.

On 27 January 1941 C. M. Fletcher, who was a Nuffield Research Student of Registrar status at the Radcliffe Infirmary, Oxford, and maintained liaison between Florey and the hospital during the early clinical trials, cautiously gave an intravenous dose of 100 mg. of penicillin to a human volunteer. Although 10 mg. of the same preparation had been without any apparent effect on a mouse, the patient suffered a delayed rigor. Was this due to penicillin itself or to an impurity? Some weeks previously Abraham had begun using chromatography on alumina as a means of purifying penicillin, and to everyone's immense relief one of his active fractions was found to be

free from pyrogen when tested in rabbits by Florey. Chromatography was thereafter a routine step in production, the material being known at the time as 'therapeutic penicillin'; it typically had about 50 units/mg., though there was wide variation. Fletcher administered such material to a number of volunteers by various routes, and the resulting blood levels and urinary excretion of the drug were measured. In the light of these experiments, lasting about 2 weeks, Florey decided that continuous intravenous infusion was likely to be the most economical and efficient method of administration.

The first patient to be treated with penicillin parenterally was a desperately ill policeman, admitted to the Radcliffe Infirmary 2 months previously with suppurating abscesses resulting from a scratch on the face from a rose bush. He had lost one eye, one humerus was involved, and surgical drainage and treatment with sulphapyridine had not controlled the mixed streptococcal and staphylococcal infection. Penicillin, administered by intravenous drip from 12 February, led within 4 days to a quite dramatic improvement, but supplies ran out and after holding his own for 10 days the patient relapsed and died.

After this tantalizing result, further treatment of patients was delayed until a larger supply of the drug had been accumulated, but during the next 3 months five more patients were treated, four of them ill with serious infections. One of them suffered from an infected cavernous sinus thrombosis, a condition believed at the time to be invariably fatal; though he later died from ruptured mycotic aneurysm, healing of the infected sites was found to be well advanced. The case histories have been described in detail elsewhere (Abraham *et al.* 1941) and it must suffice to record here that the response to penicillin was considered almost miraculous. It seemed that penicillin had vast clinical potentialities, especially in time of war.

There was throughout the work at Oxford an extreme scarcity of penicillin, and it was for this reason that four of the first chosen six patients were children. All containers, including syringes, were rinsed out, and the urine from patients receiving the drug was jealously collected and the penicillin extracted from it. Probably the total production of penicillin up to the middle of 1941 was of the order of 4 megaunits, of which half might have been used therapeutically.

Laboratory production could not be increased much more and, once he had good clinical evidence to present, Florey went to America (which was then not at war) under the auspices of the Rockefeller Foundation, with the object of trying to interest the Americans and persuade one or more commercial firms to prepare enough penicillin to treat a further score or more cases, which would have enabled its clinical value to be rigorously assessed. Without such additional proof it could hardly be expected that any firm would undertake large-scale production. It seems that Florey had approached the Rockefeller Foundation by the middle of April, if not earlier, and, the necessary formalities having been completed in secrecy, he and Heatley on 27 June 1941 travelled in a blacked-out plane from Whitchurch, near Bristol, to an unknown airport, and thence to Lisbon. After 3 days of unaccustomed relaxation in this freely lit, non-rationed paradise, as it seemed, they proceeded to New York by Clipper, via the Azores and Bermuda. At the Rockefeller Foundation on Thursday, 3 July, Florey gave an account to Dr Alan Gregg of the results so far obtained with penicillin. Quiet and factual, the report was nevertheless intensely stimulating as to matter, while its mode of delivery was equally revealing of Florey's stature as a scientist.

Since likely contacts could not be pursued on Independence Day, Florey visited his

friends, Dr and Mrs J. F. Fulton, at New Haven, Connecticut, where his two children had been staying. Fulton took Florey to Dr Ross Harrison, who recommended that he contact Dr Charles Thom, the mycologist. Florey saw Thom at Washington on 9 July, and was taken by him to Dr P. A. Wells of the U.S. Department of Agriculture, who suggested that the Department's Fermentation Division in the scarcely completed Northern Regional Research Laboratory at Peoria was the obvious place to seek expert help; he sent a telegram to Peoria asking that a cabinet of fermentation trays be put at Florey's disposal at once. Florey and Heatley reached Peoria on Monday, 14 July, and the Director, Dr E. O. May, and the head of the Fermentation Division, Dr R. D. Coghill, promised full co-operation. Heatley was to remain at Peoria, and started work at once with Dr A. J. Moyer. The lyophil-dried cultures brought from England germinated slowly and with difficulty, and it was at first feared that they might have died out at the high temperatures encountered since leaving England, over 2 weeks earlier. Since fresh yeast was not readily available, Moyer suggested that corn-steep liquor be tried, instead of yeast extract, since it was cheap, available, and already used as a source of growth factors in some fermentations. It not only accelerated growth of the penicillium, as did the English yeast extract, but increased the yield of the antibiotic; further increases were obtained when Moyer gradually adjusted the trace metal and carbohydrate composition of the medium. Shortly before Heatley left Peoria on 30 November 1941, having trained Mrs L. Robertson to carry out the penicillin assays, the yield had been raised from the 1 to 2 u./ml. commonly obtained at Oxford to 20 u./ml. Later, the yield was vastly increased by improving cultural conditions and selecting better strains of the fungus. (For some years most commercial penicillin was obtained from a strain of *Penicillium chrysogenum* originally isolated from a mouldy cantaloupe in a Peoria market, or from substrains derived from it.)

Florey meanwhile was busy seeing people and firms in connexion with penicillin and other projects. He returned to Oxford in the middle of September 1941 with the promise of the Chairman of the Committee for Medical Research of the U.S. Office of Scientific Research and Development that he would back the production of penicillin to his fullest power. One wonders whether this fateful assurance would have been so freely given had the Chairman, A. N. Richards, not known Florey's calibre from the time some 15 years previously when the two had collaborated in Richards's laboratory.

Florey has often been criticized for 'giving away to America' a project which might have earned this country millions of dollars. It is true that huge sums have been made from penicillin, but the criticism will not bear examination. In the first place the Oxford process for growing the fungus and extracting the penicillin only became commercially practicable when the yield had been greatly increased by the work at Peoria and elsewhere in America. It is doubtful whether useful patents could have been obtained in England in 1941, and there was no mechanism by which the University, for instance, could have assisted. Finally, when Florey sought high-level advice on the question he was told authoritatively that the patenting of a potentially beneficial medical discovery or invention was against medical ethics and from that point of view out of the question.

Those living and working in Oxford during the war had cause to be, and were, profoundly thankful that Oxford escaped the serious bombing suffered by some other parts of England at the time. To be sure, queueing for food, etc., wasted much time, but Civil Defence duties such a 'firewatching' could be put to use since those sleeping at

the laboratory could—and did—work until a late hour. In the spring of 1941, when invasion of Britain was no longer unthinkable, some of those engaged in the work rubbed freely-sporing mycelium in their pockets; the hope was that if the Germans invaded, someone might escape to a neutral country and, recovering the fungus from their clothes, enable the work to continue.

On his return from America in September 1941 Florey and his wife, Dr M. E. Florey, carried out a second series of clinical trials, which provided more precise information on dosage and methods of administration (Florey & Florey, 1943). The penicillin for these trials was at first made mainly in the Dunn School, but by the beginning of 1942 small amounts were contributed by Imperial Chemical Industries, Ltd. From September of that year, for some months, Kemball, Bishop & Co., a small east London firm making citric acid by fermentation, brought 150 to 200 gal. lots of formalinized crude penicillium culture fluid to Oxford in milk churns by lorry for extraction in the Oxford plant. Later, penicillin was also supplied by the Therapeutic Research Corporation.

With the prospect of increased production of the drug came the question whether it could be successfully used in the treatment of war wounds. In North Africa, preliminary treatment of 15 battle casualties had given moderately encouraging results (Pulvertaft, 1943).

During the summer of 1943 (he was away from Oxford from 23 May to 2 September) Florey took part in an extensive investigation in North Africa, the purpose of which was to find out how best limited amounts of penicillin could be used to treat battle casualties, and at what point(s) in the army medical organization it could be most effectively used. The penicillin was provided from the four sources mentioned above, and because the supply was scanty and likely to be so for some time, the emphasis was on local, rather than systemic, administration, e.g. as a powder (mixed with sulphonamides) or in solution. A radical departure from established surgical practice was made, namely early suture of wounds, with penicillin irrigation through small indwelling tubes. This enabled healing to take place in a fraction of the time which would otherwise have been required, and with far better results. Florey, who insisted on remaining a civilian, and Brigadier Hugh Cairns submitted a 114-page preliminary report of this work jointly to the Medical Research Council and to the War Office. This report was published in October of the same year (Florey & Cairns, 1943) and received high praise from Major-General Poole, Directory of Pathology, War Office.

By 1944 penicillin production was in the hands of commercial firms, while the Medical Research Council's Penicillin Clinical Trials Committee were handling this aspect. However, contributions were still made from Oxford laboratories, notably on its chemistry and, under Dr Dorothy Hodgkin, its crystallography. Florey continued to to supervise local clinical trials of various kinds and to work personally on biological aspects of penicillin and other antibiotics. He extracted and crystallized claviformin, and had a hand in the detection, extraction and assessment of several others, including helvolic acid, proactinomycin, mycophenolic acid, a colicine (later shown to be identical with colicine V), tardin, hirsutic acid, ayfivin (later known as bacitracin), cephalosporins P1 and N, and micrococcin. The latter was isolated by T. L. Su from one of a large number of antibiotic-producing organisms he had obtained from Oxford sewage. It was not very soluble in aqueous media, nor was it toxic, but the growth of several strains of *Mycobacterium tuberculosis* was inhibited by one part in 100,000 or more. Quite large

amounts could be injected in finely divided form into rabbits and mice without any apparent ill effect and were rapidly removed by the reticulo-endothelial system. Florey thought it worth investigating whether an antibacterial substance such as this, taken up by the phagocytic cells, might act upon intracellular tubercle bacilli which would by their position be protected from any soluble agent that could not cross the cell membrane; or might, at any rate, form a protective zone of 'fortified' phagocytes around a lesion. However, this concept, centred on what was termed by a colleague 'Florey's Fortified Phagocytes', was not fruitful with micrococcin, for a variety of possible reasons (Florey, 1954). Nevertheless, a good deal of quantitative information was obtained on the distribution of micrococcin among, and its persistence in, various organs of the body, and, qualitatively, in individual cells. It was still measurable in liver, spleen and kidney more than 6 months after a single intravenous injection.

As was to be expected, Florey received much correspondence from the lay public, some of it bizarre, e.g. a letter from Scotland drawing attention to a possible antibacterial effect of oat bran was accompanied by a raw herring, undeniably well preserved, packed in this material. The most fruitful contact, mediated by Dr C. S. Blyth Brook in 1948, was with Professor Guiseppe Brotzu, formerly Rector of the University of Cagliari, Sardinia, who unreservedly passed on to Florey an antibiotic-producing cephalosporium which he had isolated from the sea near a sewage outlet at Cagliari. This organism produced a range of antibiotics, and years of patient study by Abraham, the late G. G. F. Newton and others in Florey's department led to the discovery and characterization of the cephalosporins, perhaps the most interesting and important antibiotics to be discovered since the natural and semisynthetic penicillins.

The discovery of penicillin by Fleming—another original member of our Society—and its production and use are easily understood by the layman, and this, together with its amazing effect (now taken for granted) in certain diseases, led to many articles and items about it in the Press, even in children's magazines and books. 'Penicillin' has been set as a school 'project' on both sides of the Atlantic, and it, and antibiotics in general, have inspired a number of popular and semipopular books.

What was Florey's role in the development of penicillin as a chemotherapeutic agent? He himself pointed out on several occasions that he and Chain had extreme good luck in choosing penicillin as one of the first natural antibacterials to be studied in their programme; however, the programme was intended to be a wide one, and penicillin, though merely at that time a laboratory curiosity like the others, would inevitably have been one of the earliest to be studied.

Florey was once maliciously described as a physiologist who happened to have in his department the right people to work through the project. He would have been the first to disclaim expert knowledge in, for example, chemistry or bacteriology, but those who knew him know also what shrewd comments and suggestions he could make in fields other than his own. He repeatedly stressed that the outcome was the product of a team, and was scrupulous in giving all possible credit to the other members of it. But he had prepared the ground at Oxford by building up during previous years a laboratory intentionally staffed with workers trained in a variety of disciplines. This was a logical extension of his conviction—shared by few others when he came to Oxford in 1935—that advances in medicine were most likely to spring from rigorous experiment in the basic medical sciences.

At the purely practical level his own contributions were substantial and crucial. His firsthand experience of the pathological processes of inflammation, necrosis, the role of small blood vessels and so on, as well as his grasp of physiological principles, enabled him to plan the biological experiments on toxicity and pharmacological properties with economy and elegance. A colleague remarked of this phase that 'not an experiment was wasted', and these early animal experiments provided a firm basis for planning human therapy. Without them, the results from the treatment of the first half-dozen human patients might well have been inconclusive instead of clear-cut, with who knows what frustration and delay in the introduction of penicillin into medicine.

Important as this work was, it is in his other role, as leader of the team, that Florey was irreplaceable. One remembers his humour, his infectious enthusiasm, and his genial and constructive cynicism. Certain decisions he made boldly and swiftly on his own initiative and responsibility; but in general he constantly consulted his colleagues and allowed them virtually complete freedom to pursue their own branch of the work as they thought best—or rather, as they thought best after discussion with him or with another colleague. There were few meetings of 'the team' as such, but many informal discussions with individuals or small groups enabled Florey constantly to reassess the course of the work and to suggest revisions of the immediate programme. In retrospect, though there were many disappointments and failures, there were few if any occasions when some remedy or alternative approach could not be suggested. How remarkably effective was his firm but unobtrusive leadership is shown by the fact that the first clinical trials were completed after no more than 18 months of sustained work.

N. G. HEATLEY

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Role of Chitinase and Other Lysosomal Enzymes of *Coprinus lagopus* in the Autolysis of Fruiting Bodies

By W. ITEN AND P. MATILE
Department of General Botany, Swiss Federal Institute of Technology, Zürich, Switzerland

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SUMMARY

The autolysis of mature fruiting bodies of *Coprinus lagopus* is caused by the degradation of cell walls. This process is accomplished by the action of chitinases which are newly formed shortly before spore release begins. Chitinase activity is localized intracellularly in vacuoles together with other hydrolytic enzymes. It is released into the wall upon cessation of metabolic activity in senescing gills.

INTRODUCTION

Members of the genus *Coprinus* are characterized by a rapid autolysis of the mature fruiting body. Elucidation of such a lytic process should involve a study of the relevant hydrolytic enzymes as well as their intracellular localization and function in the living organism. Constitutive digestive enzymes which eventually autolyse yeasts are localized in the vacuoles (Matile & Wiemken, 1967). Thus, autolysis appears to represent an extreme case of intracellular lysis which normally takes place in a special compartment, the lysosome (see Matile, 1969). The present study concerns the intracellular localization of hydrolases in *Coprinus lagopus*. Special attention has been paid to the role of lysosomes and lysosomal enzymes in autolysis in mature gills.

METHODS

A strain of *Coprinus lagopus* Fr. isolated from horse-dung was used. Mycelia were grown in a liquid medium containing, in g./l.: glucose, 10; Hammerstencasein, 2·5; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0·2; thiamine, 0·005 and trace elements [in mg./l.: $Fe(NH_4)(SO_4)_2.6H_2O$, 5; $ZnSO_4.7H_2O$, 25; $CuSO_4.5H_2O$, 1·25; $MnSO_4.H_2O$, 0·25; H_3BO_3, 0·25; $Na_2MoO_4.H_2O$, 0·25]. 500 ml. conical flasks with 200 ml. medium were inoculated with a suspension of spores and agitated on a reciprocal shaker at 28°. Fruiting bodies were obtained in cultures in glass cylinders (10 cm. diameter) containing 100 ml. of solid medium containing (g./l. water): malt extract, 10; Difco-yeast extract, 4; glucose, 4; Difco-agar, 10. A piece of vegetative mycelium of about 1 cm^2 grown on a solid medium was placed on this nutrient agar and an incandescent lamp (tungsten filament bulb, 75 W) was installed 80 cm. from the culture vessel. Illumination of the cultures for 8 hr/day at 28° led to the first fruiting bodies maturing 7 days after inoculation.

Fractionation. Cell-free extracts used for preparing subcellular particles and for the determinations below were obtained by squeezing gills with pestle and mortar in a sorbitol medium (0·3 M-sorbitol, 0·02 M-tris-HCL buffer pH 7·4, 1 mM-EDTA).

Quartz was added for extracting vegetative mycelia. No buffer was added if extracts were used only for the determinations below.

Preparation of vacuoles. Cell-free extracts were centrifuged at a low speed (10 min. 500*g*) which removed unbroken cells and walls. A mitochondrial fraction containing vacuoles was obtained by differential centrifugation at 10,000*g* for 10 min. Two post-mitochondrial fractions sedimented at 40,000*g* (10 min.) and 150,000*g* (30 min.) respectively. The final supernatant represented the soluble fraction. The sediments were washed once with sorbitol medium. Vacuoles were isolated using isopycnic gradients of Ficoll (Pharmacia Uppsala) ranging from 20 % (w/v) Ficoll (in 0·2 M-sorbitol) to 0·5 M-sorbitol. The gradients (4·5 ml.) were loaded with 1·0 ml. of mitochrondrial fraction suspended in sorbitol medium. 0·01 % (w/v) Triton X-100 was added to prevent aggregation of particles. Centrifugation was carried out for 2 hr at 39,000 rev./min. in a Spinco SW 39 rotor. A large proportion of vacuoles was trapped at the top of the Ficoll gradients. These particles were purified by flotation in the presence of 8 % (w/v) Ficoll (in 0·3 M-sorbitol) through a layer of 6 % (w/v) Ficoll prepared in 0·3 M-sorbitol.

Table 1. *Lysosomal enzymes of Coprinus: pH-optima and references to the conditions of assay*

Enzyme	Substrate	Buffer	pH optimum	Remarks
Acid protease	Haemoglobin	Acetate	2·5	Acid-soluble products determined according to Lowry *et al.* (1951)
Alkaline protease	Casein	Carbonate	9·5	—
β-Glucosidase	*p*-Nitrophenyl-β-Glucoside	Acetate	5·0	Formation of *p*-nitrophenol
Phosphatase	*p*-Nitrophenyl phosphate	Acetate	5·0	—
RNase	Purified yeast RNA	Acetate	5·2	u.v. Absorbancy of acid-soluble products
Chitinase	Purified cell walls from gills of *Coprinus*	Acetate	5·0	Method described in the text

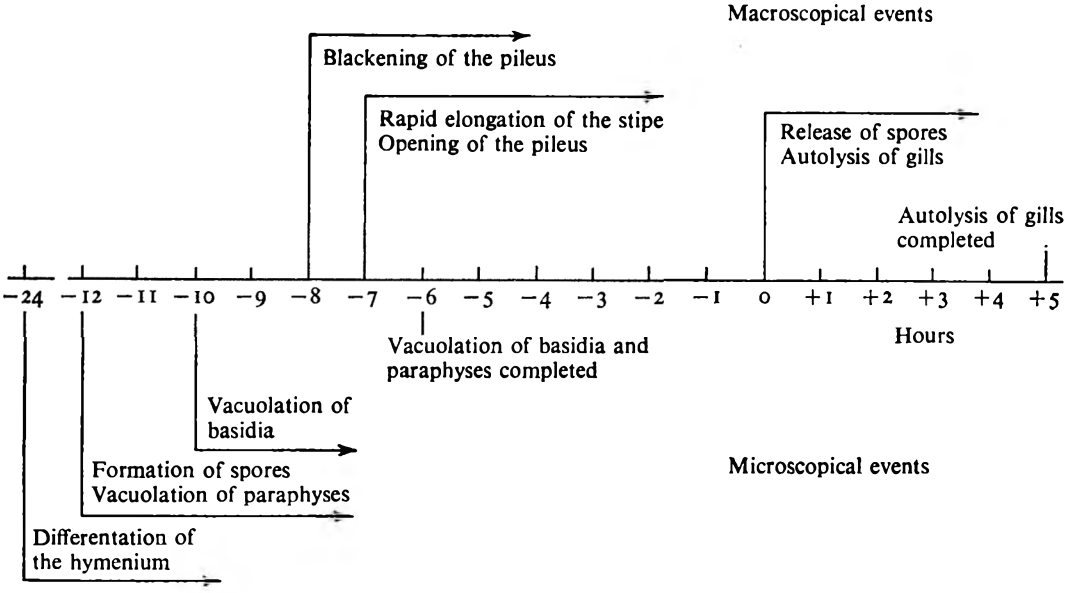
Determinations. Activities of hydrolytic enzymes were measured at 37° using the substrates and buffers listed in Table 1. Chitinase from Coprinus hydrolyzes the chitin present in extensively purified cell walls from gills of Coprinus fruiting bodies isolated according to Crook & Johnston (1962). Dry wall powder (25 mg.) suspended in 1 ml. 0·1 M-acetate buffer pH 5·0 was the substrate. The reaction was stopped by heating 2 min. at 100°. After centrifuging down the insoluble material, *N*-acetylglucosamine was determined according to Reissig, Strominger & Leloir (1955). Alternatively, reducing groups were measured using the reagent of Nelson (1954). In controls, either walls or enzyme were omitted. Cytochrome oxidase, succinic dehydrogenase and aldolase activities were assayed by standard procedures.

For protein and RNA, samples of extracts were treated with 10 % (w/v) TCA. The precipitated protein was determined according to Lowry, Rosebrough, Farr & Randall (1951), and RNA by the orcinol reagent of Schneider (1957).

Isolated walls hydrolysed with 5N-HCl for 5 hr at 100° were qualitatively analysed on thin layers of Kieselgur impregnated with 0·02 M-sodium acetate. The solvent was a mixture of 6 vol. ethylacetate and 4 vol. of isopropanol–water (2:1 v/v). Anisaldehyde was used as the spray reagent (Stahl, 1962).

Table 2. *Development of the fruiting body of Coprinus lagopus under the experimental conditions used*

The zero point of the time axis coincides with the commencement of spore release, an event which can be easily observed.



RESULTS

Morphology of the developing fruiting body. Mycelia of *Coprinus lagopus* placed on solid nutrient media developed into mats about 5 cm. in diameter within 5 days. At this time small primordia were formed which subsequently developed into fruiting bodies. This process has been described in detail by Buller (1924) and Borriss (1934). The macroscopical and microscopical events which were observed under the conditions used are summarized in Table 2. The zero point of the time axis is the time at which the first spores were released.

The first spores released originated from the edges of the gills adjacent to the stipe. Soon after a region released its spores its autolysis began. The resulting liquor was sucked by capillary forces into the intact regions of the gills where it dissolved the cells. A zone of spore release followed by a zone of autolysis moved peripherally and reached the edge of the pileus within about 5 hr.

Physiology of the developing fruiting body. Slices of intact gills were placed in drops of liquor from autolysing fruiting bodies and incubated at 25°. It appeared that the cell walls were degraded. This process could be followed in the phase contrast microscope. It was completed within about 3 hr. By using the digestive capacity of autolysate liquor, naked protoplasts could be prepared from undifferentiated hymenia. If young gills (corresponding to a developmental stage of −20 hr; Table 2) were first incubated

with autolysate for 2 hr and then gently squeezed, protoplasts containing two nuclei and two large vacuoles were released. They originated from hymenial cells which would later have differentiated into paraphyses. We could not prepare protoplasts from vegetative mycelia by this technique; the reason is unknown. The walls of gills and

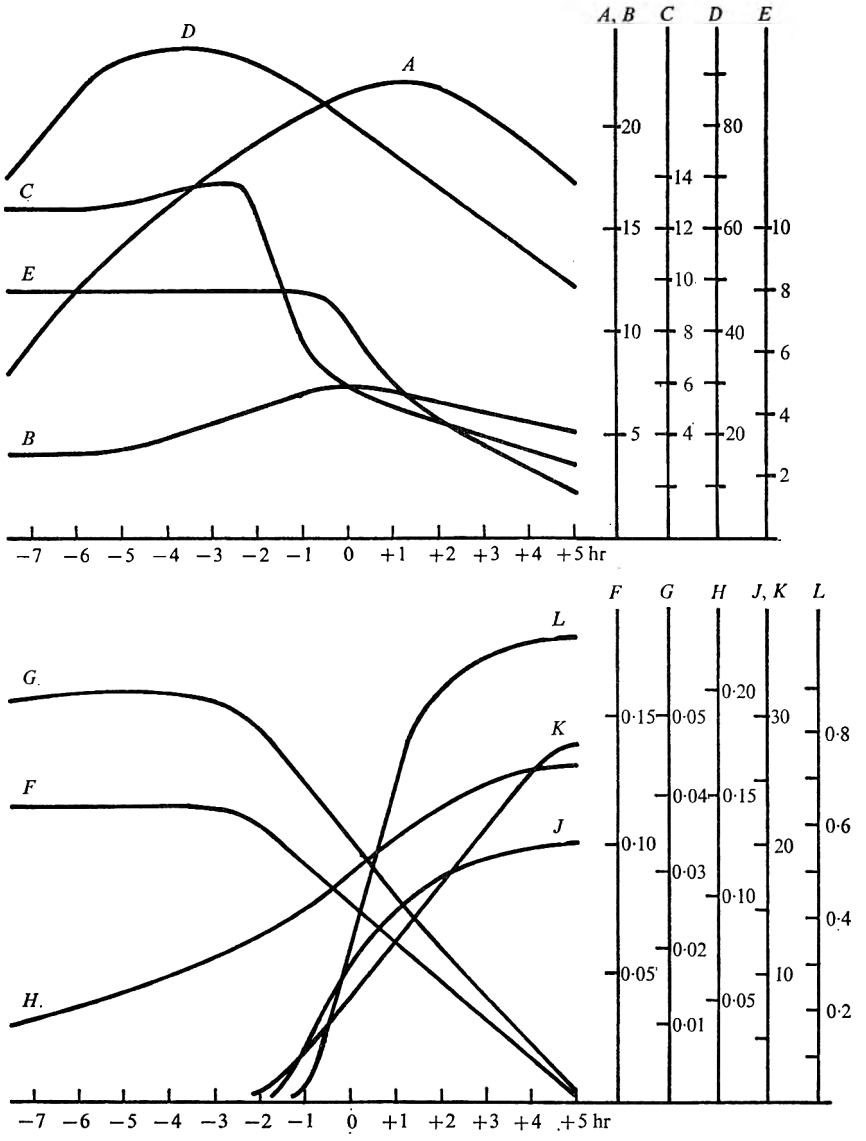


Fig. 1. Changes of some enzyme activities and compounds in cell-free extracts from gills during the development of fruiting bodies. Samples were collected at the following time points (see Table 2): −7·5, 5, 2·2, 0·5 hr and +5 hr RNA and protein are given in μg./mg. of dry matter of the extracts. *A*, alkaline protease (μg. tyrosine equiv./30 min.); *B*, acid protease (μg. tyrosine equiv./30 min.); *C*, acid RNase (μg. RNA/60 min.); *D*, protein (μg.); *E*, RNA (μg.); *F*, aldolase (ΔE_{340}/min.); *G*, cytochrome oxidase (ΔE_{550}/min.); *H*, acid β-glucosidase (μg. glucose/10 min.); *J*, *N*-acetylglucosamine (μg.); *K*, acid chitinase (μg. *N*-acetylglucosamine/20 min.); *L*, acid chitinase; determination of reducing groups (mg. *N*-acetylglucosamine equiv./20 min.).

vegetative mycelium appear to be chemically similar: thin layer chromatography of hydrolysates revealed only glucosamine. If isolated walls were incubated with autolysate liquor, *N*-acetylglucosamine was formed. Thus, chitin is a prominent wall constituent which has, in fact, been identified by Frey (1950) in walls of *Coprinus atramentarius*. In addition, chitinase activity appears to be present in the autolysing fruiting bodies of *C. lagopus*.

Using a viscosimetric method Tracey (1955) demonstrated chitinase activity in *Coprinus comatus*. Upon incubation of *C. lagopus* cell walls with autolysate liquor the formation of total reducing groups was about 30 times more active than the production of the monomer *N*-acetylglucosamine. It thus appears that *C. lagopus* formed mainly chitinase; only low activities of chitobiase seemed to be present in the autolysing fungus.

Chitinase activity was lacking in the vegetative mycelium and young fruiting bodies. The enzyme was synthesized about 2 hr before spores were released (Fig. 1). The synthesis of chitinase proceeded rapidly for about 3 hr; thereafter the activity decreased gradually towards the end of autolysis. Fig. 1 also shows that, unlike chitinase, proteases and RNase were present in extracts of gills of immature fruiting bodies and also in the vegetative mycelium. The process of differentiation of fruiting bodies was characterized by an increase of protease activities towards the beginning of autolysis and by a subsequent decrease. The protein content of gills began to decrease about 3 hr before the beginning of autolysis. Moreover, the activities of two respiratory enzymes decreased much faster than the total protein and were almost zero at the end of autolysis. Similarly the RNA content began to fall sharply at -2 to 3 hr.

Table 3. *Sedimentability of alkaline protease and cytochrome oxidase in a cell-free extract from vegetative mycelium grown for 45 hr*

Enzyme activities in arbitrary units.

			Sediment-able activity (%)	Differential sedimentation		
	Cell-free extract	Sediment 30 min. 150,000*g*		10 min. 10,000*g*	10 min. 40,000*g*	30 min. 150,000*g*
Alkaline protease	7·74	1·78	23	1.38	0·34	0·35
Percentage of total sedimentable activity				77·5	19	19·5
Cytochrome oxidase	16·0	16·0	100	14·0	0	0
Percentage of total sedimentable activity				87	0	0

The above estimations concern a heterogeneous tissue containing cells at different stages of development; nevertheless they may reflect physiological events taking place during development of fruiting bodies. The data suggests that autolysis is not exclusively responsible for the decreasing protein and RNA contents in fruiting bodies which approach maturity. Since these events take place several hours before autolysis begins it seems to be likely that they are caused by cellular lytic processes which take place in lysosomes.

Subcellular localization of digestive enzymes. Lysosome-like structures were extracted from both vegetative mycelia and fruiting bodies. One criterion used for demonstrating the existence of lysosomes was sedimentability of hydrolases. These enzymes were partially sedimentable from extracts of vegetative mycelia but a large proportion occurred in the soluble fraction. The data in Table 3 indicate that a large

proportion of the sedimentable activity was contained in a 'mitochondrial fraction'. Similar results were obtained with extracts from gills.

Upon centrifugation of mitochondrial particles loaded on gradients of Ficoll the activities of several lysosomal enzymes were trapped at the top of the gradient but some activity was distributed throughout the gradient (Fig. 2). If gills containing chitinase activity were used for preparing lysosomes, the distribution of this enzyme in the gradient corresponded with that of other hydrolases tested, viz. acid and alkaline protease, RNase, phosphatase and β-glucosidase. Coinciding distribution curves of chitinase and other hydrolases were also obtained upon centrifugation in density gradients of sucrose or Urografin. Thus chitinase appeared to represent a lysosomal enzyme.

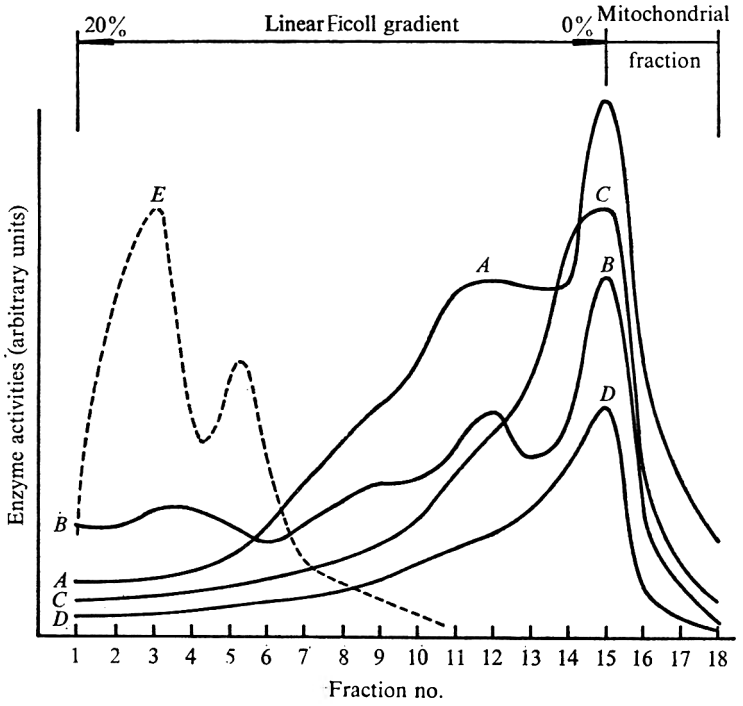


Fig. 2. Distribution of lysosomal enzymes in a linear density gradient of Ficoll. The mitochondrial fraction (suspended in 0·5 M-sorbitol medium) loaded on the gradient was obtained from an extract prepared from gills shortly before the commencement of spore release. To maintain the osmotic stabilization of particles the Ficoll gradient (20 to 0% w/v) was superimposed by a sorbitol gradient (0·2 to 0·5 M).

Phase contrast microscopy of particles trapped at the surface of a Ficoll gradient showed vacuoles 0·5 to 4 μm. in diameter (Pl. 1 *c*). Particles present in gradient zones of higher density were smaller (1 μm.). To demonstrate that the enzymes were enclosed in the vacuolar structures, it was necessary to test the respective preparations for free hydrolase activity. Some activity appeared to be free when osmotically stabilized particles were incubated, but the disintegration of these structures previous to incubation resulted in a doubling of the activities measured. If the vacuoles were further purified by flotation in Ficoll the percentage of latent activity increased markedly (Table 4). It thus seems that the vacuoles represent lysosomes. The relatively

high proportion of hydrolase activities present in the soluble fraction was undoubtedly due to rupture of large vacuoles during grinding or squeezing of hyphae. Extracts from young mycelia, grown for 45 hr, contained about 23 % of sedimentable protease activity, as compared with mycelia grown for 10 days which yielded only 11·4 % protease activity. The number of large vacuoles which cannot be extracted in intact form is much larger in old than in young hyphae. The micrograph in Pl. 1 *a*, *b* shows that the first small vacuoles occur at about 100 μm. from the growing tip of the hypha. With increasing age the vacuoles gradually inflate and at about 2 mm. from the tip the cells appear to be completely vacuolated. The size of the isolated vacuoles suggests that only a zone between 100 and 500 μm. from the tip yielded vacuoles extractable under the conditions used. A similar situation must be assumed in lamellar hyphae.

Table 4. *Latency of acid phosphatase and alkaline protease in vacuoles isolated from vegetative mycelium*

The particles were incubated isotonically in substrate containing 0·5 M-sorbitol. Total activities were measured using ultrasonicated particles. The time course of the reaction (20 min. 25°) in both untreated and sonicated preparations was strictly linear. Enzyme activities in arbitrary units.

	Acid phosphatase				Alkaline protease			
	Free activity	Total activity	Latent activity	(%)	Free activity	Total activity	Latent activity	(%)
Cell-free extract	0·254	0·266	0·012	4·5	111·3	114·0	2·7	2·4
Crude vacuoles	0·037	0·084	0·047	56	18·3	35·1	16·8	48
Purified vacuoles	0·0074	0·023	0·015	68	—	—	—	—

Isolated vacuoles could also be identified by freeze-etching. The fine structure of their membrane corresponded with that of vacuoles observed *in situ* (Pl. 1 *d*, *e*).

The role of vacuoles in intracellular lytic processes not only appears from their content of hydrolytic enzymes but also from a feature of the tonoplast which appears from the examination of freeze-etchings. Tonoplasts often show invaginations which encapsulate cytoplasmic material (Pl. 2 *a*, *c*). This process results in the formation of intravacuolar vesicles which are occasionally very numerous in vegetative and hymenial cells (Pl. 2 *b*). They can also be observed in isolated vacuoles by freeze-etching or phase-contrast microscopy. The relatively high amount of protein present in preparations of isolated vacuoles is probably due to cytoplasmic material in the cell sap. Intravacuolar vesicles probably decay soon after cleavage of their membrane from the tonoplast; subsequently their cytoplasmic content would be degraded by the action of lysosomal enzymes.

DISCUSSION

The lysosomal enzymes localized in vacuoles of Coprinus fall into two categories. A first group of enzymes comprises hydrolases which are present throughout the life cycle of the fungus. Acid and alkaline protease, RNase, phosphatase and β-glucosidase have been found in vacuoles isolated from vegetative mycelium as well as from gills. Coprinus vacuoles represent a type of lysosome which does not contain acid hydrolases exclusively; the alkaline protease has an even more pronounced activity than its acid counterpart. The group of enzymes mentioned above appears to

be involved in lytic processes which represent a conspicuous feature of metabolism. The cytoplasm of old hyphae is almost completely replaced by large vacuoles; its mobilization takes place during the process of vacuolation which begins in a zone near the tip of the hyphae. The mechanism of formation of digestive vacuoles seems to correspond essentially to that found in other organisms such as root-tip cells (Matile & Moor, 1968) and yeast (Matile, 1970). It involves a pinocytosis-like activity of the tonoplast that results in the formation of intravacuolar vesicles which are presumed to be labile. After the decay of these structures their cytoplasmic content is digested by the action of the vacuolar hydrolases.

A second group of lysosomal enzymes is represented by chitinolytic enzymes, mostly chitinase. They seem to have no function in intracellular digestion since they are synthesized shortly before autolysis begins in gills. It is not yet clear whether the cells actively secrete these enzymes into the walls and thus induce their own autolysis or whether passive release occurs from cells whose metabolic activity has ceased completely. The second possibility seems to be more likely because the maturation of the fruiting body is accompanied by a rapid decrease of the activity of respiratory enzymes. This suggests that in those cells which autolyse first the respiratory activity is extinguished at the moment of spore release. The functional significance of autolysis with regard to the release of spores in Coprinus has been extensively described by Buller (1924).

Certain hydrolases belonging to the first group have a dual, intracellular and extracellular function. If vegetative mycelia are nourished with a proteinaceous nitrogen source such as casein the secretion of acid and alkaline protease into the culture medium can be observed. The release of hydrolases then is most probably not accomplished by autolysing cells. Active secretion via secretory granules is more likely involved as has been observed in *Neurospora crassa* (Matile, 1965; Matile, Jost & Moor, 1965).

We should like to thank Dr E. Müller for kindly supplying the Coprinus strain used in this study, and also Professor H. Moor and Miss C. M. Berger for preparing the freeze-etchings.

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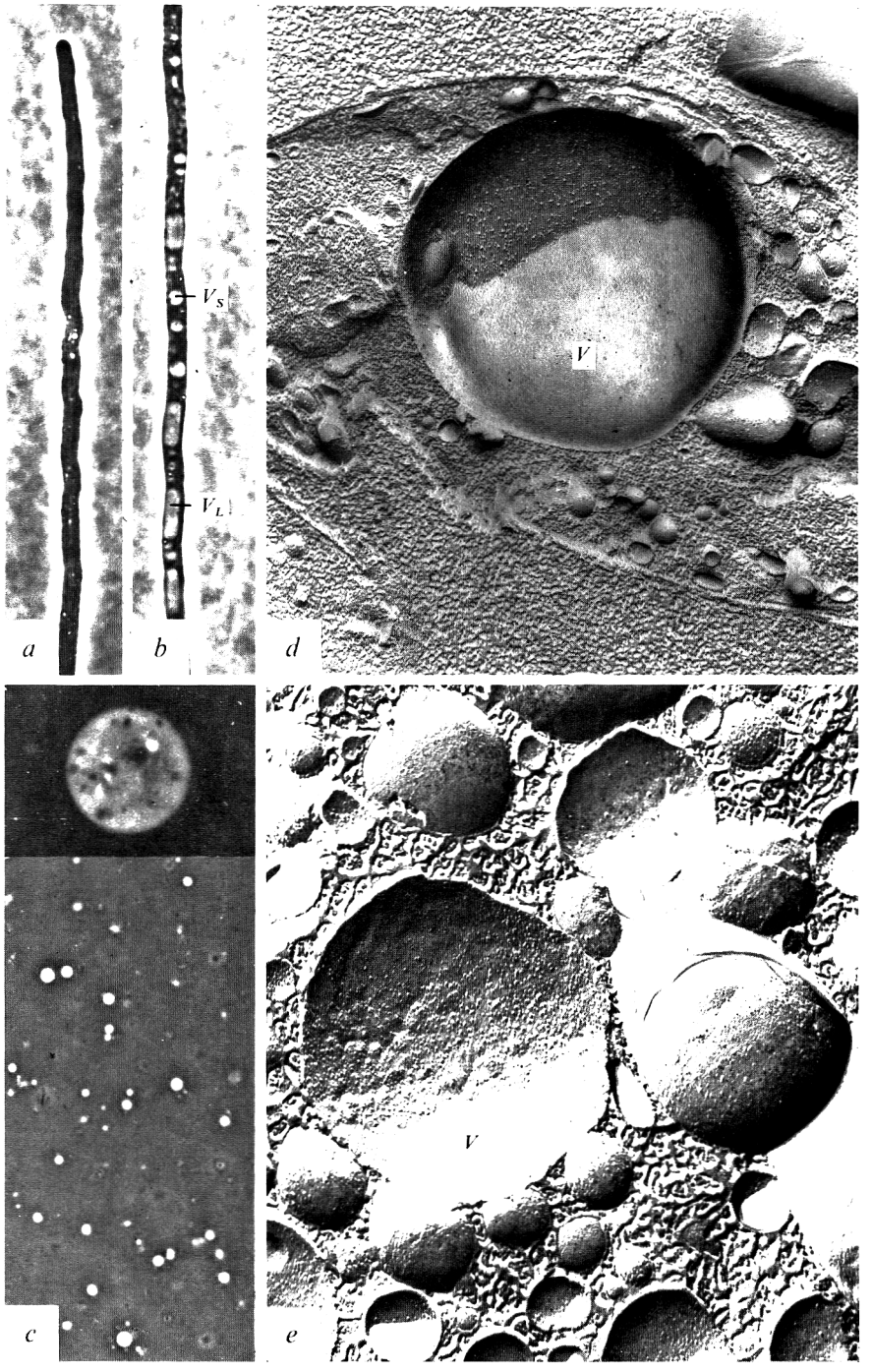
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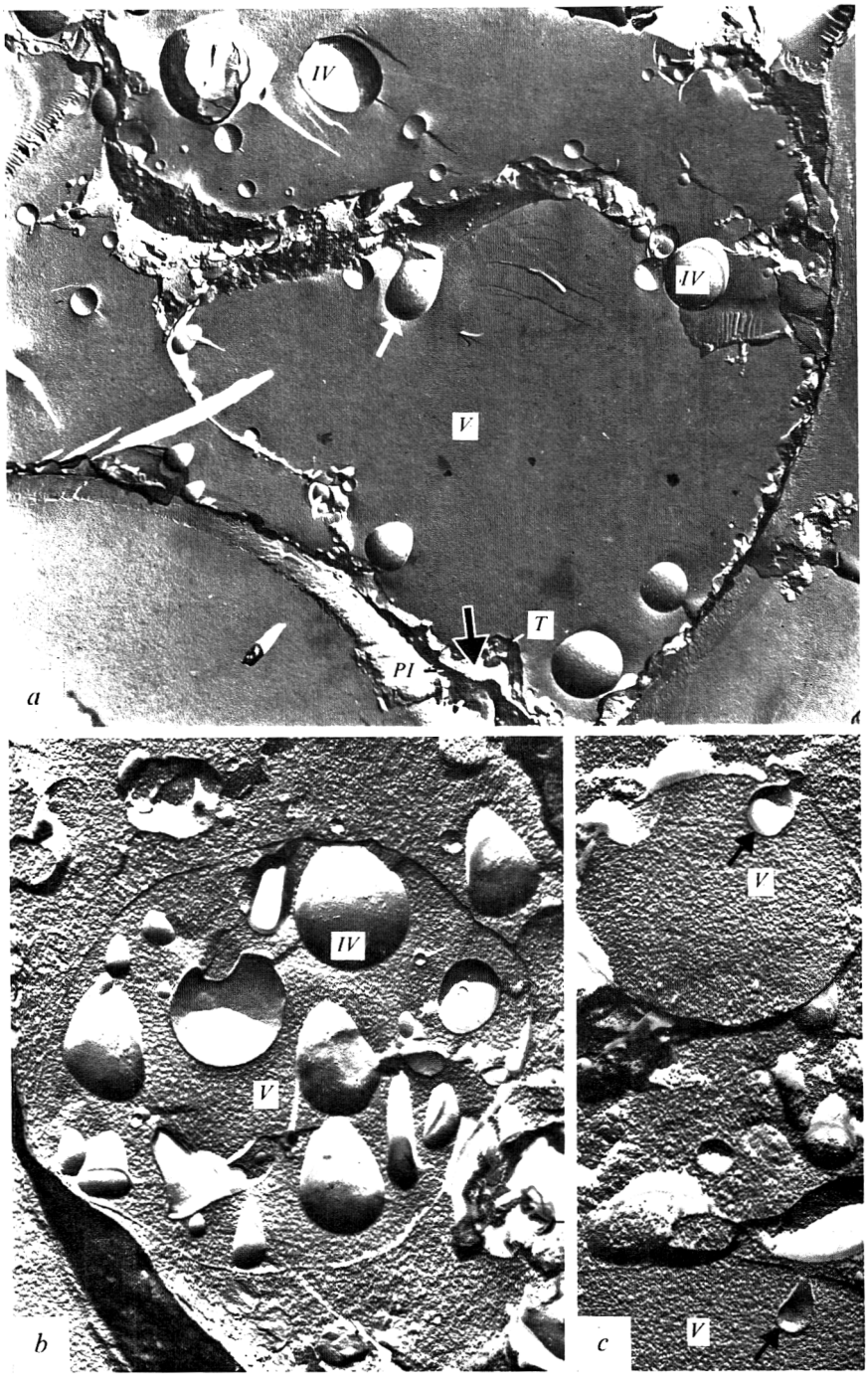
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EXPLANATION OF PLATES

PLATE 1

a, *b*. Vegetative hyphae grown on a microscope slide covered with a film of solid medium, *a*, Tip of a hypha; *b*, adjacent section of the same hypha. Note the small vacuoles (V_s) which correspond to the isolated vacuoles. Larger vacuoles (V_L) are destroyed upon homogenization. (Phase contrast, ×460.) *c*, Vacuoles isolated from vegetative mycelium (phase contrast, ×440). Insert shows vacuole containing numerous intravacuolar vesicles (×1400). *d*, Freeze-etching of a vegetative hypha. Note the vacuole (V) viewed on the outer surface of the tonoplast. *e*, Freeze-etched preparation of isolated vacuoles (×36,000).

PLATE 2

Freeze-etchings of gills at the stage of spore formation (12 hr before beginning of spore release). *a*, Highly vacuolated cells; the central vacuole (*V*) occupies almost all the cell. A thin layer of cytoplasm (black arrow) can be recognized between the plasmalemma (*Pl*) and the tonoplast (*T*). The vacuole contains a number of intravacuolar vesicles (*IV*). The white arrow points to an invagination of the tonoplast which results in the formation of an intravacuolar vesicle (×4600). *b*, Vacuole containing numerous intravacuolar vesicles (×20,600). *c*, Formation of intravacuolar vesicles; vacuoles showing invaginations (arrows) of the tonoplast (×27,600).

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Profiles of Soluble Protein During Sporulation of *Bacillus subtilis*

By W. M. WAITES* AND D. G. WILD
Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU

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SUMMARY

Electrophoresis in polyacrylamide gels of soluble proteins from *Bacillus subtilis* showed changing patterns of bands during sporulation. Somewhat different patterns were obtained from extracts of three asporogenous mutants exposed to a sporulation medium but the differences did not correlate well with the stages in sporulation at which the mutants were believed to be blocked.

INTRODUCTION

Spore formation in bacilli is accompanied by complex metabolic changes (reviewed by Murrell (1967) and Mandelstam (1969)). These include synthesis of enzymes directly involved in formation of spore-specific components and of others whose appearance is correlated with the seven morphologically distinct stages (Ryter, Schaeffer & Ionesco, 1966) of sporulation. More indirect alterations in enzymic machinery may result from experimental procedures used to induce commitment to spore formation; for example, derepression of synthesis of certain enzymes may occur if a nutritionally defective medium is used. Moreover, sporulating bacteria contain one or more proteases responsible for a high rate of turnover of protein; a consequence is that, in *Bacillus subtilis*, nearly all the protein in the spore is newly made during sporulation (Mandelstam & Waites, 1968).

Electrophoresis in polyacrylamide gels provides a sensitive indication of differences in the composition of the soluble protein fraction of bacteria at different stages in the growth cycle and in exponential growth in different media (Raunio & Sarimo, 1965; Moses & Wild, 1969). We have used this technique to monitor changes that occur after suspension in sporulation medium of a sporogenic organism and of asporogenous mutants derived from it. Such an electrophoretic technique might supplement biochemical and morphological criteria to determine the point in spore formation at which a particular mutation leading to asporogeny is expressed. In particular, a mutant blocked at a very early stage of spore formation has been compared with the corresponding wild-type organism in an attempt to distinguish changes in the soluble protein fraction due to sporulation itself from changes less directly associated with the process.

* Present address: Agricultural Research Council Food Research Institute, Colney Lane, Norwich, Norfolk, NOR 70F.

METHODS

Organisms. Bacillus subtilis (Marburg strain 168) requires indole or tryptophan for growth but is sporogenic and is called the 'wild-type'. Three asporogenous mutants from this parent were: E22, which forms neither the protease nor antibiotic characteristic of Stage I of sporulation. An unpublished morphological examination by D. Kay shows that mutant E22 retains the characteristic appearance of vegetative organisms under conditions in which the wild-type sporulates. Mutants E31 and A23 synthesize protease, antibiotic, alkaline phosphatase and glucose dehydrogenase in a sporulation medium but form only 'phase-grey' refractile bodies and do not make dipicolinic acid. On biochemical grounds (Warren, 1968) they therefore probably complete only Stages I to III of sporulation. This view is supported by unpublished electron microscopy by D. Kay.

Growth and preparation of extracts. Organisms were shaken overnight at 37° in the hydrolysed casein medium of Donnellan, Nags & Levinson (1964) with glucose omitted. A culture (containing about 0·25 mg. dry wt organisms/ml.) was centrifuged and the organisms resuspended in the same volume of the defined sporulation medium of Donnellan *et al.* (1964) except that glucose was omitted and $MgSO_4$ was 40 mM. This latter medium normally causes a high incidence of spores in about 8 hr. At intervals, 200 ml. samples were harvested and the organisms resuspended in 0·5 or 1·0 ml. ice-cold buffer containing 10 mM-tris-HCl, 100 mM-KCl, 10 mM-magnesium acetate, pH 7·4. Samples were maintained at 0° and the organisms broken with an ultrasonic disintegrator (M.S.E. Ltd.) used intermittently for a total of 5 min. The suspension was then centrifuged at 40,000 rev./min. for 1·5 hr at 4° in a M.S.E. 'Superspeed 50' centrifuge to remove cell debris and ribosomes. Protein in the supernatants was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). In a control experiment, portions of bacterial suspension were taken after 0 and 6 hr, centrifuged, and the organisms resuspended in 10 ml. buffer and broken with a Braun MSK cell homogenizer (Shandon Scientific Co.). After removal of debris and ribosomes, extracts prepared by this latter method were concentrated by pressure dialysis. Profiles of soluble protein were not significantly different from those obtained by sonication.

Polyacrylamide gel electrophoresis. An apparatus was used in which flat slabs of gel (about 16 × 18 cm.) were cast between plates cooled with tap water during electrophoresis. Up to 10 samples could be examined in the same gel from parallel slots. The gels were made from 7·5 % 'cyanogum 41' and 0·1 % (v/v) N,N,N′,N′-tetramethylethylenediamine in 150 ml. of tris (25 mM)-glycine (190 mM) buffer, pH 8·3, and were polymerized by adding ammonium persulphate (0·12 %, w/v). The same buffer was used in the electrode vessels. Electrophoresis was carried out for about 2 hr, at first with a current of 90 mA (200 V). During a run, the voltage rose to 400 V and was then kept constant; as a consequence, the current had decreased to about 60 mA by the end.

Extracts for electrophoresis were diluted when necessary with the tris-KCl-magnesium acetate buffer to 3 mg. protein/ml. To 0·1 ml. portions were added 10 mg. sucrose and 10 µl. 0·1 % (w/v) bromophenol blue. A sample (30 µl.) was loaded for electrophoresis. At the end of a run protein bands were stained by immersing the slab of gel for 1 hr in a solution of naphthalene black 12B (0·1 %, w/v) in acetic acid-methanol-water (2:5:13, v/v/v); excess stain was removed by washing with frequent

changes of 7·5 % (v/v) acetic acid. The gels were subsequently sliced and the protein patterns of the individual samples scanned by reflectance in a Joyce–Loebl 'Chromoscan' using the 5-042 filter.

RESULTS

Profiles from wild-type

Extract of the wild-type gave a pattern in which discrete protein bands were superposed on background stain. This background was minimized by a visual inspection but was much more apparent when gels were scanned photoelectrically in a semi-quantitative manner. More bands could usually be seen by eye than were clearly resolved by the densitometer. Figure 1 contains a visual representation of protein distribution for comparison with that given by a densitometer tracing of the same gel: some fine detail was lost in redrawing the densitometer curves.

Traces for samples derived from the wild-type organism after, 0, 1, 2, 4 and 6 hr incubation in the sporulation medium are shown in Fig. 1. The major peaks are numbered and components of the same mobility (confirmed by visual inspection of the gels) are given the same number in the sequential samples. There was a constant change of protein profile during incubation although some peaks (for example, 6, 9, 10) varied little in relative prominence. Some components (11, 12, 13) declined markedly in the samples taken at early times, and peaks 12 and 13 decreased throughout the experimental period. Peak 3 was most prominent in the sample taken at 2 hr; peak 8 also reached a maximum after 1 to 2 hr. Peak 18 was particularly noticeable in the 6 hr sample. New protein bands (e.g. peaks 16 and 17) appeared at different times and thereafter increased in prominence. The major protein band in all samples except the last was peak 7. At 6 hr this region of the profile was much less prominent and the major peak (19) had a mobility slightly greater than peak 7. This change was observed in a number of different experiments. The most striking change in these profiles was, however, in peak 1. At first this was minor, but it increased steadily in prominence until in the 6 hr sample it was the major component of the profile. Similar changes in peak 1 were found consistently in different experiments, as were many of the other alterations referred to above. However, the degree of resolution of these complex patterns varied from one electrophoresis run to another. It was therefore desirable to compare samples in the same gel slab. Extracts prepared from organisms grown for separate experiments also differed somewhat in their profiles because slight alterations in cultural conditions may have affected the proportions of the different proteins in the cytoplasm.

Profiles from mutant organisms

Figure 2 shows the densitometer traces of samples taken following incubation in the resuspension medium of the asporogenous mutant E22. Samples were examined in the same gel slab as those of Fig. 1. Where possible, peaks have been numbered to correspond with those of the same mobility in the wild-type strain (this equivalence was confirmed by visual comparison of the gels) but are distinguished by a superscript (′). The protein profile at zero time was very similar to that of the wild-type organism. Mutant E22 grew rather less well in the casein hydrolysate medium than the wild-type, so that some differences were to be expected. The samples taken after resuspension in sporulation medium showed marked changes, several of which were similar to those in the wild-type. For example, peaks 11′, 12′ and 13′ decreased in

later samples; peak 16′ was not visible at zero time but appeared subsequently; peak 3′ at first increased in relative intensity but then almost disappeared. However, the profiles of mutant E22 differed from those of the wild-type in several respects. For example,

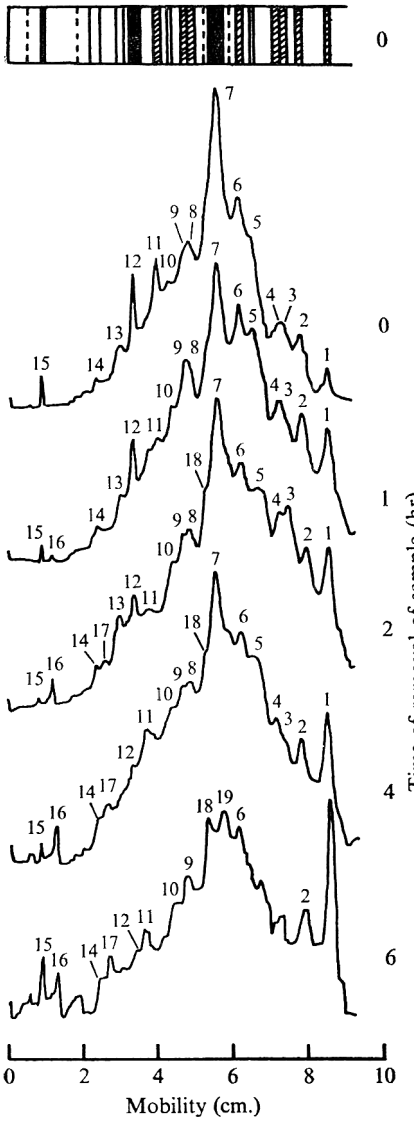

Fig. 1

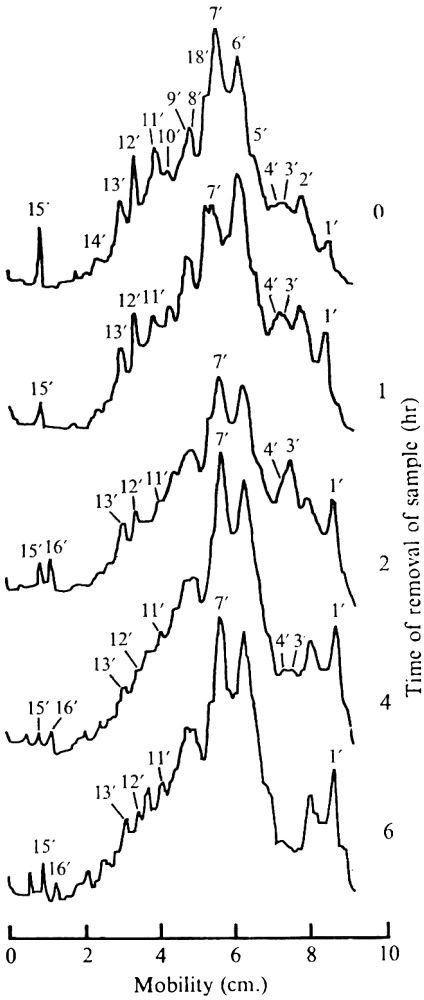

Fig. 2

Fig. 1. Profiles of soluble proteins from *Bacillus subtilis* Marburg strain 168 taken at different times after resuspension in sporulation medium. Preparation of samples and electrophoretic separation of proteins are described in the text. After electrophoresis, the bromophenol blue marker dye had migrated about 14 cm. Some very minor diffuse components of mobility greater than that of peak 1 are not shown. For the sample taken at zero time the densitometer trace is compared with a visual representation of the gel.

Fig. 2. Profiles of soluble proteins from mutant E22 taken at different times after resuspension in sporulation medium. Electrophoresis was in the same gel as that used in Fig. 1. Experimental procedures are given in the text.

peak 13′ was present in all samples of mutant E22 whereas in the wild-type the corresponding component was not resolved after 4 hr. The considerable changes in peak 7 of the wild-type that occurred between 4 and 6 hr did not take place in mutant E22; in general, the profile of this mutant after 6 hr differed much less from the previous (4 hr) sample than for the wild-type. This was evident when the overall distribution of proteins in the samples were compared but applied particularly to peak 1′. At 6 hr peak 1 was the major component in the wild-type and increased very considerably in prominence from 4 hr; in contrast, although peak 1′ at first increased, there was little difference between the 4 and 6 hr samples.

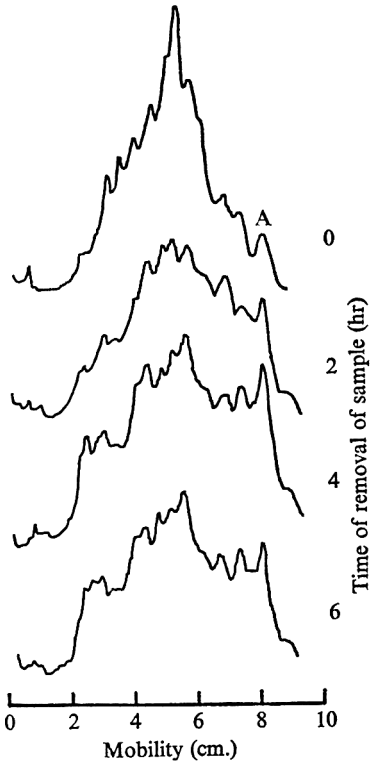

Fig. 3

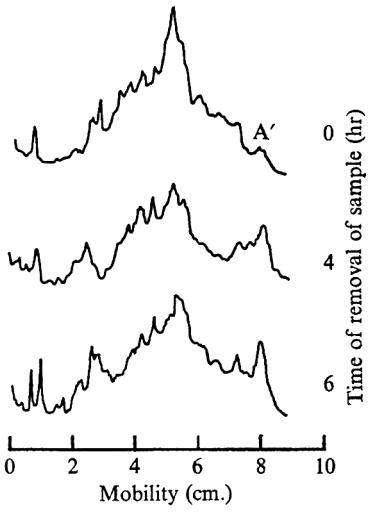

Fig. 4

Fig. 3. Profiles of soluble proteins from mutant E31 taken at different times after resuspension in sporulation medium. Experimental procedures were as for Fig. 1 and as described in the text.

Fig. 4. Profiles of soluble proteins from mutant A23 taken at different times after resuspension in sporulation medium. Experimental procedures were as for Fig. 1 and as described in the text, except that the cell-free extracts used for electrophoresis contained 1·5 mg. protein/ml.

Two other asporogenous mutants were examined in less detail. Results with E31 are in Fig. 3. This mutant is able to proceed as far as Stage IV of spore formation; sporulation processes should thus be almost normal for about 4 hr. The protein profile at zero time was similar to those of mutant E22 and the wild-type and changed considerably in the sample taken after 4 hr. This redistribution of proteins again involved an increase in the prominence of peak A which corresponds to peaks 1 and 1′

of Fig. 1 and 2. The 4 and 6 hr samples were extremely similar so that, as with mutant E22, the late changes apparently characteristic of the wild-type were not observed. Results with mutant A23 (Fig. 4) were rather similar in that the protein profile altered greatly during the first 4 hr. However, there were rather more changes in the following 2 hr than with the other two mutants. Peak A′ was minor at zero time and at first increased considerably. Although this component was rather more prominent in the 6 hr sample, the increase was much less than in the wild-type organism.

DISCUSSION

Electrophoresis of soluble proteins prepared at intervals from a sporogenic organism reveals a pattern of complex changes. These experiments show that electrophoresis in polyacrylamide gels provides a sensitive indication of changing cytoplasmic conditions during spore formation. Interpretation of the changes observed is rendered difficult by several factors. Although many components are satisfactorily resolved in the gels, each of these is likely to be a mixture of otherwise dissimilar proteins. Moreover, like many other analyses of events during spore formation, the relevance of the observed changes to sporulation *per se* is unknown; for example, some of the alterations in the profiles could be a consequence of the growth conditions necessary to induce commitment. An unexpected finding is that, during incubation of mutant E22 in sporulation medium, considerable changes occurred in the profiles during the first 4 hr, some of which closely parallelled those observed in the wild-type strain. Mutant E22 is, as stated in Methods, thought to be unable to participate in the first stages of spore formation and to synthesize none of the proteins associated specifically with the various morphological events (Warren, 1968). In particular, this mutant lacks the protease normally responsible for a high rate of protein turnover in organisms in sporulation medium (Mandelstam & Waites, 1968). The considerable redistribution of proteins in the absence of turnover is presumably made possible by a slow increase in net protein content under the conditions of our experiments. The close association of the protease with the ability to sporulate is puzzling and factors that may be involved have been discussed by Mandelstam (1969). That there are considerable changes in the profiles of mutant E22 similar to some of those found in the wild-type suggests that many of the alterations observed in all the organisms tested may be more a consequence of altered nutritional conditions consequent upon resuspension of organisms in sporulation medium than a result of changes directly concerned with sporulation itself.

The major difference noted between the mutants and the wild-type was that profiles from the former after 4 and 6 hr differed little; in particular, the large increase in peak 1 characteristic of the wild-type did not occur. The increase in prominence of this electrophoretic component may therefore be more directly related to events in sporulation than some of the changes observed. But because the mutants can effect some increase in the proportion of material of this mobility during the early stages of incubation, the relevance of this observation to spore formation requires further clarification. Moreover, the time during incubation at which the 'freezing' of profiles of soluble protein takes place is not directly related to the point in spore formation at which, on biochemical and morphological grounds, the mutants are thought to be blocked. Thus, although asporogenous mutants differ from the wild-type in their behaviour, these

differences are not yet sufficiently well defined to enable mutants to be ordered by an examination of their profiles of soluble protein.

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Lysogenic Conversion of *Rhizobium trifolii*

By YVONNE M. BARNET AND J. M. VINCENT
School of Microbiology, University of New South Wales, Kensington, New South Wales 2033, Australia

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SUMMARY

Rhizobium trifolii strain SU 297, when lysogenized with phage 7 or its clear-plaque mutant 7*cr*, underwent lysogenic conversion that resulted in loss of ability to adsorb phages 7 and 7*cr* and the related phage 8. The same conversion was reflected in changes in the surface of the bacterium by which a somatic antigen, characteristic of the parent strain, was modified and a new, non-crossreacting, antigen produced.

INTRODUCTION

The nature of their somatic antigens is one of several properties of bacteria that have been found to be subject to 'lysogenic conversion', i.e. modified as a result of the presence of a phage genome within the host. Almost all of these investigations have been concerned with the somatic antigens of Salmonella and have been comprehensively reviewed by Lüderitz, Staub & Westphal (1966). One case involving a strain of Pseudomonas has also been reported (Holloway & Cooper, 1962). The present paper arises from a further investigation of a defective lysogen (*Rhizobium trifolii* strain SU 298) which requires an inducing phage (phage i_s) liberated from *R. trifolii* strain SU 297, for the production of active phage (Takahashi & Quadling, 1961; Barnet, 1969). In the course of this work it was found that *R. trifolii* strain SU 297 had its antigenic surface modified when it was lysogenized by phage 7 (one of two phages released by the induction of *R. trifolii* strain SU 298), or by phage 7*cr* (a clear-plaque mutant of phage 7).

METHODS

Bacteria and bacteriophage. Rhizobium trifolii strain SU 297/32 was used as a representative of the small-colony, non-gummy variant of SU 297 (Vincent, 1962). The lysogens of SU 297/32 have been designated SU 297(7) and SU 297(7*cr*), involving phages 7 and a clear-plaque mutant, 7*cr*, respectively. Phage 7 was obtained by the induction of the defective lysogen (*R. trifolii* strain SU 298) with phage i_s, and detected by means of plaque formation on SU 297. Phage 8, obtained similarly from SU 298, formed plaques on the strain from which it was derived (Takahashi & Quadling, 1961). We have reverted to the original numbering of these hosts with which Marshall (1956) first demonstrated lysogenicity in the genus *Rhizobium*.

Media and cultivation. Phage broth, used for liquid culture and as the diluent for phage and bacterial dilutions, had the composition: 2·5 g. sucrose; 0·5 g. K_2HPO_4; 0·2 g. $MgSO_4.7H_2O$; 0·1 g. NaCl; 0·16 g. $CaSO_4$; 0·02 g. $FeCl_3$; 0·5 g. Oxoid yeast

extract; distilled water, 1 l. Mannitol nitrate agar, used for the poured plates for phage assay, contained: 0·45 g. $Na_2HPO_4.12H_2O$; 0·06 g. $Na_2SO_4.10H_2O$; 0·6 g. KNO_3; 0·01 g. $FeCl_3$; 10·0 g. mannitol; 0·06 g. $CaCl_2$; 0·1 g. $MgCl_2.6H_2O$; 100 μg. thiamine hydrochloride; 0·5 μg. biotin; 7·5 g. Oxoid 'Ionagar'; distilled water, 1 l. The bacteria used as a source of antigens and for the absorption of agglutinins were grown in liquid mannitol nitrate medium. Incubation was carried out at 26°. Liquid cultures were grown in flasks shaken in a bath.

Adsorption of bacteriophage. The bacterial suspension was freed from observable clumps by centrifugation for 5 min. at 1200 *g* and the supernatant standardized to contain between 250×10^6 and 500×10^6 bacteria/ml. The bacteriophage suspension was obtained by incubating the infected bacteria overnight in low salt broth (0·25 % sucrose, 0·05 % Oxoid yeast extract) to avoid difficulties due to clumping and inhibition of liberated phage (Barnet, 1969). Residual bacteria were removed from the lysate by membrane filtration. Adsorption was carried out at 26° and the reaction stopped at the required times by 100-fold dilution of the samples in ice-cold broth containing 10 % (v/v) chloroform to kill the bacteria. Bacteria and adsorbed phage were removed by centrifugation and the residual titre of the supernatant determined in the usual way.

When using a lysogenic strain of SU297/32 it was necessary to distinguish residual (unadsorbed) phage from the normal background count of phage produced spontaneously from the lysogen. When the added and resident phage were of different plaque types (phage 7 and 7*cr*) the distinction could be made directly. Otherwise a control had tc be set up to establish the background count due to spontaneous induction.

Serological methods. Antisera against whole or broken bacteria were produced in rabbits using a combination of intramuscular and intravenous injections (Humphrey & Vincent, 1965). Quantitative absorption of agglutinins followed the method of Vincent & Humphrey (1968). Whole cell antisera were diluted with saline (0·85 % NaCl) to give an initial standard agglutinating titre of 400. The absorbing cells were grown for 72 hr at 26° in mannitol nitrate medium, sedimented, washed with distilled water and resuspended in saline to give an initial concentration of 300 μg. dry wt/ml. The required absorbing doses were then sedimented from appropriate volumes of the initial suspension, resuspended in antiserum diluted 1/25, shaken for 1 hr at 37° and held at 4° for 18 hr. The bacteria were then sedimented and the residual somatic agglutination titre of the supernatant determined with the strain of bacterium that had been used for developing the antiserum under test. Gel diffusion followed the detailed procedure of Humphrey & Vincent (1965), based on that adapted for use with Rhizobium by Dudman (1964). Oxoid Ionagar no. 2 (0·75 %) was used with 0·85 % NaCl and 0·25 % sodium azide to give a layer 4 mm. deep. Wells were 4 mm. diameter and 8 mm. apart. The test bacteria were sedimented, resuspended in saline, broken by shaking with ballotini beads on the Mickle disintegrator for 20 min. at 4° and used as antigen against undiluted antisera.

RESULTS

Adsorption of phage

Lysogenization of *Rhizobium trifolii* strain SU 297/32 with phage 7 prevented adsorption of phages 7, 7*cr* and 8 (Fig. 1 *a* to *c*). Similarly, lysogenization with phage 7*cr* prevented the uptake of phage 7 (Fig. 1 *d*). These results with the two independently developed, though related, lysogens indicate that lysogenization was indeed responsible for the alteration of the receptor sites of the parent bacterium.

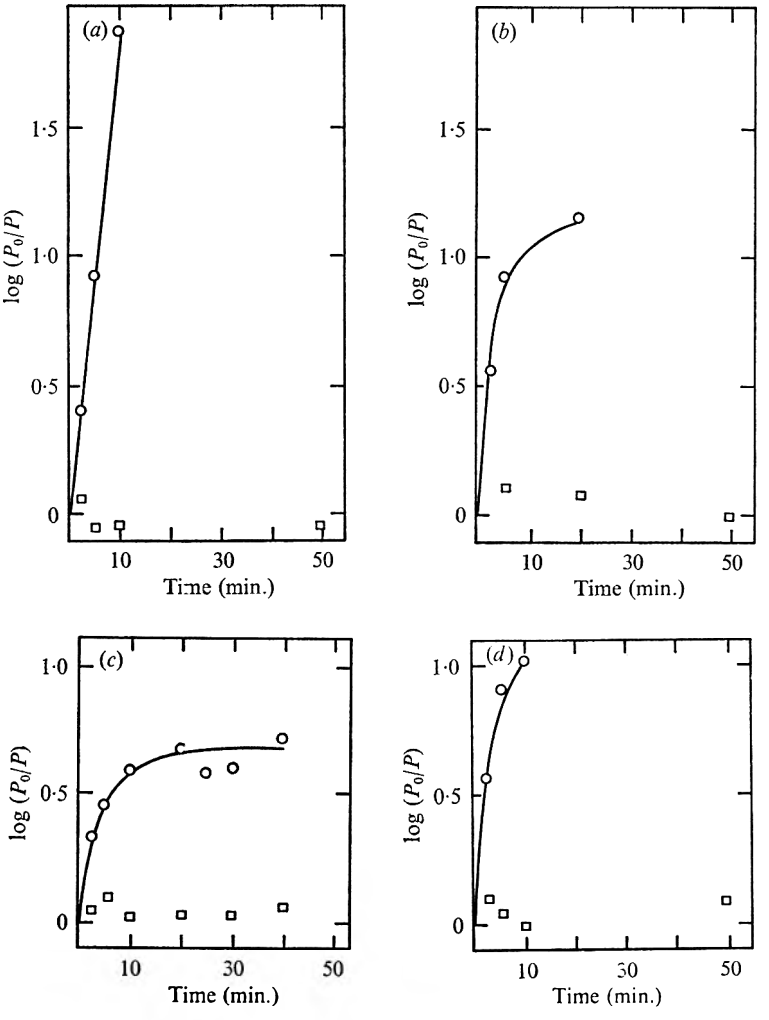

Fig. 1. Effect of lysogenization on the adsorption of phage 7 (*a*), phage 7*cr* (*b*) and phage 8 (*c*) to *Rhizobium trifolii* strain SU 297/32 (○), and strain SU 297(7) (□). In (*d*), phage 7 adsorbed to strain SU 297/32 (○) and strain SU 297(7*cr*) (□). P_0 phage titre at commencement, P = phage titre after t min.

Surface antigens

It seemed likely that changes in receptor sites would be accompanied by changes in the surface antigens of the bacterium. This was tested by the technique of quantitative absorption of agglutinins and by gel diffusion. Figure 2 shows the reciprocal quantitative absorption test with the two kinds of bacteria and their antisera. From Fig. 2*a* (antiserum for non-lysogenized strain SU 297/32) it can be concluded that the lysogenized culture had sufficient of the same, or similar, antigen as the non-lysogenized to remove virtually all of agglutinins when used in a sufficiently large absorbing dose (> 480 μg./ml.). However, the nature of the homologous and non-homologous curves in the range of less concentrated absorbing dose shows that in strain SU 297(7) the responsible antigen after lysogenization is either qualitatively different or less concentrated than in the parent strain.

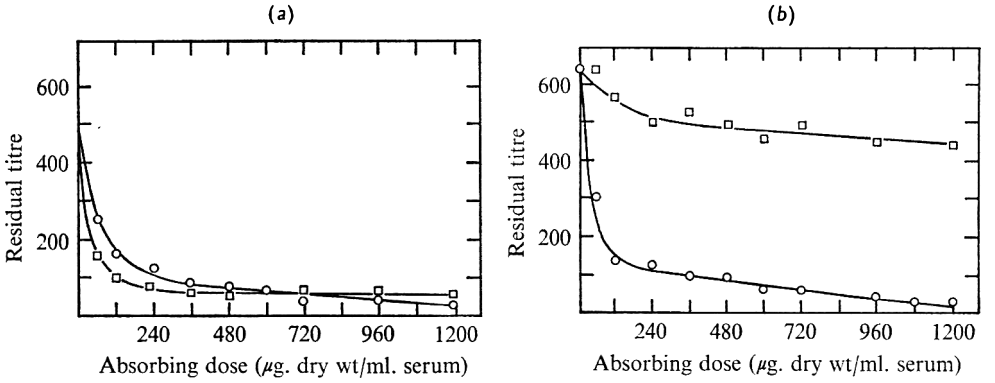

Fig. 2. Absorption of antiserum to *Rhizobium trifolii* strain SU 297/32 (*a*) or strain SU 297(7) (*b*), with *R. trifolii* SU 297/32 (□) and SU 297(7) (○).

The difference in the reciprocal test (using antiserum prepared against the lysogenic strain SU 297(7); Fig. 2*b*) is more striking. The non-lysogenized strain (SU 297/32) left a large amount of antibody unabsorbed. showing that lysogenization had resulted in the appearance of at least one new antigenic determinant. In addition, the common antibody was removed less efficiently. If the difference in the homologous and non-homologous curves of Fig. 2*a* had resulted from quantitative differences in a common antigen, the non-lysogenic strain SU 297/32 should have remained the more efficient absorbent in this reciprocal experiment. The results therefore support a qualitative difference in the cross-reacting antigens of lysogenized and non-lysogenized strains.

Diffusing antigens

Gel diffusion between broken rhizobia as antigen and the corresponding antisera yield precipitation lines that have been identified (Humphrey & Vincent, 1965) with somatic antigens ('a' lines nearest the antigen well) and internal antigens ('b' and 'c'). This technique was applied to obtain more information about changes in antigenic properties caused by lysogenization. Figure 3 shows the 'a' line patterns obtained with graded amounts of non-lysogenized and lysogenized bacteria against the antisera to each. The 'b' and 'c' lines were unaffected and have been omitted to simplify the diagram.

It can be concluded from the continuity of the line at higher antigen concentrations in Fig. 3*a* that the lysogenized strain carried a similar somatic antigen to that of the non-lysogenized parent. However, the failure of the line to appear at lower concentrations (20 and 5 mg./ml.), like the agglutinin absorption evidence, indicated that this antigen was either less concentrated in the lysogenized culture or had less affinity for antibodies formed against the normal strain. Spur formation in the reciprocal test (Fig. 3*b*) supported evidence from quantitative absorption that lysogenization had led to the production of a new antigenic group. This conclusion was substantiated by the diffusion pattern of Fig. 4, in which the diagonal line reflected an antigen unique to the lysogenized strain.

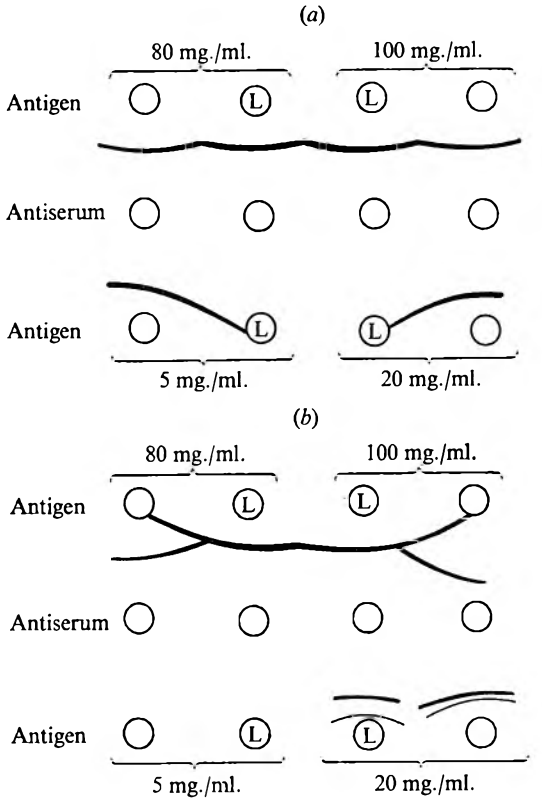

Fig. 3. Schematic representation of gel-diffusion patterns using antigenic material from *Rhizobium trifolii* strain SU 297/32 and strain SU 297(7) and antiserum against (*a*) *R. trifolii* strain SU 297/32 and (*b*) SU 297(7). Broken cells were suspended in saline to the concentrations shown (dry wt/ml.). Wells shown as open circles; those containing material from lysogenic bacteria (SU 297(7)) labelled L.

DISCUSSION

Phage 7 released from *Rhizobium trifolii* strain SU 298 after exposure to phage i_s, for which strain SU 297 is already lysogenic, is probably the result of the formation of a genetic recombinant between defective prophage carried in SU 298 and phage i_s (Barnet, 1969). The fact that phage 7 adsorbs normally on both SU 298 and normal SU 297 shows that neither the prophage precursor to phage 7 (carried by SU 298) nor

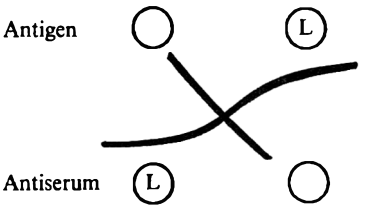

Fig. 4. Schematic representation of gel diffusion patterns obtained with broken cells of *Rhizobium trifolii* strain SU297/32 and strain SU297(7) (100 mg. dry wt/ml.) diffused against antisera to SU297/32 and SU297(7). Wells shown as open circles. L = wells con-containing antigenic material from or antiserum against lysogenic bacteria (SU297(7)).

phage i_s (for which SU297 is already lysogenic) is able to cause this modification of the surface of these strains. It is possible that genes necessary for such surface modification are in fact present in the prophage of SU298 but are not expressed in that host. On the other hand, genetic information might have to be provided by both prophages, as could operate in SU297 lysogenized by phage 7. The first situation is found in the results of Uetake, Nakagawa & Akiba (1955) and LeMinor, Ackermann & Nicolle (1963); the second in those of Uetake & Hagiwara (1961), who reported that two prophages (ϵ^{15} and ϵ^{34}) were needed in *Salmonella anatum* before antigen 34 was synthesized.

The parallel loss of phage-adsorbing capacity and the change in surface antigen found with lysogenized SU297 suggests that the receptor site for phages 7, 7*cr* and 8 is associated with the lipopolysaccharide somatic antigen of these bacteria (Humphrey & Vincent, 1969). Receptor sites for several T phages and Shigella phages have been found in the lipopolysaccharide surface layer (Jesaitis & Goebel, 1955; Beumer, Beumer-Jochans, Dirkx & Dekeil, 1966) and some earlier work with rhizobial phages showed a relationship between antigenic constitution and susceptibility to specific phages (Marshall & Vincent, 1954).

Modification of the phage sensitivity of cells by lysogenic conversion of the phage receptors has already been reported, for example in a group E_1 strain of Salmonella, after infection with phage ϵ^{15} and subsequently with phage ϵ^{34} (Uetake, Luria & Burrows, 1958). As pointed out by Neubauer (1967), such conversions may provide a safeguard, additional to that of cytoplasmic immunity in lysogenic cells, preventing lysis by superinfecting virulent mutants of the homologous phage. Phage maintained in such protected lysogenic cells would have advantage in survival. Conversion of the immune lysogenic cell to a non-adsorbing form would also increase the efficiency of phage multiplication by preventing wasteful abortive infections.

We are indebted to Mrs B. A. Humphrey of this Department for advice on the techniques of quantitative absorption and gel diffusion, and for helpful discussion as to their interpretation. One of us (Y.M.B.) carried out this work as Commonwealth Post-graduate Scholar.

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Invertase and Disulphide Bridges in the Yeast Wall

By D. K. KIDBY* AND R. DAVIES
Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge

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SUMMARY

Invertase released from *Saccharomyces fragilis* (Jorgensen) by either breakage or thiol treatment exhibited identical electrophoretic characteristics. Evidence was obtained against an indirect chemical effect of breakage on the release of invertase from yeasts. Electron microscopy revealed no obvious differences between walls of *S. fragilis* and other yeasts. A model is proposed in which invertase is not bonded to the wall but is retained by structures requiring intact S—S linkings.

INTRODUCTION

There is no reason to doubt that yeast invertase is mainly associated with the wall (Myrback, 1960). However, opinion differs on whether or not this enzyme is associated with the wall by chemical bonds or is simply contained by a permeability barrier. The former view has been most recently expressed in a model for the yeast wall (Lampen, 1968) in which either a covalent linkage to the wall phosphomannan components is affected *via* the mannan moiety of the enzyme or other non-covalent bonds hold the enzyme in place. The latter view (Burger, Bacon & Bacon, 1961) is founded upon the readiness with which the enzyme is released by breakage but retained in ethyl acetate-treated organisms (Burger, Bacon & Bacon, 1958).

This paper describes results relevant to the elucidation of the nature of the association of invertase with the yeast wall. The observations of other workers, which have not previously been clearly related to this question, are considered. A model for the yeast wall (Kidby & Davies, 1968) is discussed.

MATERIALS AND METHODS

Yeasts. Saccharomyces fragilis (Jorgensen) was grown as previously described (Kidby & Davies, 1970). *Saccharomyces cerevisiae* was obtained as compressed baker's yeast from C. Holland & Sons, Cambridge, and Sigma Chemical Co., St Louis, Mo., U.S.A., but essentially the same results were obtained for yeasts from both of these sources.

Electrophoresis. Invertase was released by treating suspensions with 5 mM-2-mercaptoethanol at pH 8·0 for 30 min. at 30°, which released 88 % of the total enzyme; or by treatment in a sonic oscillator at a 500 W input for 60 sec. at 10°, which released 84 % of the total enzyme. Suspensions were then centrifuged at 4° and 40 µl. samples of

* Present address: Department of Microbiology, University of Guelph, Guelph, Ontario, Canada.

supernatant fractions, diluted with an equal volume of a 10 % (w/v) solution of mannitol in the electrophoresis buffer, were applied to 5·2 % (w/w) polyacrylamide gel (Cyanogum 41, British Drug Houses, Poole, Dorset) columns. The samples were electrophoresed in a discontinuous buffer system containing 0·3 M-boric acid, adjusted to pH 7·0 with sodium hydroxide, in the cathode tank; and 0·05 M-tris buffer, adjusted to pH 7·5 with citric acid, in the anode tank. A potential of 170 V was applied for 2·5 hr to obtain a substantial migration of the enzyme (*c.* 6 cm.). The gels were then extruded and sectioned into 0·2 cm. fractions, which were eluted by shaking overnight in 1·0 ml. of 0·2 M-acetate buffer (pH 5·0). The eluants were assayed for invertase activity by the method described previously (Kidby & Davies, 1970).

Preparation of disrupted and untreated mixtures of organisms. A 0·6 % and 0·4 % (dry wt) suspension of *Saccharomyces cerevisiae* and *S. fragilis* respectively were treated in a sonic oscillator at a 130 W input for 5 min. at 10°. This treatment released 36 and 43 % of the invertase respectively but broke very few organisms. Supernatant and sedimented fractions were obtained by centrifugation at 30,000*g* for 30 min. at 4°. Equal volumes of the unfractionated treated organisms, the supernatant from fractionated treated organisms, or the resuspended sediment from treated organisms were incubated with untreated organisms of each species at either 4 or 30° for 30 min. Following incubation the total invertase released was determined in yeast-free filtrates as previously described (Kidby & Davies, 1970). Single components of the above mixtures were also incubated to calculate the quantity of invertase released from the untreated organisms by the treated organisms.

Electron microscopy. Material for sectioning was fixed according to the Glauert & Thornley (1966) modification of the Ryter–Kellenberger technique and embedded in Epon (Kidby & Goodchild, 1966), except that prefixation with glutaraldehyde was omitted for half of the material sampled. This omission appeared to yield a better definition of intracellular detail but this distinction was not critically examined. Negatively stained material was first treated in a sonic oscillator at a 500 W input for 60 sec. at 10°. The disrupted yeasts were washed with 0·1 M-sodium chloride three times, then once in deionized water. The sediment was resuspended in 0·2 % (w/v) phosphotungstic acid, neutralized to pH 7·0 with KOH, and samples dried on to grids.

Specimens were photographed at a magnification of ×4000 using 35 mm. film on a Philips EM 75 microscope.

RESULTS

The electrophoretic mobilities of invertase released by either disruption or thiol treatment were identical (Fig. 1.) Recoveries of enzyme activity from each gel were also identical. A low mobility system in which the enzyme was electrophoresed in a cationic form also yielded identical mobilities but as the mobilities were extremely low, this was not acceptable as convincing evidence of similarity.

The single activity peak encountered on electrophoresis of these samples was repeatable when the enzyme was electrophoresed within a day or two of preparation. Storage of unfrozen enzyme for longer periods produced electrophoretically distinguishable species. Purification did not seem to overcome this problem as the fractionation procedures themselves appeared to induce heterogeneity (D. K. Kidby, unpublished).

Yeast which had been mechanically disrupted did not release invertase from either untreated *Saccharomyces cerevisiae* or *S. fragilis*, when incubated with these for 30 min. at either 4 or 30°.

The sectioned yeasts revealed walls of variable thickness but examples of thin-walled yeasts (Pl. 1, fig. 1) were rare. The pronounced convolutions at the inner margin of the wall were common but were characteristic of thick rather than thin walls (Pl. 1, fig. 2). Yeasts which had been subjected to sonic oscillation for short periods frequently exhibited large tears but the walls retained considerable structural integrity, as indi-

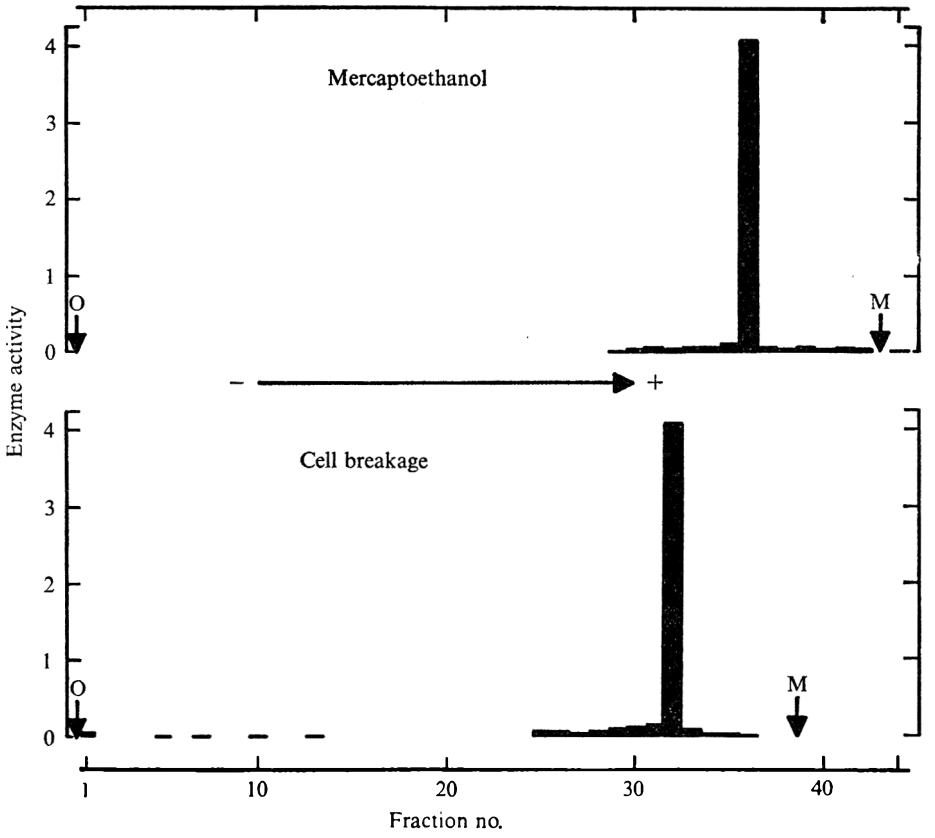

Fig. 1. Electrophoretic mobility of invertase released by either mercaptoethanol or cell breakage. O, Origin; M, position of anionic marker dye (bromophenol blue); Fraction, eluant from 0·2 cm. lengths of gel; Enzyme activity, μmoles reducing sugar produced/assay tube.

cated by retention of shape (Pl. 1, fig. 3), The majority remained unbroken, as indicated by retention of cytoplasmic contents, even though most of the invertase was released.

DISCUSSION

The equivalent electrophoretic mobilities of invertase released by either thiol treatment or by sonic disruption indicate that the release mechanism is essentially equivalent in each case. If, for example, there is a covalent linkage between the enzyme and the wall, then the same linkage would appear to be broken by both thiol treatment and by sonic disruption. While this possibility cannot be rejected, it would be a remarkable coincidence.

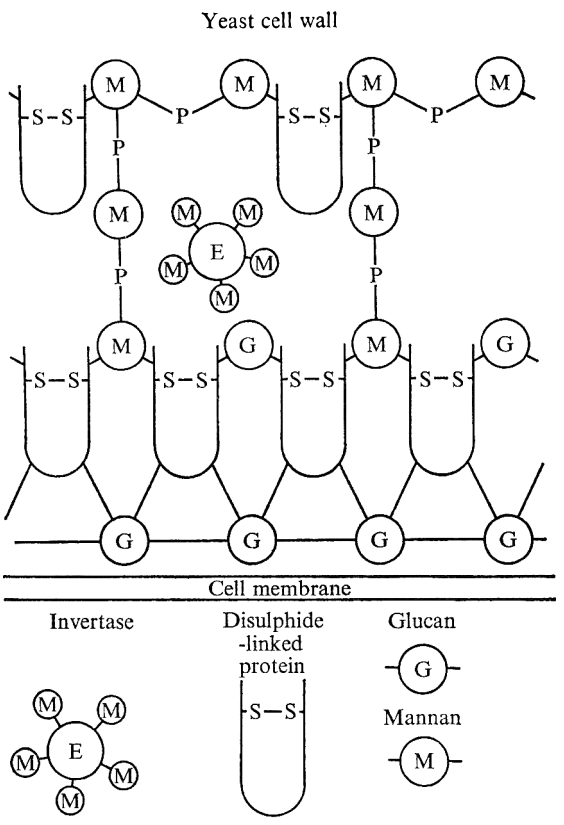

Fig. 2. A model for the yeast cell wall. The inner region of the wall is identified by the adjacent cell membrane. —P—, Phosphodiester link; other essential features are identified at the foot of the figure.

Invertase release resulting from the action of a chemical factor released by either mechanical disruption or thiol treatment was considered as a possible explanation for the apparent similarity between the release mechanisms. However, the failure of disrupted yeasts to release invertase from untreated yeasts is evidence against release of a chemical factor.

Sectioned *Saccharomyces fragilis* reveals features similar to those of other yeasts (Phaff, 1963). The wall region was not obviously different from that of *S. cerevisiae* (Agar & Douglas, 1955) or *Schizosaccharomyces octosporus* (Conti & Naylor, 1959). The walls varied in thickness but no more than those of *S. cerevisiae* (R. Sentandreu, personal communication). It therefore appears that neither the fragility nor the susceptibility to thiols (Davies, 1966) of *S. fragilis* can be attributed to thin walls.

The retention of a high molecular weight invertase in Neurospora is correlated with wall permeability (Trevithick & Metzenberg, 1966; Trevithick, Metzenberg & Costello, 1966) and suggests that an impermeable wall structure may be an effective barrier. It may therefore be unnecessary to invoke wall-to-enzyme bonds, as is suggested by Lampen (1968), for retention of wall enzymes. The operation of a permeability barrier is also supported by the suggested release mechanism of invertase from thiol-treated cells of *Saccharomyces fragilis* (Kidby & Davies, 1970). The thiol- and KCl-induced

invertase release from yeasts, observed by Weimberg & Orton (1966), is difficult to interpret but is possibly a related phenomenon. Studies on the digestion of yeast wall have frequently employed thiols to sensitize the wall to the attacking enzyme. Davies & Elvin (1964) showed that the thiol acted upon the wall rather than enhanced the activity of the digesting enzyme. Bacon, Milne, Taylor & Webley (1965) established that thiol treatment rendered regions of the wall containing mannose and glucose more susceptible to enzyme digestion. The simplest interpretation of this is that greater quantities of the substrate are physically accessible to the enzyme. This explanation is supported by the observation that autoclaving yeasts also enhanced enzyme attack (Bacon *et al.* 1965).

The foregoing considerations together with the model for the yeast wall proposed by Lampen (1968) led to the proposal of a modified model (Kidby & Davies, 1968). The major modifications contained in this model (Fig. 2) are: (i) an invertase not chemically bonded to the wall, in accordance with our own observations and those of Burger *et al.* (1961); and (ii) an external region in the wall which depends upon disulphide bridges for maintenance of its structural integrity as a permeability barrier to large molecules. The distribution of these disulphide bridges between inter- and intramolecular linkages is unknown and the model does not elucidate this question. The phosphodiester linkages are retained in accordance with the model of Lampen (1968), as are other features of the model. Observations on the degradation of walls by enzymes have led to the suggestion of a 'third membrane' (Bacon *et al.* 1965). However, provided a system exists to maintain disulphide bridges in either the oxidized or reduced state, according to physiological demand (see Nickerson, 1963), there is no reason to invoke a third membrane.

Because invertase is not released from *Saccharomyces cerevisiae* (Davies, 1966) it might be argued that the assumptions upon which the current model is based do not apply generally. However, thiols increase the accessibility of underlying regions of the wall of *S. cerevisiae* to enzyme attack (Bacon *et al.* 1965) and there is independent evidence for the invertase of *S. cerevisiae* being not bonded to the wall (Burger *et al.* 1961). Furthermore, there is evidence to suggest that the *S. fragilis* invertase escapes more readily than that of *S. cerevisiae* because the enzyme molecule is smaller. The electrophoretic mobility of the *S. fragilis* extracellular enzyme approximates the high mobility intracellular low molecular weight enzyme described by Gascon, Neumann & Lampen (1968). The *S. fragilis* invertase also differs from invertase of other yeasts in its substrate specificity (Snyder & Phaff, 1960).

Disulphide bridges in the outer regions of the wall and free invertase confined by a permeability barrier provide a basis for explaining all of the previously observed effects of thiols on yeast walls. It is conceivable that retention of extracellular enzymes by an impermeable wall is not peculiar to yeasts; the evidence that wall permeability is involved in Neurospora was mentioned earlier. Bacterial walls also offer similar possibilities since wall permeability (Mitchell, 1959; Gerhardt & Judge, 1964) appears to be limiting for particles of protein size. The release of surface enzymes from bacteria (Heppel, 1967; Neu & Chou, 1967; Rogers, 1968) may reflect wall impermeability. The concept of the wall as a molecular sieve has been suggested by Payne & Gilvarg (1968) on the basis of differential permeation of peptides in *Escherichia coli*.

The extent to which disulphide bridging may contribute to such impermeability is conjectural but this appears to be a possibility in cases where wall protein layers form

effective barriers (Weinbaum, Rich & Fischman, 1967) or where thiols facilitate the release of protein from a wall (Sargent, Ghosh & Lampen, 1968).

The helpful comments of Dr R. Sentandreu and Dr D. H. Northcote are gratefully acknowledged. One of us (D.K.K.) is indebted to the Commonwealth Scientific and Industrial Research Organization of Australia for a post-doctoral studentship, and to Professor E. F. Gale, F.R.S., for support and research facilities.

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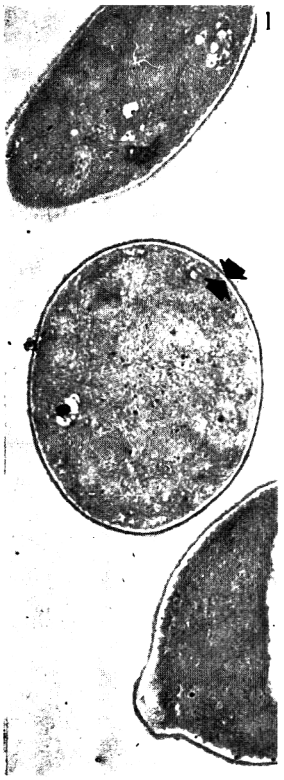
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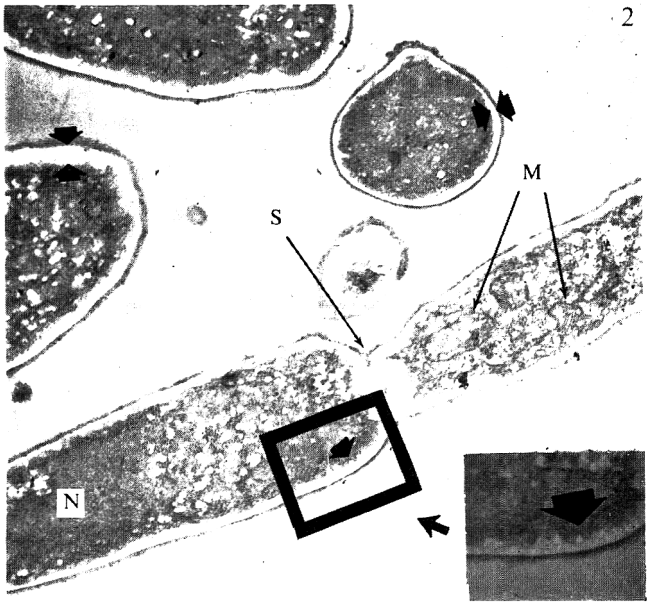
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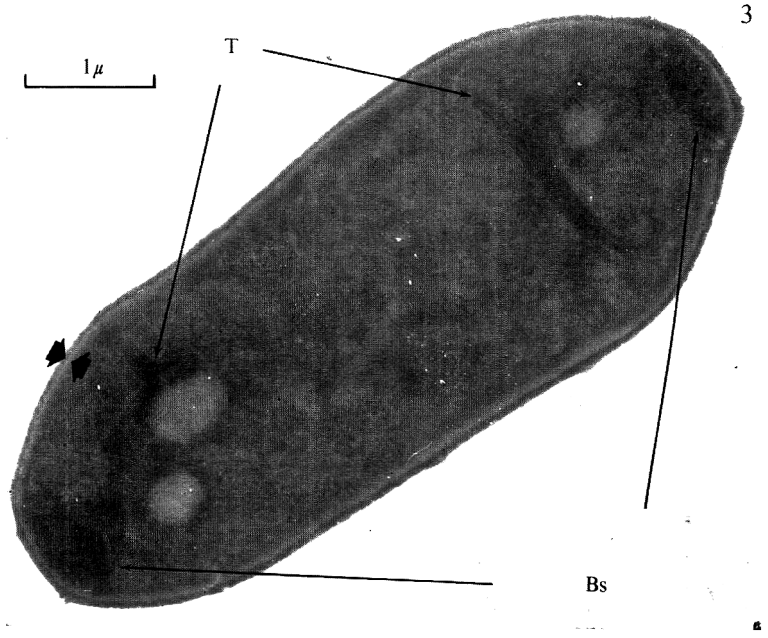
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EXPLANATION OF PLATE

Electron micrographs of cells fixed in 2% (w/v) osmium tetroxide and stained with uranyl acetate or negatively stained with phosphotungstic acid.

Symbols: S, septum; M, mitochondrion; N, nucleus; T, tears in cell wall; Bs, bud scars.

Fig. 1. Sectioned cell exhibiting a thin cell wall.

Fig. 2. Sectioned cell exhibiting typical variations of cell-wall thickness. An example of convolution of the inner cell-wall margin is shown in inset.

Fig. 3. Negatively stained sonic oscillator-treated cell exhibiting tears in cell wall but retention of gross structure.

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Biochemical Studies on Walls Synthesized by *Candida utilis* Protoplasts

By MONIQUE NOVAES-LEDIEU
AND CONCEPCION GARCIA-MENDOZA
Instituto de Biologia Celular, C.S.I.C. Madrid-6, Spain

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SUMMARY

Protoplasts of *Candida utilis* obtained by treatment with helicase built a new wall in a liquid medium. The spherical protoplast was converted to a tubular form which later changed to an ellipsoidal yeast. Walls isolated at different stages of regeneration were analysed. Glucan (42 to 48 %), mannan (25 to 31 %) and protein (8 to 9 %) were the main constituents of walls of normal and completely regenerated ellipsoidal forms. Walls of the tubular forms differed markedly by having a high content of chitin (12 to 18 %) and of protein (20 %); mannose was present only as traces. The glucan content was similar in normal, tubular and ellipsoidal regenerating yeasts. These results demonstrate that some modifications occur in the structural polysaccharides of the yeast wall which can be correlated with morphological changes in the reversion process. The significance of glucose, mannose and acetylglucosamine polymers is discussed in relation to the biosynthesis of regenerated walls.

INTRODUCTION

Necas (1956) and Eddy & Williamson (1959) found that yeast protoplasts were capable, under well defined conditions, of forming new wall structures and of regeneration. However, relatively few chemical studies on this phenomenon have been reported. Recently, Garcia-Mendoza & Novaes-Ledieu (1968) described the *de novo* synthesis of wall structures by *Candida utilis* protoplasts incubated in a liquid medium and noted a high content of chitin in walls of the first regenerating tubular forms. The aim of the present study was to investigate the composition of the wall synthesized newly by protoplasts throughout the regeneration process and to discuss the chemical differences in relation to the morphology.

METHODS

Growth conditions and preparation of protoplasts

As in our previous experiments (Garcia-Mendoza & Novaes-Ledieu, 1968), *Candida utilis* CECT 1061 was grown in Winge medium in a reciprocal shaker at 28°. Yeast was harvested by centrifugation at 10,000 *g* for 10 min., when an optical density of O.D.$_{600\,m\mu}$ of 0·6 was reached, and washed with distilled water. Then the yeast was washed once with 0·01 M-2-mercaptoethanol in 0·01 M-citrate buffer (pH 5·8). Treated yeast was washed twice with this buffer, resuspended in the digestion medium containing 0·8 M-mannitol in citrate buffer (pH 5·8), containing 1 to 2 mg./ml. dried helicase

(Industrie Biologique Française, Gennevilliers, France) and shaken at 28°. After 3 hr all the yeast was converted into protoplasts as seen by examination under a phase microscope.

Cultivation of protoplasts in nutrient medium

Protoplasts prepared from different quantities (5 to 100 mg. dry wt) of normal yeast, prepared as described above, were washed twice with 0·8 M-mannitol, suspended in 500 ml. of Winge medium containing 0·55 M-$MgSO_4 . 7H_2O$ (iso-osmotic for this strain) and incubated at 28° with gentle shaking. In a series of experiments the initial protoplast inoculum used was minimal (about 5 mg. dry wt) and regenerating yeast was collected after 18, 30, and 64 hr of incubation. In other series larger amounts of initial protoplast inoculum (about 100 mg. dry wt) were used and samples were collected after 4, 18, and 36 hr; moreover, regenerating yeasts in their final logarithmic phase (after about 20 hr incubation) were centrifuged and a portion of the deposit was incubated again for 6 or 16 hr in a fresh Winge medium to obtain sufficient yeast at different stages of logarithmic reproduction. During incubation, wall regeneration and cell budding was followed under a phase microscope.

Preparation of walls

Yeast at different stages of regeneration was collected by centrifugation and treated in a Braun cell homogenizer using no. 12 ballotini beads. To purify the material, the crude wall preparations were suspended several times in 60 and 10% sucrose in water and centrifuged at 10,000 *g* for 20 min. Subsequent treatments with 10 and 1% NaCl were performed. Finally, the walls were washed with distilled water and dried in a vacuum.

Analytical methods

Nucleic acids were estimated from the ultraviolet spectra of extracts obtained by treating samples with 0·5 N-perchloric acid at 75 to 80° for 30 min. (Gale & Folkes, 1953). All preparations showing more than 0·5% nucleic acids were eliminated because they could have been contaminated with cytoplasmic materials.

Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) performed on cold N-KOH extracts of walls: casein was the reference standard.

Amino acids were characterized by chromatography of hydrolysates (6 N-HCl at 105° for 24 hr) on Whatman paper no. 1; excess HCl was removed in vacuum over NaOH. Chromatograms were run for 18 hr with *n*-butanol:formic acid:water (75:15:10, v/v); amino acids were detected with ninhydrin (0·2 %, w/v) in acetone. Three groups of amino acids: arginine, lysine and histidine; aspartic acid, serine and glycine; glutamic acid and threonine were eluted separately with water from chromatograms and characterized by electrophoresis on Whatman no. 1 paper at pH 11·5, 2·25 and 6·5 respectively.

Neutral carbohydrates were determined using an anthrone reaction (Chung & Nickerson, 1954). Solutions containing known quantities of glucose and mannose were estimated simultaneously. Identification of monosaccharides was by paper chromatography of hydrolysates prepared in sealed tubes at 105° with N-HCl for 4 and 6 hr. Excess HCl was removed in a desiccator over NaOH. The paper chromatograms (Whatman no.1) were run for 24 hr with *n*-butanol:acetone:water (4:5:1, v/v). Sugars were detected with aniline hydrogen phthalate in *n*-butanol. Semiquantitative deter-

mination of monosaccharides was made on chromatograms as described by Gottschalk & Ada (1965).

For total amino sugars, samples were hydrolysed in sealed tubes with 6 N-HCl at 105° for 6 hr. The insoluble materials were separated by filtration and HCl was removed in a vacuum over NaOH pellets. Neutral sugars, neutral amino acids and condensation products of amino acids were removed by passage of the hydrolysate through a column of Dowex 50-X8 (H^+) resin prepared by the method of Boas (1953), then total hexosamine was determined by the method of Rondle & Morgan (1955) using glucosamine HCl as a standard. Amino sugars were resolved by chromatography on Whatman no. 1 paper with *n*-butanol:pyridine: 0·1 N-HCl (5:3:2, v/v) as solvent. Hexosamine was located by using a modification of the Elson–Morgan reagent (Partridge, 1948).

For estimation of chitin, yeast walls (5 to 10 mg.) were shaken for 6 hr at 37° with 2 mg. fungal chitinase (Koch Light) in ammonium acetate buffer 0·03 M (pH 5·8). Chromatograms on Whatman no. 1 paper were run with *n*-butanol:acetic acid:water (3:1:1, v/v) for 24 hr. N-Acetylglucosamine, chitobiose and chitotriose were detected with diphenylamine trichloroacetate reagent (Hough, Jones & Wadman, 1950). The intensity of colour and the size of N-acetylglucosamine (NAGA) spot were compared with spots produced by known amounts of standard NAGA. The spots corresponding to chitobiose and chitotriose were eluted from chromatogram with water and hydrolysed with 2 N-HCl for 3 hr at 105°. Glucosamine-HCl, liberated by acid hydrolysis, was estimated semiquantitatively on chromatograms (for conditions, see above). Part of the enzymic hydrolysate was dried in a vacuum and then hydrolysed with 2 N-HCl for 4 hr at 105°. Excess HCl was removed by repeated evaporations over NaOH and the residue was passed through cationic resin (Dowex 50). Glucosamine-HCl was estimated by means of a modification of the method of Rondle & Morgan as described by Ghuysen, Tipper & Strominger (1966).

Lipids were isolated by ether extraction after a preliminary treatment with methanol to disrupt any lipoprotein complexes. The extracts were centrifuged to remove traces of suspended material, evaporated and dried in a vacuum over P_2O_5.

Solubility of chitin in acids and alkalis

Samples of yeast walls regenerated for 18 hr were treated for 30 min. with cold N-HCl, then treated 30 min. at 100°. The residual walls were incubated with N-HCl at 100° for 30 min. Residues and supernatants were dried in a vacuum.

Enzymic treatments of walls of regenerating cells

Parallel to the chemical studies, regenerating yeasts were treated with helicase or with chitinase and the attack followed under a microscope. Conditions of enzymic digestion were the same as those described above for the treatment of normal yeast and walls.

Enzymic pretreatments of yeast protoplasts

To eliminate possible contaminants attached to the surface of protoplasts and to study the consequences of this cleaning on successive regenerating phases, the following two procedures were used.

Chitinase treatment: Large amounts of protoplasts prepared with helicase were

incubated for 3 hr in 0·8 M-$MgSO_4.7H_2O$ in 0·03 M-acetate buffer (pH 5·8), and 1 mg. chitinase/ml. The reaction mixture was centrifuged and the pellet treated twice more in the same way. The three supernatants were collected and kept to analyse.

Lysozyme treatment. Large amounts of protoplasts prepared with helicase were incubated once for 30 min. in 0·8 M-sucrose and 0·05 M-$MgCl_2$ in 0·03 M-tris-HCl (pH 7·5) and 200μg. lysozyme/ml.

Controls without enzyme were included. Both preparations of protoplasts were washed to eliminate the residual enzymes and inoculated in reversion medium as described above.

RESULTS

Chemical analyses

Examination under a light microscope of different stages of protoplast regeneration showed that all the initially spherical protoplasts showed signs of growth, seeming to bud after 2 to 4 hr; afterwards they changed to tubular forms from which new normal

Table 1. *Chemical composition of Candida utilis walls*

About 5 mg. protoplasts were inoculated in 500 ml. Winge medium.

	Normal cells 24 hr growth	18 hr regenerating cells* (tubular form)	30 hr regenerating cells† (yeast form)	64 hr regenerating cells‡ (yeast form)
Total carbohydrate (anthrone)	78–80	50–51	75–78	78–79
Glucose	48	48	46	47
Mannose	30	1–2	29	30
Total hexosamine (Rondle & Morgan)	0·2–0·5	16	2–3	2–3
Protein (Lowry)	8–9	20	9	9
Chitin (chitinase)	0·1–0·2	18	1–3	1–2
Free and bound lipids (gravimetrically)	1	0·5–1	1	1

* Logarithmic phase yeast.
† Final logarithmic phase yeast.
‡ Stationary phase yeast.

shaped yeasts later originated. A homogeneous population of ellipsoidal-shaped yeast was not complete until after 30 to 36 hr of incubation although the first ellipsoidal forms appeared after 20 to 24 hr.

Garcia-Mendoza & Novaes-Ledieu (1968) showed the composition of walls of tubular forms (18 hr incubation) to be very different from that of the parent yeast. These results have been confirmed (see Table 1): a high content of chitin (18%) and a small amount of mannose were found in the tubular form. Normal yeast walls contained large amounts of mannose and small amounts of chitin. The samples between 20 and 30 hr incubation were discarded because the cultures were showing a heterogeneous appearance.

In contrast with the above finding, the wall material from minimal inocula, prepared from final logarithmic phase cells (after 30 hr incubation) and stationary phase cells (64 hr incubation) had a composition grossly similar to that of normal yeast (Table 1).

Walls of 4 hr regenerating yeast were therefore analysed. Preliminary experiments

performed with these structures showed three principal components: glucan, protein and chitin (this last over 12%). This suggested that a complex of glucan plus chitin could be formed during the early stage of wall biosynthesis.

Wall material, from large inocula, prepared from stationary-phase yeast from the 36 hr culture differed largely from material obtained above with 30 hr walls (see Tables 1, 2). In the former there was as high a proportion of mannose as in normal walls, but the chitin content remained approximately the same as that in tubular forms, possibly as a consequence of the exhausted medium, so there were few opportunities for the regenerating yeast to bud. In these preparations the protein content was reduced to the value of normal yeast walls. Chemical analyses of logarithmic phase yeast walls (20 hr plus 6 hr, and 20 hr plus 16 hr), both from ellipsoidal cells, revealed compositions similar to that of normal yeast walls, suggesting that the newly formed generation contained only traces of chitin, as in normal walls.

Table 2. *Chemical composition of Candida utilis walls*

About 100 mg. protoplasts were inoculated in 500 ml. of Winge medium.

	Normal cells 24 hr growth	18 hr regenerating cells* (tubular form)	36 hr regenerating cells† (yeast form)	20+6 hr regenerating cells‡ (yeast form)	20+16 hr regenerating cells‡ (yeast form)
Total carbohydrate (anthrone)	78–80	50–51	65–68	78–80	78–80
Glucose	48	48	42	48	48
Mannose	31	1–2	25	30	30
Total hexosamine (Rondle & Morgan)	0·2–0·5	16	16	1–1·2	0·4–0·8
Protein (Lowry)	8–9	20	9	9	9
Chitin (chitinase)	0·1–0·2	18	18	1·5–1·8	0·3–0·5
Free and bound lipids (gravimetrically)	1	0·5–1	0·5–1	0·6–1	0·5–1

* Logarithmic phase yeast.
† Stationary phase yeast.
‡ Logarithmic phase yeast from two-stage culture.

Analyses of the various stages of regenerated yeast walls and normal walls have shown that all the usual amino acids were present and in similar proportions. The $MgSO_4.7H_2O$ in the regeneration medium did not have an influence on the growth of yeast and on the composition of walls because analyses of normal cell walls incubated in Winge medium and in the same medium but containing $MgSO_4$ were similar.

Solubility of regenerated cell walls in acids and alkalis

Treatment with N-HCl, in cold and at 100° solubilized only a small portion of tubular walls. The soluble material corresponded to 15 to 20% of total wall and was glucan and protein. In the insoluble part an acid-insoluble glucan and the chitin were found. Treatment of the residue with N-KOH at 100° dissolved the glucan alone, the glucosamine polymer being extraordinarily resistant toward acid and alkali. In normal walls, 70% of anthrone-positive products were soluble in hot HCl.

Effect of selected enzymes on regenerated walls

Phase-microscope examination showed that the walls of 18 hr tubular forms treated with helicase were damaged to some degree and that a small proportion of these

tubes were transformed into protoplasts. Treatment with chitinase damaged the wall structures to a lesser degree. Regenerating ellipsoidal cells harvested during the logarithmic phase of growth (30 hr incubation, 20 hr plus 6 hr incubation, or 20 hr plus 16 hr incubation) and treated with helicase were markedly attacked, giving a high percentage of protoplasts, suggesting that the carbohydrate chains present in their walls are similar to those in normal cells. As was expected, treatment with chitinase did not appear to affect the wall structure.

Regeneration of enzymic pretreated protoplasts

After pretreatment with chitinase, examination under a phase microscope showed that protoplasts had difficulties in building a new wall. Growth was slower and after 18 hr incubation in the usual conditions, numerous protoplasts were observed; these were often deformed but without clear appearance of reversion. Some tubular forms with thick and less compact walls were also encountered. After 24 hr incubation the preparations appeared more normal, showing a high percentage of tubular forms. The first yeast forms were observed after 40 to 44 hr incubation. Cultures which had regenerated for 18 hr and 24 hr presented generally a heterogeneous picture, thus making it difficult to obtain a pure preparation of tubular form walls. However, preliminary chemical analyses were carried out on samples of walls obtained from tubular yeasts and from 36 hr regenerating yeasts (stationary phase forms described in Table 2). We expected that both walls would be rich in chitin; however, the chitin content was lower than that expected.

Microscopic examination showed that pretreatment with lysozyme also interfered with reversion of protoplasts. Estimation of chitin in the walls of 18 hr tubular forms and 36 hr regenerating ellipsoidal yeasts (stationary phase forms described in Table 2) gave percentages lower than those found when protoplasts were incubated without pretreatment with lysozyme.

DISCUSSION

Protoplasts of our strain of *Candida utilis* synthesized walls *de novo* despite earlier reports that a solid medium was necessary for regeneration of protoplasts of yeasts whose walls contain mannan. Protoplasts of mannan-free yeasts regenerate either in liquid or on solid media (Svoboda, 1965; Necas & Svoboda, 1965). The composition of the regeneration medium is probably important; presumably the osmotic stabilizer plays a role preventing leakage of intermediates of mannan synthesized extracellularly (Kozak & Bretthauer, 1968). This may be one reason why Eddy & Williamson (1959) did not obtain reversion of protoplasts of *Saccharomyces carlsbergensis*, though they observed tubular forms like those we saw. Alternatively, their regeneration medium may have been nutritionally inadequate.

The walls of *Candida utilis*, when fully regenerated, had the chemical constitution of normal walls, but the intermediate tubular forms were rich in glucan and chitin but deficient in mannan. Mannan formation was associated with restoration of the normal ellipsoid form. We suggest, therefore, that primary formation of a glucan–chitin matrix gives the organism rigidity and provides a basis for mannan deposition, which confers the ellipsoid form. This view is consistent with Bartnicki-Garcia & Nickerson's (1962) report of extra mannan in walls of yeast-like forms of *Mucor rouxii* and with Chattaway, Holmes & Barlow's (1968) report that the mycelial form

of *C. albicans* is relatively rich in chitin. Chitin formation is associated with budding in yeasts (Bacon, Davidson, Jones & Taylor, 1966; Bacon, Jones & Ottolenghi, 1969).

The glucan–chitin matrix is probably identical with the fibrilar network revealed by electron microscopy of early regenerating protoplasts (Kopecka, Ctrvrtnicek & Necas, 1965; Necas, Svoboda & Havelkova, 1968; Uruburu, Elorza & Villanueva, 1968) and previously believed to be composed of glucan; it is presumably closest to the protoplast membrane in the normal yeast.

The regenerating protoplasts in our work and in other reports probably possessed residual wall components which acted as primers for synthesis of new polymer (Glaser & Brown, 1957; Garcia-Mendoza, Garcia Lopex, Uruburu & Villanueva, 1968; Streiblova, 1968; Bacon *et al.* 1969; Darling, Theilade & Birch-Andersen, 1969). The delaying effect on regeneration of pretreatment with lysozyme or chitinase is probably due to removal of primer by these enzymes (Cherkazov & Kravchenko, 1968).

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The Production of Opacity in Serum by Group A Streptococci and Its Relationship with the Presence of M Antigen

By JEAN P. WIDDOWSON, W. R. MAXTED AND DORIS L. GRANT

Cross-Infection Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

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SUMMARY

The ability to produce opacity in horse serum is a characteristic of certain M types of group A streptococci. The types that produce the opacity factor (O factor) are generally those for which it is difficult to produce good anti-M sera. M-positive (M+) and true M-negative (M−) variants of strains that belong to serotypes in which the serum opacity reaction (s.o.r.) is positive both possess the O factor, but there is a difference in binding of the factor to other cellular components in the two variants. The O factor is closely bound to the wall-membrane fraction of M− cells, whereas in M+ cells it is easily extracted by Lancefield's method or 1% sodium deoxycholate. It is detectable in broth culture supernatants, in the cytoplasm and in the areas surrounding colonies in pour plates of M+ but not of M− cultures. The O factor is poorly antigenic, but when it is possible to obtain a good antiserum the inhibitory action is type specific. The O factor produced by several M types appears to be inhibited by normal rabbit serum.

INTRODUCTION

The production of opacity in horse serum by group A streptococci is believed to be mediated by a lipoproteinase which acts upon the α_1-lipoprotein fraction of the serum (Krumwiede, 1954; Rowen & Martin, 1963). Streptococci of groups other than A apparently do not produce opacity in serum, and within group A the serum opacity reaction (s.o.r.) is positive only in certain serotypes. Gooder (1961) and Köhler (1963) grew streptococci in a liquid medium consisting of 3 parts horse serum and 1 part Hartley digest broth, and recorded the presence of opacity in the culture supernatant as a positive s.o.r. Consistently s.o.r.-positive serotypes were those in which no M antigen was detectable, or for which satisfactory anti-M sera were difficult to prepare; on the other hand, members of a number of easily recognizable M serotypes were almost invariably s.o.r.-negative. Both workers suggested that there was an inverse relationship between a positive s.o.r. and the production of M antigen. Top & Wannamaker (1968*a*) incubated killed cells or deoxycholate extracts of fractions consisting predominantly of cell walls and cell membranes ('wall-membrane fractions') in horse serum They confirmed that opacity was frequently produced by members of 'difficult' M types, but could find no true inverse relationship between M antigen and opacity production, except among type 12 strains.

Most of the evidence concerning the supposed inverse relationship was obtained by examining collections of M-typable and M-untypable strains with common T-antigen

patterns (Gooder, 1961; Top & Wannamaker, 1968*a*). Evidence based on the examination of M+ and M− variants from the same culture appears to be lacking, except possibly in the case of type 12 strains.

We have now examined, by a variety of methods, the serum opacity reactions of paired M+ and M− variants, each pair isolated from the same culture. The results indicate a different relationship between M antigen and O factor production from that suggested by previous workers. We have also obtained some information about the location within the streptococcal cell of the factor responsible for the production of opacity.

METHODS

Strains of group A streptococci were classified by T typing (Griffith, 1934) and M typing (Swift, Wilson & Lancefield, 1943). The strains in Table 1 were used for the selection of M+ and M− variants. In addition, strains SF59 (M type 2), R68/1115 (M type 4), T9/101/4 (M type 9), T11/54/4 (M type 11), R68/3116 (T type 12, M type 22), T22/83/2 (T type 22, M type 22), T25/PZH small (M type 25) and Lancefield's strain B737/34/3 (M type 49) were used as vaccine strains for the production of anti-s.o.r. sera. Representatives of a variety of different serotypes, selected from stock laboratory strains and from cultures submitted for serotyping, were used in preliminary screening experiments.

Table 1. *Strains of group A streptococci used in selection of M+ and M− variants*

Strain number	T agglutination pattern	M type	Source
SF130/13 (NCTC8198)	1	1	S (see Griffith, 1934)
SF59	2	2	S (see Griffith, 1934)
R68/1115	4	4	R
T5B/PS	5	5	S
T11/54/4	11	11	S
R69/2408	11	11	R
1130	12	12	S (see Maxted, 1955)
R47/3722 (NCTC8300)	12	12	S
R69/1804	22	22	R
R69/1805	22	22	R
R69/3116	12	22	R
A703	49 (14)	49	S (Lancefield, Rockefeller Institute, New York, U.S.A.)
B3264 original	3/13/B3264	NT	S (see Griffith, 1934)

S = Colindale stock strain; R = strain received for routine typing; NT = not typable.

Selection of M+ and M− variants. Colonies of virulent group A streptococci often have a typically 'matt' appearance on blood agar. Loss of M antigen, associated with loss of virulence, is often accompanied by a change to the 'glossy' colonial form (Lancefield & Todd, 1928). For the selection of variants, cultures were streaked on plates of Hartley Digest agar (Burroughs Wellcome & Co., London N.W.1) containing 5% (v/v) horse blood, incubated at 37° for 24 hr and then examined for differences in colonial morphology. Most of the strains used were matt initially, but it was possible in some to detect occasional glossy variants. The majority of the strains

chosen for investigation (Table 1) were ones in which it appeared relatively easy to do this but many of the strains examined in preliminary experiments did not produce glossy variants even on repeated subculture. Some strains were examined on serum glucose agar (Hartley Digest agar + 5% (v/v) horse serum + 0·2% (w/v) glucose). A few variants were picked by their 'opaque' (M+) or 'blue' (M−) appearance on this medium. The selected pairs of variants of each strain were retyped by T-agglutination and M-precipitin tests to establish that they shared a common T pattern and that the glossy variant lacked the appropriate M antigen.

Survival in human blood. M+ and M− variants were examined for their ability to survive and multiply in human blood. Heparinized samples of normal blood were obtained from at least three individuals, and the tests were carried out as described by Maxted & Valkenburg (1969). A 4 hr culture of organisms in Todd–Hewitt broth was diluted so that 0·02 ml. contained 100 to 200 colony-forming units. This volume was inoculated into 0·3 ml. blood in an ampoule, which was sealed and rotated slowly for 3 to 4 hr at 37°; 0·02 ml. samples were then withdrawn from the ampoule and spread on the surface of blood agar plates. The plates were incubated at 37° for 24 hr and the extent of survival or growth scored on a scale: −(no colonies) to 4+ (confluent growth).

Preparation of fractions for use in s.o.r. tests

Washed cells. In screening experiments, in which large numbers of strains were examined, strains were grown overnight at 37° in 3 ml. Todd–Hewitt broth (Oxoid Todd–Hewitt broth + 1% (w/v) Neopeptone (Difco Ltd., Detroit, Michigan, U.S.A.). The bacteria were deposited by centrifugation and washed once in normal saline. In all other experiments involving washed cells, standardized suspensions of cells were prepared by suspending the washed growth from 50 ml. of a 24 hr culture to an extinction (A) = 1·0 at 600 nm. (Unicam SP600 spectrophotometer) in normal saline containing 1 in 5000 (w/v) thiomersal.

Supernatant fluids. These were obtained by centrifugation of overnight cultures in Todd–Hewitt broth.

Lancefield extracts. N/5 hydrochloric acid extracts were prepared from the overnight growth in 50 ml. Todd–Hewitt broth by the method described by Swift *et al.* (1943).

Deoxycholate extracts. Wall-membrane fractions were extracted with 1% sodium deoxycholate for 16 hr at 4° (Top & Wannamaker, 1968*b*).

Wall-membrane fractions. Washed cells were disrupted in a Mickle disintegrator at 4° (Michel & Gooder, 1962). Centrifugation at 20,000 *g* for 1 hr sedimented the wall-membrane fraction. After two washings in normal saline the opacity of the suspension in saline was adjusted to A_{600} = 1·0.

Cytoplasmic fractions were obtained as the supernatant fluids from the first high-speed centrifugation of the Mickle-disintegrated material.

Serum opacity reactions with cell fractions and extracts

The s.o.r. of cell fractions and extracts were tested by incubating 0·2 ml. material with 1·0 ml. horse serum (Wellcome Research Laboratories, Beckenham, Kent) containing 1 in 5000 (w/v) thiomersal, for 16 to 18 hr at 37°. After incubation, 1·2 ml. saline was added to each tube and the extinction read in a Unicam SP600 spectrophotometer at 475 nm. No increase in opacity compared with the control (1·4 ml.

saline + 1·0 ml. horse serum) was recorded as a negative s.o.r. An increase of up to 0·05 was recorded as ±, 0·05 to 0·20 as 1+, 0·20 to 0·30 as 2+, an increase of greater than 0·30 as a 3+ reaction. In screening tests for the s.o.r. of whole cells, the washed cell pellet from 3 ml. of a 24 hr culture was suspended in 1·0 ml. of horse serum containing 1 in 5000 (w/v) thiomersal, and the opacity developed after 16 to 18 hr at 37° was graded by eye from −(no opacity) to 3+ (very opaque). All tubes were centrifuged at 2500 *g* for 10 min. to remove particulate matter before opacities were read.

Serum opacity reactions of growing cultures

In serum broth (Gooder, 1961). One loopful of an overnight Todd–Hewitt broth culture was transferred to 3 ml. of serum broth (1 part Hartley pancreatic digest of beef + 3 parts horse serum) and incubated at 37° for 18 to 24 hr. Tubes were read at 475 nm, and an increase in extinction up to 0·1 was recorded as a ± s.o.r., 0·1 to 0·25 as 1+, 0·25 to 0·45 as 2+, and greater than 0·45 as 3+. No increase in extinction of the medium was recorded as a negative s.o.r.

In pour-plates. A method similar to that of Maxted & Valkenburg (1969) was used, except that Oxoid Ion agar no. 2 replaced Davis agar; 1 % (w/v) of this in a mixture of equal parts of Wright broth (Mackie & McCartney, 1960) and horse serum gave a clear medium in which zones of opacity around colonies were easily visible. Plates were incubated at 37° for 48 hr and then allowed to remain at room temperature for 24 to 48 hr before reading.

Preparation of protoplast membranes by means of phage-associated lysin

Protoplasts were induced by incubating washed streptococci in a mixture of equal parts of reduced phage-associated lysin and 4 M-sodium chloride for 30 min. at 37°, as described by Gooder & Maxted (1958). The mixture was centrifuged at 3500 *g* for 15 min. and the supernatant fluid kept for s.o.r. testing. The protoplast pellet was suspended in distilled water and the lysed protoplast envelopes were sedimented at 20,000 *g* for 60 min. The supernatant fluid (cytoplasm) was also kept for the s.o.r. test. The protoplast envelopes were washed twice in water before testing for s.o.r.

Preparation of antisera against the opacity factor

Wall-membrane fractions were given intravenously to rabbits as described by Top & Wannamaker (1968*b*). Two rabbits were inoculated with each strain. They were bled after a series of 3 × 1·0 ml. injections per week given over a period of 5 weeks, and their sera were tested for inhibitory activity against the O factor of the vaccine strain. When this was present, the rabbits received 1·0 ml. injections of vaccine weekly for a further 4 weeks to maintain the increased antibody level. When the s.o.r.-inhibiting capacity was low or absent at the first bleeding 3 × 1·0 ml. injections per week were given during this 4-week period. Pre-immunization serum was obtained from all rabbits for use as controls in inhibition tests.

Measurement of the inhibition of s.o.r. by antisera. The method used was similar to that of Top & Wannamaker (1968*b*); 0·2 ml. quantities of standardized (A_{600} = 1·0) cell suspensions were pre-incubated for 15 min. at 37° with 0·2 ml. quantities of the antiserum before the addition of 1 ml. horse serum containing 1 in 5000 (w/v) thiomersal. After incubation at 37° for 16 to 18 hr, 1·4 ml. saline was added to each tube and the A_{475} was read spectrophotometrically. Two controls were set up for each

strain, one containing 0·2 ml. normal rabbit serum, and the other 0·2 ml. normal saline in place of immune serum. A saline control was necessary to assess the extent of inhibition of opacity by normal rabbit serum. Blanks containing 0·2 ml. saline in place of the cell suspensions were set up for each serum used.

In inhibition tests involving material other than whole cells, e.g. cell fractions, supernatant fluids or extracts, 0·2 ml. samples of the materials were incubated with 0·2 ml. rabbit serum, and the test was done exactly as described for whole cells.

RESULTS

Preliminary examination of a large number of laboratory stock strains and strains received for routine typing revealed a distinct association between M type and ability to give a positive s.o.r. Our findings were in general agreement with those of Gooder (1961) and Top & Wannamaker (1968*a*). M serotypes could be divided into two main groups with respect to their s.o.r.: the first group included M types 2, 4, 9, 11, 13, 22, 25, 28, 48 and 49, which were invariably s.o.r.-positive; the second, much larger group included the majority of other M types, which were never s.o.r.-positive. We selected representatives from both groups for further investigation, and from some of the strains were able to pick M+ and M− variants. It was important at this stage to establish, by the blood survival test, that the supposed M− variants selected from initially M+ cultures were indeed truly M− versions of the original strain, and not strains with different M antigens (Maxted & Valkenburg, 1969).

Ability of M+ and M− variants to survive in human blood. All M+ variants gave 3+ or 4+ survival in the blood of at least three persons known not to possess antibody to the serotype under test. M− variants were killed, or at least prevented from multiplying during 4 hr incubation, in normal human blood (−, ±, or 1+ survival). Presumed M+ and M− variants that did not satisfy these criteria were rejected.

Serum opacity reaction of M+ and M− variants

M+ and M− variants of strains belonging to both the s.o.r.-positive and s.o.r.-negative groups of serotypes were examined in detail for ability to produce serum opacity. Preliminary investigations showed that when cell suspensions in which multiplication was prevented by the addition of 1 in 5000 (w/v) thiomersal were incubated in undiluted horse serum, both variants of a particular strain were either s.o.r.-positive or s.o.r.-negative, depending on the serotype. However, tests in serum pour-plates showed a striking difference between the M+ and M− variants of the s.o.r.-positive group of strains. M+ variants in each case gave in pour-plates diffuse zones of opacity around the colonies. These zones had a characteristic appearance and seemed to flow to one side of the colony (Pl. 1, fig. 1). In some strains, e.g. members of types 9, 11 and 13, M+ colonies were surrounded by a defined opaque ring in addition to the more diffuse zone of opacity we associate with s.o.r. (Pl. 1, fig. 2). Certain strains belonging to the group of serotypes that were never s.o.r.-positive also had these defined rings but never showed the more diffuse type of opacity (Pl. 1, fig. 3). It was concluded that the presence of a defined opaque ring round colonies in serum pour-plates was not associated with the serum opacity reaction. Strains that produced these rings were invariably mucoid, and the addition of hyaluronidase to the medium

Table 2. *The occurrence of the opacity factor in M+ and M− variants of strains of group A streptococci*

Strain no.	Antigenic pattern T	Antigenic pattern M	Survival in human blood*	Production of opacity by: Growth in: Serum broth†	Growth in: Serum agar plates‡	Incubation in horse serum of: Washed cells§	Culture supernatants	Lancefield extract	Deoxycholate extract	Wall-membrane fraction	Cytoplasm
SF 59 matt	2	2	++++	++	Yes	+	+++	++	++	++	+
SF 59 glossy	2	−	−	−	No	+	−	−	−	++	−
1115 matt	4	4	++++	++	Yes	+	++	++	++	+	++
1115 glossy	4	−	±	±	No	+	−	−	−	+	−
M 11 matt	11	11	++++	++	Yes	+	+	+	+	+	+
M 11 glossy	11	−	±	−	No	±	−	−	−	+	±
2408 (*a*)‖	11	11	++++	+++	Yes	++	+	+	...	...	...
2408 (*b*)	11	−	−	±	No	++	−	−	...	...	...
3116 matt	22	22	++++	+++	Yes	+	+++	+++	+++	++	+++
3116 glossy	12	−	±	−	No	+	±	−	−	++	+
1805 matt	22	22	++++	...	Yes	+	+++	+++	...	...	...
1805 glossy	22	−	±	...	No	++	±	−	...	...	...
1544 matt	49(14)	49	++++	...	Yes	+	+++	++	...	...	...
1544 glossy	49(14)	−	±	...	No	++	±	−	...	...	...
B 3264(*a*)‖	3/13/B 3264	NT	++++	++	Yes	±	++	+	+	...	+++
B 3264(*b*)	3/13/B 3264	NT	−	−	No	++	−	−	−	...	−

* ++++, Confluent growth after incubation in human blood; ±, few survivors; −, no survivors.
† Opacity of serum broth supernatants (A_{475})—see Methods.
‡ Yes = presence of characteristic diffuse zone of opacity round colonies. No = no diffuse opaque zone round colonies.
§ Opacity produced in horse serum (A_{475})—see Methods.
‖ Variants selected by picking s.o.r.+ and s.o.r.− colonies from serum pour-plates.

appeared to decrease ring formation in some cases, though this was by no means a consistent finding.

The difference between M+ and M− variants with respect to ability to produce zones of opacity in serum agar plates prompted us to investigate other methods of detecting the opacity factor and to look for activity in different extracts and subcellular fractions of M+ and M− variants. Table 2 shows the results of an investigation of the behaviour of four sets of paired variants of different s.o.r.-positive serotypes. Also included in Table 2 are the results of a smaller series of tests with other paired variants. The results showed striking differences between M+ and M− variants in all of the pairs tested. The O factor was detectable in Lancefield extracts and 1% sodium deoxycholate extracts of M+, but not of M− cultures. The culture supernatant fluids of M+ cultures and of the cytoplasm of disrupted M+ cells were invariably s.o.r.-positive; on the other hand, the O factor was not detected in the culture supernatant fluids nor in the cytoplasm of M− cells. When the O factor was tested for in growing cultures, i.e. in serum pour-plates or in a liquid medium containing 75% (v/v) horse serum, only M+ cultures produced the opacity factor. However, it was clear from tests in which whole cells or wall-membrane fractions were incubated in horse serum that both M+ and M− variants possessed O factor, since both gave a positive s.o.r. under these conditions. Therefore the difference between M+ and M− variants appeared to be a difference in binding of the opacity factor to other cellular components. We also tested for the O factor in paired M+ and M− variants of strains belonging to the group of M serotypes that we had found in preliminary screening tests to be s.o.r.-negative (e.g. types 1, 12, 5). The O factor was completely absent from both variants of these strains, irrespective of the method of testing.

Location of the opacity-producing factor

Investigations were made to locate the opacity factor in M+ and M− cells by preparing protoplast envelopes of both variants of a type 2 strain, and testing at various stages of preparation for the opacity factor. The results are summarized in Table 3. Most of the activity in M− cells appeared to be bound to the cell membrane. The O factor was also found in fractions containing cell wall fragments, but

Table 3. *Location of the opacity factor in M+ and M− variants of strain SF59 Type 2*

Fraction	*Opacity produced in horse serum by M+ variant	M− variant
Washed whole cells	+	+
Culture supernatant fluid	+++	−
Supernatant fluid after lysin treatment (contains cell wall fragments)	+++	+
Supernatant fluid from lysed protoplasts (cytoplasm)	++	−
Protoplast membranes (unwashed)	+	++
Protoplast membranes (washed)	+	++
Washings from protoplast membranes	++	−

* Opacity produced in horse serum (A_{475}); −, no increase in opacity; ± increase of up to 0·05; +, increase of 0·05 to 0·2; ++, increase of 0·2 to 0·3; +++ increase of greater than 0·3.

cytoplasmic constituents and washings from the protoplast membrane fraction were s.o.r.-negative. Although washed protoplast envelopes of M+ cells had some s.o.r. activity, the greater activity of supernatant fluids, cell wall fragments and washings at every stage during the preparation showed that O factor was easily removed from the cell membrane of M+ cells. Matt and glossy variants of other s.o.r.-positive serotypes behaved in a similar manner.

Table 4. *Inhibition of the opacity factor by rabbit sera in washed cell suspensions of group A streptococci*

		*Intensity of serum opacity reaction in the presence of				
		Immune rabbit antiserum against strain			Normal rabbit serum	Serum opacity reaction of control (no rabbit serum)
Strain	M type	SF 59	R 68/1115	T 25/PZH small		
SF 59	2	−	++	++	++	++
Other type 2 strains	2	−	NT	NT	++	++
R 68/1115	4	++	−	++	++	++
Other type 4 strains	4	NT	−	NT	++	++
T 9/101/4	9	−	−	−	−	+
T 11/54/4	11	±	±	±	±	+
T 22/83/2	22	+++	+++	+++	+++	+++
T 25 PZH small	25	++	++	±	++	++
Other type 25 strains	25	NT	NT	±	++	++
Small	28 R	±	±	±	±	++
B 403/48/1	48	++	++	++	++	++
C 274/1	49	+	+	+	+	++

* Opacity produced in horse serum (A_{475}); −, no increase in opacity; ±, increase of up to 0·05; + increase of 0·05 to 0·2; ++, increase of 0·2 to 0·3; +++; increase of > 0·3; NT, not tested.

Inhibition of serum opacity reaction by type specific antisera

Eight different vaccine strains were used in attempts to prepare anti-s.o.r. sera in rabbits. Three strains, i.e. R 68/1115 (type 4 M+), SF 59 (type 2 M+) and PZH (type 25 M+), gave sera capable of inhibiting the O factor of the vaccine strain; we were unsuccessful in preparing antisera for the other five strains tested. The antisera that showed some activity (two type 4 sera, one type 2 serum, one type 25 serum) were each tested against several strains of the vaccine type and nine different s.o.r.-positive heterologous types. Washed cell suspensions (A_{600} = 1) were used as sources of opacity factor, and 0·2 ml. quantities were pre-incubated with the antiserum under test before the addition of horse serum (see Methods). Tubes were read at 475 nm. on a Unicam SP600. In each case inhibition seemed to be specific for the vaccine type, except that all rabbit sera tested (including normal rabbit sera) inhibited the s.o.r. of types 9, 11, 28R, 49 to a greater or lesser extent (Table 4). Inhibition by normal rabbit serum appeared to be a constant feature of the s.o.r. of these types. All samples of normal rabbit serum tested were inhibitory; the inhibition was most marked with type 9, where the s.o.r. could virtually be eliminated by adding 0·2 ml. normal rabbit serum.

Inhibition of serum opacity reaction by antisera in M+ and M− variants

An antiserum prepared against the wall-membrane fraction of a type 4 matt variant was used to investigate the identity of the opacity factor of different fractions and extracts of M+ and M− variants of strain R68/1115. These tests (Table 5) established that the O factors detected in different fractions were antigenically identical, in that they were all inhibited by the specific antiserum. Plate 1, fig. 4 shows the inhibition by specific antiserum of s.o.r. zones surrounding M+ colonies in serum pour-plates. The O factor of the Lancefield extract of M+ cells seemed to be the exception in that it was inhibited only to a small and variable extent by the antiserum prepared against

Table 5. *Inhibition, by specific antiserum, of the opacity factor in fractions and extracts of M+ and M− variants of a type 4 strain*

Source of opacity factor	*Intensity of serum opacity reaction in the presence of	
	Normal rabbit serum	Specific antiserum
Washed M+ variant	++	−
Washed M− variant	++	−
Culture supernatant fluid of M+ culture	+++	−
Lancefield extract of M+ culture	+++	++
Deoxycholate extract of M+ culture	+++	±
Wall-membrane fraction of M+	+	−
Wall-membrane fraction of M−	+	−
Cytoplasm of M+	+++	−
Colonies of M+ variant in serum agar	Yes†	No
Culture of M+ variant in serum broth	+++	−

* Increase in opacity of horse serum (A_{475}); −, no increase in opacity; ±, increase of less than 0·05; +, increase of 0·05 to 0·2; ++, increase of 0·2 to 0·3; +++, increase of >0·3.

† Yes = presence of diffuse zones of opacity around colonies. No = no diffuse zones of opacity around colonies.

a type 4 wall-membrane vaccine. Extraction with more dilute acid (N/20) or at lower temperature (60°) gave extracts in which the O factor was inhibited by the specific antiserum. A specific antiserum prepared against the O factor of the wall-membrane fraction of a type 2 M+ variant gave similar results with the O factor of M+ and M− variants of a type 2 strain.

DISCUSSION

Gooder (1961) suggested that there was an inverse relationship between the production of opacity in serum and the presence of M antigen. His evidence was a type 12 strain that was s.o.r.-negative when it was in the matt (M+) form and s.o.r.-positive when it occurred as an apparently M− variant. Our results with M+ and M− variants of a variety of serotypes suggest a different relationship. Strains with the 12M antigen (T12, M12) belong to the group of M types that are always s.o.r-negative; true M− variants of members of this group are also s.o.r.-negative. However, it is possible for type 12 strains to give variants that possess an M antigen other than M12 (Maxted & Valkenburg, 1969, 1970, and to be published. Strains with these

'12 variant' M antigens are invariably s.o.r.-positive. The s.o.r. of type 12 and its variants will be discussed in detail elsewhere (Widdowson, Maxted & Grant, 1970).

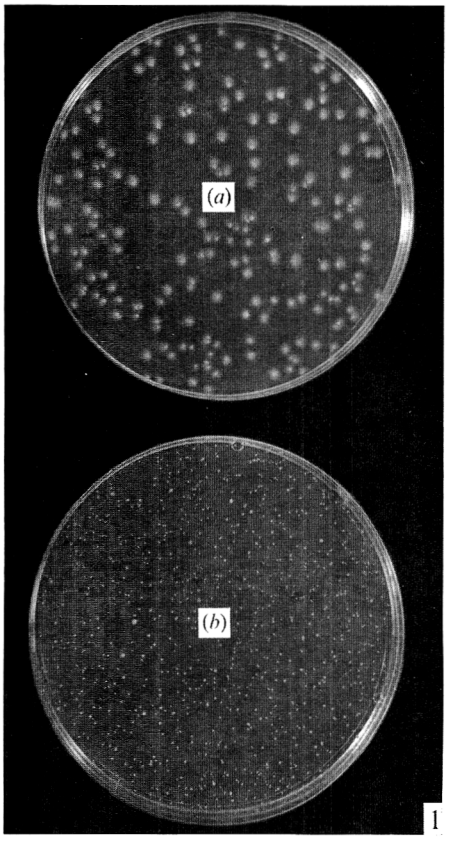
Both M+ and M− variants of the s.o.r.-positive group of serotypes (e.g. types 2, 4, 11, 22, 49) possess the opacity factor. This could be shown by incubating whole cells or wall-membrane fractions from M+ and M− cultures in horse serum. However, other methods of testing for the O factor that were dependent on ease of extraction of the factor from cells, or its release into supernatant fluids during growth, gave s.o.r.-positive results only with the M+ variants. The site of binding of O factor in M− cells appeared to be on the cell membrane, though some activity was always present in the cell-wall fraction released by phage-associated lysin.

There is evidence that in s.o.r.-positive serotypes, M antigen and the O factor are closely associated chemically (J. P. Widdowson, W. R. Maxted & D. L. Grant unpublished). Thus the presence of O factor in the wall-membrane fraction of cells which had apparently lost the ability to produce M antigen, raises the interesting possibility that M antigen itself might also be present in these cells in a bound or inactive form.

The studies of Top & Wannamaker (1968*b*), showed the remarkable type specificity of the lipoproteinases of group A streptococci. They prepared antisera with specific inhibitory activity by the injection into rabbits of wall-membrane fractions of s.o.r.-positive types. Twelve antigenically distinct lipoproteinases were identified in this way, though many of the strains tested did not give detectable amounts of antibody even after 9 weeks' immunization. In a limited series of experiments in which a similar method of immunization was used, we were able to prepare anti-s.o.r. sera to three strains out of eight tested. The anti-s.o.r. sera (against a type 4, a type 2 and a type 25 strain) were specific for the vaccine type, and to this extent our findings are in agreement with those of Top & Wannamaker. With other vaccine strains (types 9, 11, 49) the problem in assessing the anti-s.o.r. activity of the sera was that all normal rabbit sera tested gave some inhibition of opacity factor. The antigenicity of the O factor of some types appears to be weak (in the vaccines we used) and the investigation of other types of vaccine seems warranted.

With the anti-s.o.r. serum prepared against strain R68/1115 (type 4) it could be shown that the O factor present in matt cells was antigenically identical with that in glossy cells. The O factor detected in the culture supernatant fluids, in the cytoplasm and in deoxycholate extracts of matt cells was also inhibited by the specific antiserum. The O factor in Lancefield extracts of M+ cultures was only partially inhibited by the antiserum. A possible explanation for this is that heating M+ cells in the presence of acid may alter the structure of the O factor so that, although it is still able to produce opacity in serum, it is unable to combine with its specific antibody. The fact that s.o.r.-positive Lancefield extracts were obtained only from s.o.r.-positive cells seems to indicate that the O factor in these extracts is derived from the native form.

The detection of opacity in pour-plates of serum agar proved a useful means of distinguishing M+ and M− variants of s.o.r.-positive serotypes. In our experience, colonies giving characteristic diffuse opaque zones in the surrounding medium are invariably found to be M+ by survival tests in human blood, even when the lack of a suitable anti-M serum makes it impossible to demonstrate the M antigen by the precipitin method.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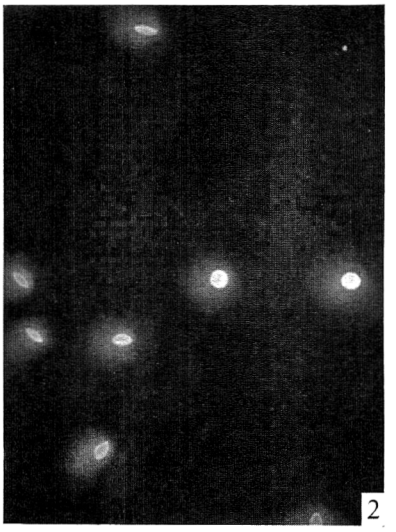
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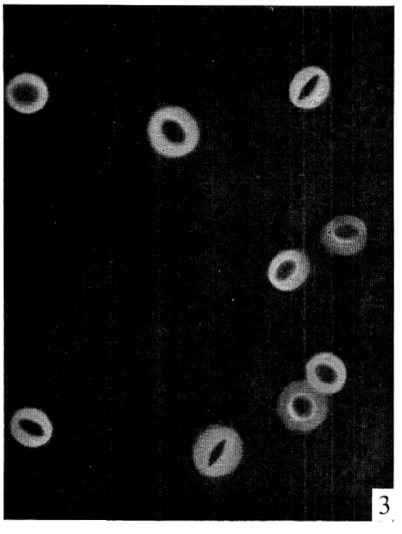
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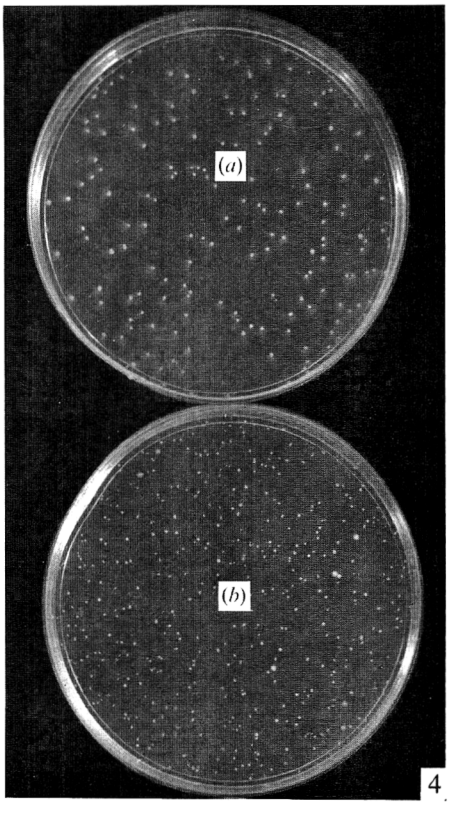
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We are very grateful to Miss C. A. M. Fraser for supplying us with M and T typing sera.

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EXPLANATION OF PLATE

Fig. 1. Serum agar pour-plates of (*a*) M+ and (*b*) M− variants of a type T 12 M 22 strain (R 69/3116) of *Streptococcus pyogenes*.

Fig. 2. Colonies of a type 11 M+ strain (T 11/54/4) of *Streptococcus pyogenes* in a serum agar pour-plate.

Fig. 3. Colonies of a type 24 (C 98/97) M+ strain of *Streptococcus pyogenes in* a serum agar pour-plate.

Fig. 4. Colonies of a type 4 M+ (R 68/1115) strain of *Streptococcus pyogenes* in serum agar pour-plates (*a*) in the presence of 1 ml. of normal rabbit serum per plate, (*b*) in the presence of 1 ml. type specific anti-s.o.r. serum per plate.

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The Fine Structure of a Nuclear Envelope Associated Endosymbiont of Paramecium

By R. A. JENKINS

Department of Zoology and Physiology, University of Wyoming, Laramie, Wyoming 82070, U.S.A.

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SUMMARY

Particles of a newly described endosymbiont of Paramecium, here referred to as epsilon, have a fine structure that is essentially identical to that of Gram-negative bacteria. The symbionts occur only within loculi formed by extension of the outer membrane of the nuclear envelopes.

INTRODUCTION

It is difficult to identify the presumed endosymbionts of Paramecium with a particular micro-organism or to determine the nature of the cytoplasmic interaction of the host cell and symbiont. The endosymbiont mu resembles Gram-negative bacteria (Beale & Jurand, 1960, 1966). More recently, Jurand & Preer (1969) established that the symbionts of all stocks known to kill by rapid lysis are flagellated, Gram-negative bacteria. Beale, Jurand & Preer (1969) reviewed the biology of the better known endosymbionts of *Paramecium aurelia* and concluded that the symbionts represent a miscellaneous collection of bacteria which are adapted to an intracellular niche. It is generally accepted that the existence and perpetuation of an endosymbiont is highly dependent upon the genetic and cytoplasmic constitution of the host cell; however, only in the case of alpha, which is macronuclear, has a direct association of nucleus and symbiont been demonstrated. The present study developed from a general investigation of the fine structure of nuclear processes in ciliate protozoa; the first Paramecium micronucleus examined showed numerous bacterium-like particles associated with the nuclear envelope. This paper reports the specific association of that symbiont, which structurally resembles Gram-negative bacteria and perhaps rickettsiae, primarily with the micronucleus but also with the macronucleus of Paramecium.

METHODS

The original culture identified as *Paramecium multimicronucleatum* was obtained in 1962 from Carolina Biological Supply Company, Elon College, North Carolina, U.S.A. Since that time organisms have been maintained with bacteria in a standard hay + lettuce medium. Paramecia were collected from newly established cultures by micropipette and fixed by exposure to osmium tetroxide vapour as previously described (Jenkins, 1964). Following rapid ethanol dehydration, the protozoa were embedded singly in methacrylate which contained 2% (v/v) of divinylbenzene as a cross-linking

agent or an Epon–Araldite mixture according to procedures reported earlier (Jenkins, 1967). Sections were cut with an LKB Ultrotome equipped with glass knives, picked up on grids with Formvar-carbon films, doubly stained with uranyl acetate and lead citrate, and examined with an RCA 3G electron microscope operated at 100 kV and equipped with 150 μm. and 30 μm. condenser and objective apertures respectively.

RESULTS

Micronuclei of the paramecia were approximately 2·5 μm. wide in the greatest dimension and were bounded by an envelope consisting of two membranes separated by a less dense space 15 to 20 nm. wide and having numerous pores about 60 nm. in diameter (Pl. 1, fig. 1; Pl. 2, fig. 3). All micronuclei thus far examined had, just inside the nuclear envelope and at the apices of the longest dimension, a small bundle of microtubules cut in cross-section (Pl. 1, fig. 1; Pl. 2, fig. 2). This apparently consistent configuration is reminiscent of the microtubules in the marginal bundle of mammalian blood platelets as studied by Behnke & Zelander (1967). The macronucleus was very much larger, with an irregular outline, and was enclosed by an envelope similar to that of the micronucleus except that pores were perhaps more numerous and slightly larger (Pl. 1, fig. 1). Nuclear contents were essentially identical to those described for Paramecium by others (Jurand, Beale & Young, 1962; Ehret & DeHaller, 1963). In some sections loose bundles of 12 nm. fibres were seen in the central portions of the micronucleus (Pl. 1, fig. 1); at high magnification a faint periodicity was evident within the bundle. Vivier & André (1961) described similar fibrillar structures within the macronucleus of *Paramecium caudatum*.

The endosymbiont, tentatively named epsilon, occurred only within bulbous distensions (loculi) of the outer membrane of both micro- and macronuclei (Pl. 1, fig. 1), being more numerous per unit surface area for micronuclei. In fact, the macro nuclear envelope was generally devoid of symbionts except in the region near to a micronucleus. The dilated intermembrane space of the loculus was always continuous with that electron-lucid space between the membranes of the nuclear envelope, and arose by way of a 70 nm. wide channel formed by an outward extension of the external membrane of the envelope (Pl. 2, fig. 2). In some instances, continuity between the outer membranes of the envelopes of micronuclei and the macronucleus existed (Pl. 2, fig. 2); such channels might show a dilation which contains an epsilon particle (Pl. 2, fig. 2). A loculus might show multiple connexions to the nuclear envelopes and, in some cases, to the rough-surfaced endoplasmic reticulum (Pl. 1, fig. 1); however, the locular membrane itself was apparently always devoid of ribosomes. The epsilon particle was always separated from its surrounding membrane by an electron-lucid space 75 to 100 nm. wide. Such uniform separation may indicate a capsule which was not preserved by the preparative techniques employed. Only in instances where the division of a symbiont appeared to have occurred recently were two symbionts present within a single loculus.

The symbiont epsilon is best described as a very short rod, sometimes appearing nearly coccoid, approximately 0·35 μm. in diameter with longer forms reaching 0·7 μm. in length (all figures). Each particle was limited by a pair of membranes separated by an electron-lucid space (Pl. 2, fig. 3); the outer membrane, space and inner membrane were each about 10 nm. in width. The outer membrane had an irregular external

surface, while the inner membrane, contiguous with the internal content, had a smooth profile. The cytoplasm of epsilon consisted generally of closely packed granules 17 nm. in diameter; in some cases, regions appear to lack these particles and might represent nuclear areas (Pl. 2, fig. 3). The cytoplasm occasionally contained small membranous whorls similar to mesosomes (Pl. 2, fig. 3). Deeply constricted forms as shown in Pl. 2, fig 3, were presumably in division, and had a loculus with a concentric indentation. The means by which the two resulting symbionts came to lie within separate loculi has not been observed.

DISCUSSION

A recent study by Jurand & Preer (1969) led these workers to conclude that lambda and sigma particles are flagellated bacteria. Earlier, Beale & Jurand (1966) had shown that three types of mu particles are bacterium-like but did not identify the particles as bacteria. Stevenson (1969) presented biochemical evidence which seemed to establish quite clearly that the mu particle is a symbiotic bacterium. Also, Wagtendonk, Clarke & Godoy (1963) demonstrated that lambda grown *in vitro* are Gram-negative rods. The particle epsilon described in this work most resembles Gram-negative bacteria (Glauert, 1962; Bladen & Waters, 1963) or perhaps rickettsia (Ito & Vinson, 1965; Entwistle, Robertson & Juniper, 1968) although no pleomorphic forms typical of rickettsia have been observed. The convoluted cytoplasmic invaginations of the plasma membrane described here are not common to Gram-negative bacteria or rickettsia but have been described for both (Lee, 1960; Ito & Vinson, 1965). With only fine structure evidence available it is not possible to identify the exact nature of the symbiont but, in accordance with the conclusions of Beale *et al.* (1969) reached after an extensive study of endosymbionts, it seems most appropriate to identify epsilon as a bacterium. Endosymbionts of paramecia, with the exception of alpha which is found within the macronucleoplasm (Beale *et al.* 1969), are cytoplasmic, either embedded directly in the cytoplasmic matrix (for example: mu, Beale & Jurand, 1966) or enclosed either singly or in multiples within membranous vacuoles which are in turn embedded in the cytoplasm as is the case for lambda (Jurand & Preer, 1969). Sonneborn (1965) stated that mu has a capsular sheath which is in contact with the cytoplasmic matrix. Epsilon is found exclusively within bulbous extensions of the outer membrane of the nuclear envelopes. Such a localization is unique and is thought not to be a transient one since paramecia at different division stages all show the endosymbiont to be nuclear envelope-associated. Although the intracisternal space of the endoplasmic reticulum is continuous with the lumen between membranes of the nuclear envelope, epsilon is not found within the cisternae proper. Also, while extensions of granular endoplasmic reticulum might arise from an occupied loculus, the membrane of the loculus always lacks ribosomes. In this regard, gamma, which is very similar to epsilon in structure and size (Beale *et al.* 1969), is limited by a vacuole which does bear ribosomes and is thought to be granular endoplasmic reticulum.

The peculiar intracellular location of epsilon seems a most favourable one in terms of communication with both the cytoplasm and the nucleus, at the same time separating the symbiont from possible abrasive cytoplasmic factors. Epsilon is not in direct contact with the nuclear contents; the loculus and the intermembrane space of the nuclear envelope are continuous and are separated from the nucleoplasm by whatever barrier is imposed by the medial components of nuclear pores which are continuous

through the membranes. Before assuming that the intermembrane location might relate specifically to the advantage of nuclear association, it should be remembered that the lumen of a loculus is often continuous with the intracisternal space of the granular endoplasmic reticulum and could leave the symbiont in direct contact with a product of protein synthesis. The possibility certainly exists that the nuclear association is of benefit to the paramecium by providing ready nuclear access to a product of symbiont synthesis such as purines which are speculated to be a product of endosymbiote metabolism in at least one case (van Wagtendonk *et al.* 1963). The intermembrane location may not relate directly to nutritional factors alone but also to the mechanism of perpetuation for epsilon. Unfortunately it is not known whether this symbiont shows the same specificity for the genotype of the host that is known to be necessary for the maintenance of other particles (Gibson & Beale, 1961; Gibson & Sonneborn, 1964). However, on the basis of the single intracellular location, it seems most probable that epsilon requires nuclear contribution for its existence. Since the nuclear envelopes of both macronuclei and micronuclei remain intact during ciliate division, distribution of epsilon to both daughter cells seems assured and always in association with nuclear material. Whatever the advantage, it appears that epsilon has become adapted to a most unique and favourable intracellular location.

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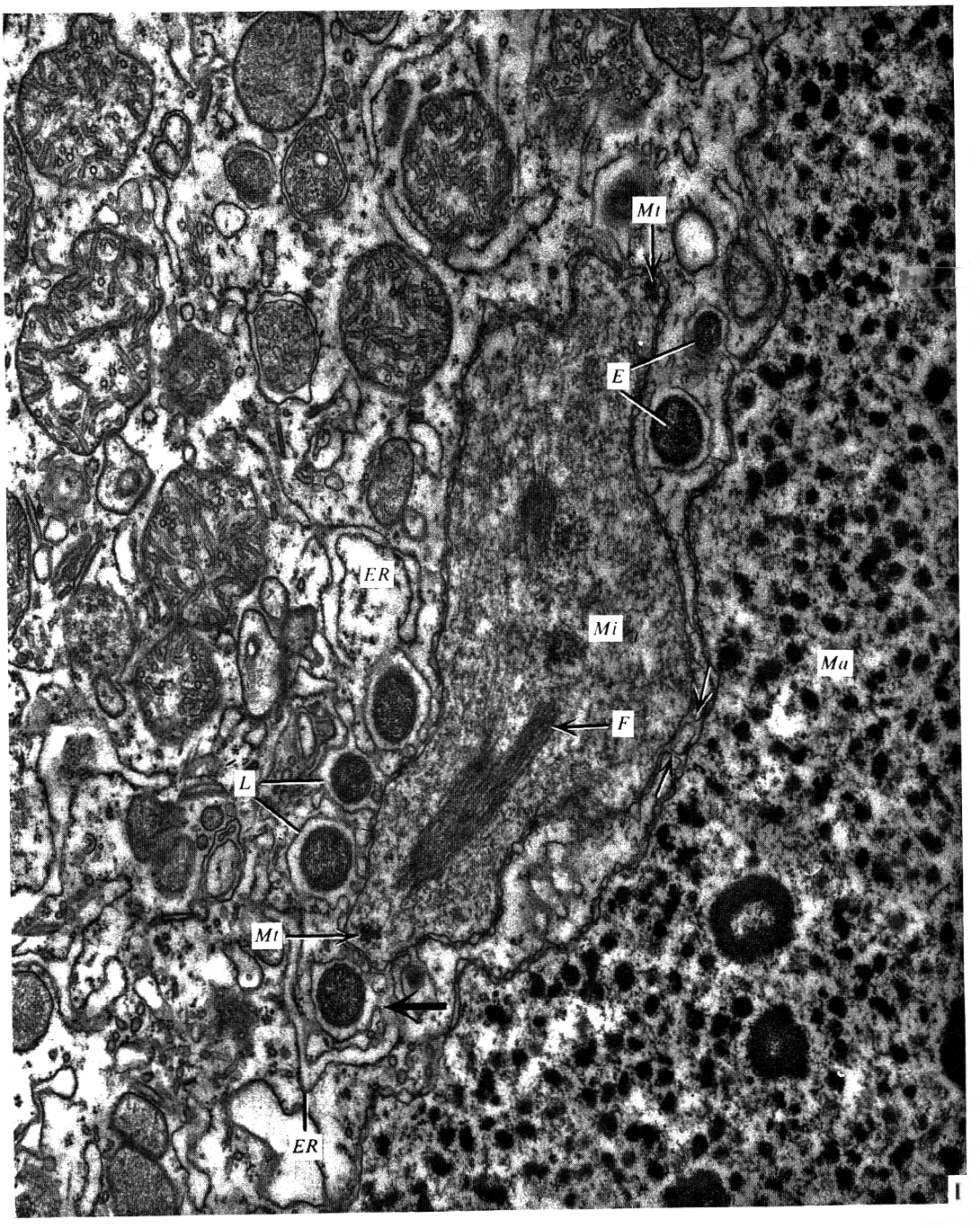

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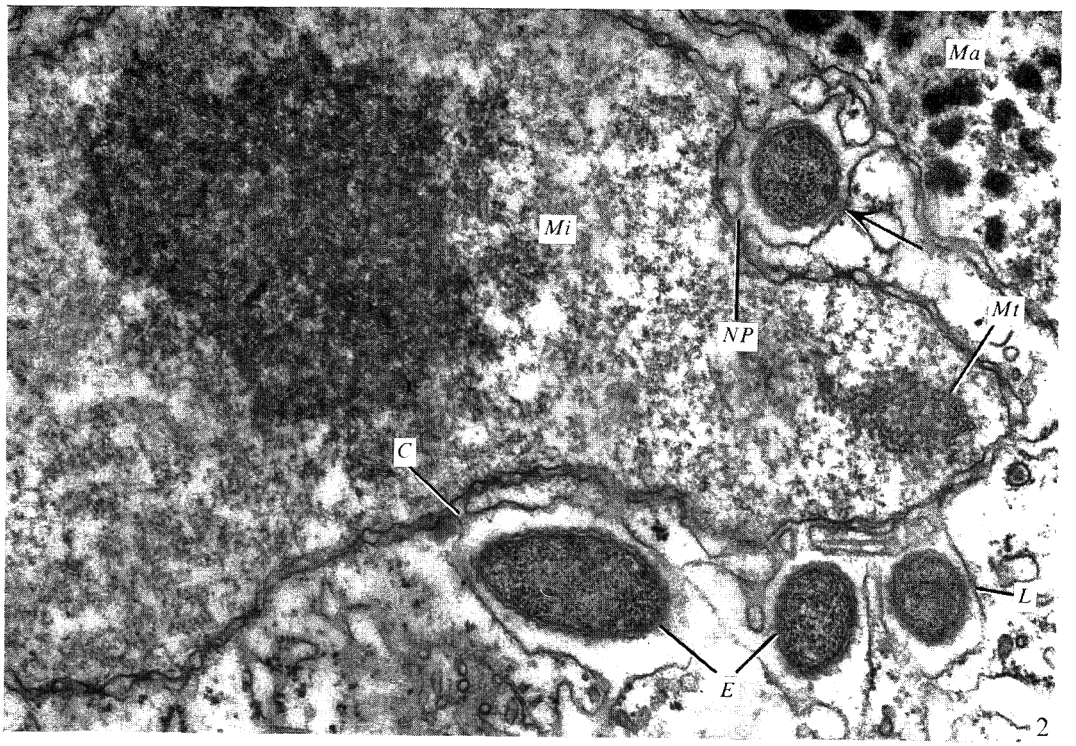

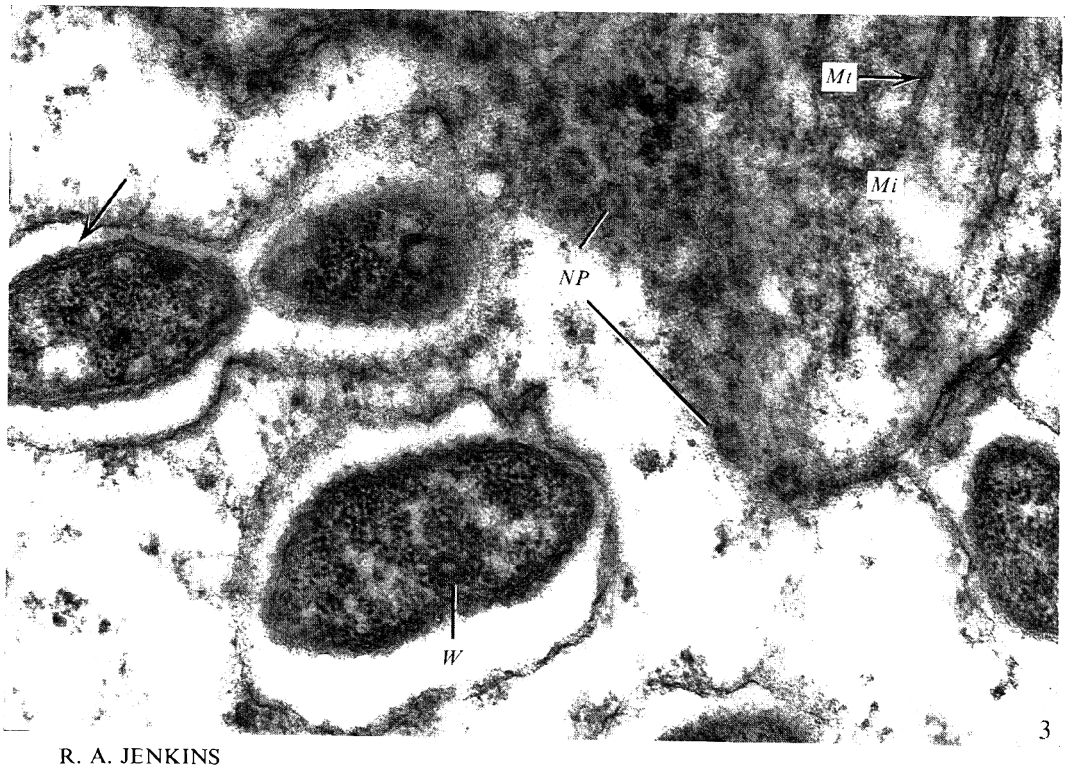

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EXPLANATION OF PLATES

Symbols: *C* = channel, *E* = epsilon particle, *ER* = endoplasmic reticulum, *F* = fibres, *L* = loculus, *Ma* = macronucleus, *Mi* = micronucleus, *Mt* = microtubules, *NP* = nuclear pore, *W* = membranous whorl.

PLATE 1

Fig. 1. A portion of a longitudinal section of Paramecium which shows the macronucleus (*Ma*) and micronucleus (*Mi*) in apposition and with points of contiguity between the outer membranes of their envelopes (arrows). Numerous epsilon particles (*E*) are present in loculi (*L*) around the micronucleus. Note the cross-sections of microtubules (*Mt*) at the opposite ends of the micronucleus; all micronuclei so far examined, whether in division or not, show such a disposition of microtubules. The most conspicuous component of the micronucleoplasm is generally the dense, central mass, presumed to be chromatin, as shown in Pl. 2, fig. 2; however, many nuclei are found to have quite extensive bundles of fibres (*F*). Whether these fibres are present at the expense of the central mass is not clear. Endoplasmic reticulum (*ER*) shows continuity with the membrane of a loculus at the broad arrow. Mitochondria and other cytoplasmic inclusions are shown at the left side of the figure. × 22,000.

PLATE 2

Fig. 2. The micronucleus (*Mi*) of Paramecium occupies the greater amount of the micrograph; epsilon particles (*E*) are present within loculi (*L*) which are formed by extensions of the nuclear envelopes via channels (*C*) initiated at the outer envelope membrane. A loculus with multiple connexions to the micronuclear and macronuclear envelopes occurs at the arrow. Cross-sections of microtubules within the micronucleus occur at *Mt*. A small portion of the macronucleus (*Ma*) is shown in the upper right. × 33,000.

Fig. 3. A high-power micrograph showing details of the epsilon particles. The two membranes of epsilon are visible at the arrow. Within a particle, a membrane whorl is discernible at *W*; the particulate cytoplasm and nuclear areas are also visible. Note the indented membrane of the loculus containing two epsilon particles. The envelope of the micronucleus (*Mi*) shows numerous annuli of nuclear pores (*NP*). Intranuclear microtubules (*Mt*) are present in this dividing micronucleus. × 74,000.

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Nature and Properties of a Cytolytic Agent Produced by *Bacillus subtilis*

By A. W. BERNHEIMER AND LOIS S. AVIGAD
Department of Microbiology, New York University School of Medicine, New York, N.Y. 10016, U.S.A.

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SUMMARY

The substance responsible for lysis of erythrocytes by cultures of *Bacillus subtilis*, designated subtilysin, was purified. It contained a peptide of leucine, aspartic acid, glutamic acid and valine and probably a lipid. Subtilysin was activated by Mg^{2+}, Mn^{2+} and Ca^{2+}. The rate of haemolysis was abruptly increased by chilling the reaction mixture. Haemolysis was inhibited by normal sera; the most inhibitory serum fractions contained α- and β-globulins. Haemolysis was inhibited by low concentrations of phosphatidylcholine, phosphatidylinositol, phosphatidic acid and sphingomyelin. Subtilysin possessed antibiotic properties and lysed protoplasts and spheroplasts derived from several bacterial species; subtilysin was identical with surfactin, a peptidelipid from *B. subtilis* cultures that inhibits fibrin clot formation. Kakinuma and co-workers found surfactin to be a heptapeptide having an N-terminal glutamic acid in amide linkage with the carboxyl group of 3-hydroxy-13-methyl-tetradecanoic acid. Surfactin (subtilysin) possesses some properties in common with two other cytolytic agents of bacterial origin, namely, staphylococcal δ-toxin and streptolysin S.

INTRODUCTION

In an earlier study a series of aerobic sporogenic bacilli was surveyed for production of haemolysin active against rabbit erythrocytes (Bernheimer & Grushoff, 1967). Among the species examined, broth cultures of *Bacillus cereus*, *B. alvei* and *B. laterosporus* were found to contain relatively potent lysins that were activated by SH-compounds and that belonged to the group of oxygen-labile haemolysins of which streptolysin O is the prototype (Bernheimer, 1970). *B. subtilis* produced a haemolysin which appeared to differ from these. What can be assumed to be the same lysin of *B. subtilis* was studied by Büsing (1950) and later by Williams (1957), who found the active moiety had the solubility properties of an organic acid. To establish more exactly its nature and properties, we have re-investigated this lytic agent; it is provisionally designated 'subtilysin'.

METHODS

Organism. Bacillus subtilis QMB 1228 was supplied by Dr H. S. Levinson.

Reagents. Phosphatidylcholine and diphosphatidylglycerol were bought from Sylvana Co. (Milburn, N.J.); phosphatidylserine, phosphatidylethanolamine, sphingomyelin and cerebrosides from Applied Science Laboratories, Inc. (State College, Pa.); phosphatidic acid from General Biochemicals (Chagrin Falls, Ohio); phosphatidyl-

inositol and serum fractions from Nutritional Biochemicals Corp. (Cleveland, Ohio); cholesterol from Matheson, Coleman and Bell (East Rutherford, N.J.).

Measurement of haemolytic activity. In the early part of the investigation, capacity of subtilysin to lyse washed rabbit erythrocytes was measured as for staphylococcal α-toxin (Bernheimer & Schwartz, 1963) but with 0·1 % (w/v) gelatin instead of bovine serum albumin. This method utilized phosphate-buffered saline solution and an incubation period of 30 min. at 37°; later, the method was altered by substituting for phosphate buffer, tris buffer + Mg and by incubating the mixtures of lysin and cells for 30 min. at 37° followed by 30 min. in an ice-bath. These changes resulted in titres about three times as great as those obtained under the conditions first used. In the method finally adopted, test preparations were diluted in 0·84 % (w/v) NaCl (buffered at pH 7·2) with 0·01 M-tris containing 0·1 % (w/v) gelatin + 0·01 M-$MgCl_2$. To 1 ml. of each of a series of dilutions increasing in about 30 % steps was added 1 ml. of a twice-washed suspension of rabbit red blood cells. The medium in which the cells were washed and suspended was 0·84 % (w/v) NaCl buffered at pH 7·2 with 0·01 M-tris. The concentration of the red cell suspension (about 0·7 %, v/v) was adjusted so that a sample, after lysis with saponin and addition of an equal volume of diluent, gave a spectrophotometric (Zeiss) extinction of 0·8 with light of 545 nm. and a light path of 1 cm. The mixtures of subtilysin dilutions and red blood cells were put in a 37° water-bath for 30 min., removed to an ice-bath for 30 min. and then centrifuged briefly. The colour of the haemoglobin in the supernatant fluids was compared visually with standard haemoglobin solutions, and the dilution haemolysing 50 % of the red cells in the suspension determined by interpolation. A unit of subtilysin is defined as that amount which liberates half the haemoglobin in the test red cell suspension under the conditions stated.

Production of subtilysin. In preliminary experiments the quantity of subtilysin produced in cultures utilizing a variety of media was estimated by titrating culture supernatant fluids for haemolytic activity; stationary and shaking cultures were used. The casein basal medium of Knight & Proom (1950) supplemented with a small amount of yeast extract gave satisfactory titres of subtilysin. 10 g. of Casamino acids (Difco Laboratories, Detroit, Michigan), 5 g. KH_2PO_4, 1 g. NaCl, 100 mg. tryptophan and 10 mg. cystine were dissolved in a litre of water, and the mixture adjusted to pH 7·0, the medium brought to a boil, filtered through paper, and distributed in 50 ml. amounts/250 ml. Erlenmeyer flask. Sterilization was at 123° for 20 min. To each flask was added 0·13 ml. 20 % (w/v) solution of yeast extract (Difco Laboratories, Detroit, Michigan). Growth and subtilysin formation in this medium are illustrated by the data of Table 1 derived from flasks which received an inoculum of 5 ml. and incubated at 37° in a shaking water-bath. Within limits of error of measurement the haemolytic activity paralleled the degree of growth. Addition to the medium of glucose, sodium ribonuclease or serum did not yield significantly higher titres.

For routine production of subtilysin a seed culture was prepared by adding a loopful of growth from an agar plate to 50 ml. medium and incubating in a shaking water-bath to an extinction of about 1·0 at 650 nm. Eight flasks containing 50 ml. medium were each inoculated with 1 ml. of a 10^4 dilution of seed culture and incubated in a shaking water-bath at 37° for 17 h. Supernatant fluids of the cultures usually contained 20 to 40 subtilysin units/ml.

RESULTS

Purification of subtilysin

Unless otherwise noted purification was at room temperature. The combined supernatant fluids from a series of cultures (stage 1, Table 2) were concentrated seven- to tenfold by pervaporation through Cellophane sacks. The decrease in volume was accompanied by formation of a precipitate (stage 2) which was collected by centrifuga-

Table 1. *Growth of Bacillus subtilis and course of appearance of subtilysin*

Time of incubation (hr)	Growth as extinction at 650 nm.	Subtilysin in culture supernatant fluid*
4	0·62	4
7	2·1	13
16	3·5	18
24	5·2	23

* Units of haemolytic activity/ml. obtained by titrations utilizing phosphate-buffered saline and incubation time of 30 min.

Table 2. *Purification and recovery of subtilysin*

	Volume (ml.)	Total units of subtilysin	Recovery of activity (%)
Stage 1. Culture supernatant fluid	1,055	35,000	100
Stage 2. Precipitate from pervaporation	134	35,000	100
Stage 3. Acid precipitate	30	15,000	43
Stage 4. Salt precipitate	30	18,300	52
Stage 5. Dialysed and lyophilized product, 234 mg.	—	20,100	57

tion and which contained virtually all the subtilysin activity. The supernatant fluid, containing less than 10 units of subtilysin/ml. was discarded. The precipitate was washed twice with 30 ml. 10 % (w/v) NaCl and then dissolved in 30 ml. distilled water. A small amount of insoluble material was removed by centrifugation, and the supernatant fluid was adjusted with N-HCl to pH 4·0. The precipitate which formed was washed twice with 30 ml. 0·1 N-acetate buffer (pH 4), and then suspended in 30 ml. 0·05 N-tris (pH 7·5). Sufficient NaOH was added to the mixture to bring to slightly above neutral pH and solution was left to occur overnight in the cold (stage 3).

A small amount of insoluble material was removed by centrifugation, and solid NaCl was added to 10 % (w/v). After standing 60 min. the mixture was centrifuged for 10 min. at 10,000 rev./min. The opalescent supernatant fluid, which contained 50 units subtilysin/ml., was discarded. The precipitate was stirred with 30 ml. 0·05 N-tris (pH 7·5) allowing several hours for solution to occur (stage 4). The solution was dialyzed at 4° against two changes of distilled water (1800 ml.) over about 20 hr, and then freeze-dried. A white solid (234 mg.) was obtained (stage 5). This product had a specific activity of 86 haemolytic units/mg. The specific activities of a series of such products varied from 55 to 95 haemolytic units/mg. Further purification was

attempted by iso-electric focusing and by use of a Sephadex G-100 column. Upon iso-electric focusing, subtilysin precipitated as it moved into the region pH 4 to 5; in Sephadex no further increase in specific activity was achieved.

Physical and chemical properties

Subtilysin was soluble in slightly alkaline water, in ethanol, acetone and chloroform; it was precipitated from aqueous solution by 10 % NaCl (w/v) or by acidification to pH 4; it did not diffuse through Cellophane. Solutions retained their haemolytic activity at 100° for 15 min. at pH 8·2 or 9·6, but at pH 3·0 two-thirds of the activity disappeared. The haemolytic activity of subtilysin was not affected by treatment with trypsin, papain or pronase. These properties suggested that the active material was of relatively large molecular size but was apparently not a protein.

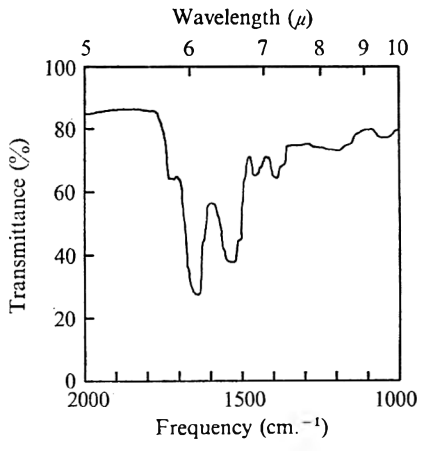


Fig. 1

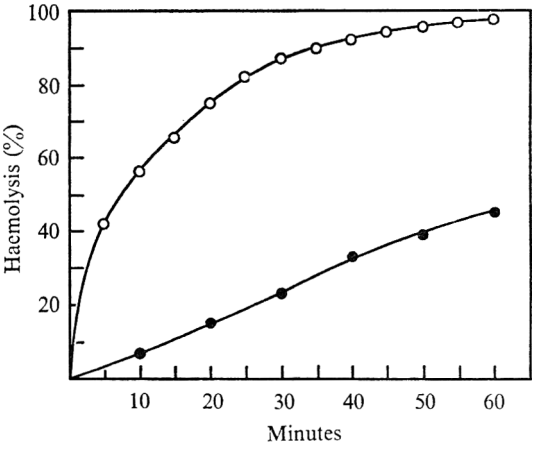


Fig. 2

Fig. 1. Infrared spectrum of subtilysin.

Fig. 2. Course of haemolysis in presence of 0·005 M-$MgCl_2$ (open circles) and in absence of added Mg^{2+} (solid circles).

A solution containing 7 mg. subtilysin/ml. 0·05 M-tris buffer (pH 7·5) appeared to be homogeneous in the analytical ultracentrifuge, giving a single peak having an $S_{20\,w}$ of about 1·2. Using a column of Sephadex G-100 (superfine) equilibrated with 0·03 M-sodium borate buffer (pH 8·5) containing 0·1 M-KCl, and calibrated with bovine serum albumin, ovalbumin and cytochrome *c*, elution volumes plotted according to Andrews (1964) gave a molecular weight of about 50,000.

Subtilysin was estimated to contain 8·8 % nitrogen (micro-Kjeldahl analysis), less than 0·3 % phosphorous (Lohmann & Jendrassik, 1926) and less than 1 % carbohydrate as glucose by the anthrone reaction (Colowick & Kaplan, 1957). When assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951) it gave a colour equivalent to 4 % of that produced by an equal weight of ovalbumin. Subtilysin after hydrolysis for 22 hr at 110° in 6·5 N-HCl yielded a ninhydrin colour equivalent to 75 % of that given by an equal weight of ovalbumin.

Analysis for amino acids (Blackburn, 1968) showed leucine, aspartic acid, glutamic acid and valine in molar ratio near 4:1:1:1. Infrared absorption (KBr) yielded a

spectrum (Fig. 1) consistent with lipid as well as of a peptide. The presence of lipid could also account for the solubility properties, the nitrogen content and the low sedimentation coefficient in relation to molecular size as determined by gel filtration. The formula weight of subtilysin is 1036; therefore the material formed aggregates of large size. Consistent with this was a tendency for subtilysin to give solutions that were opalescent.

Haemolysis and inhibition of haemolysis

Divalent cations. The following experiment shows the effect of $MgCl_2$ on haemolysis. 15 ml. tris-buffered saline (0·84 %, w/v, NaCl at pH 7·2 with 0·1 M-tris) containing 35 haemolysin units was mixed with 15 ml. 0·7 % (v/v) washed rabbit erythrocytes in tris-buffered saline; both solutions were pre-warmed to 37°; a similar mixture was prepared with 0·005 M-$MgCl_2$ present. Samples were taken at intervals and immediately centrifuged, and the percentage haemolysis estimated from light absorption readings at 545 nm. The results (Fig. 2) show that Mg substantially increased the rate of lysis. A similar effect was produced when Ca or Mn were substituted for Mg.

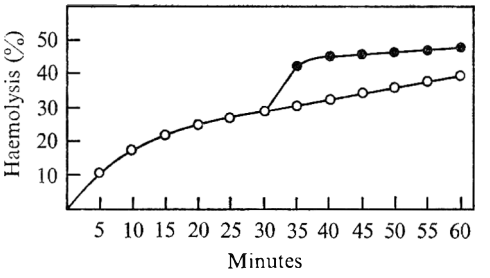


Fig. 3. Effect of chilling on course of haemolysis. Open circles: reaction mixture at 37° throughout: solid circles: reaction mixture at 37° for 30 min. and then chilled.

'*Hot–cold*' *effect.* The experimental conditions were like those described, but the concentration of subtilysin was about two-thirds as great, and 0·005 M-$MgCl_2$ was in each of two flasks of reaction mixture: one was at 37° for the duration of the experiment: the other was at 37° for 30 min. and then chilled in an ice-bath. The results (Fig. 3) show that chilling increased abruptly the rate of lysis.

Source of erythrocytes. Assays of the haemolytic activity of a single preparation of subtilysin with erythrocytes from four different rabbits agreed within 10 %; the sensitivity of erythrocytes derived from man and guinea pig resembled that of rabbit cells. About four times as much subtilysin was required to produce 50 % lysis of erythrocyte suspensions derived from calf, sheep and goat as for rabbit erythrocytes. The sensitivity of red cells from horse, pig, cat and chicken was less than that of rabbit cells but greater than that of cells from calf, sheep and goat.

Inhibition of haemolysis by serum and serum fractions. The capacity of serum to inhibit the haemolytic activity of subtilysin was assayed by mixing decreasing concentrations with a fixed amount (3 haemolysin units) of subtilysin, allowing the mixtures of 1 ml. each to stand for 10 min. at 20°, and then adding 1 ml. 0·7 % (v/v) washed rabbit red cells. After 30 min. at 37° followed by 30 min. in an ice-bath, the mixtures were centrifuged briefly and the haemoglobin in the supernatant fluids estimated colorimetrically at 545 nm. The tests were done in tris-buffered saline containing

0·005 M-$MgCl_2$ and 0·05 % gelatin; 50 % haemolysis was used as the endpoint of the titrations. About 0·005 ml. normal serum, whether from man, rabbit, calf or horse, was required to inhibit 2 haemolysin units, i.e. two-thirds the test amount of subtilysin. Assay of several Cohn fractions of human serum showed that they inhibited haemolysis in order of decreasing effectiveness as follows: α-globulin (fraction IV-1), β-lipoprotein (fraction III-0), α-globulin (fraction IV-4) and albumin; γ-globulin did not inhibit up to 5 mg./ml., the highest concentration tested.

Table 3. *Effect of lipids on subtilysin haemolysis of rabbit red cells*

	Concentration required to inhibit two-thirds of test amount of subtilysin (μg./ml.)
Phosphatidylcholine (egg)	4
Phosphatidylcholine (beef)	5
Phosphatidylinositol	6
Phosphatidylserine (brain)	100
Phosphatidylserine (plant)	70
Phosphatidylethanolamine (plant)	> 500
Diphosphatidylglycerol (beef heart)	> 500
Phosphatidic acid	10
Sphingomyelin (beef heart)	5
Cerebrosides (beef brain)	400
Cholesterol	> 500

Inhibition of haemolysis by lipids. The foregoing results suggested that lipids may play an important role in inhibition of lysis by serum. A variety of lipids suspended or dissolved in 0·85 % (w/v) NaCl at 1 mg./ml. were tested for inhibition of lysis by adding decreasing amounts to 3 haemolysin units of subtilysin. The conditions were the same as those used for testing inhibition by serum and serum fractions. To improve dispersion, most of the preparations were briefly treated by sonication before testing. The results (Table 3) show that low concentrations of several phosphatides, most notably phosphatidylcholine, phosphatidylinositol, phosphatidic acid and sphingomyelin, inhibited subtilysin haemolysis. A high concentration of cerebrosides was required to produce inhibition; cholesterol did not inhibit at all.

Bacteriolytic and antibiotic activity

Protoplasts and spheroplasts were prepared from a variety of bacteria according to methods used earlier (Bernheimer & Schwartz, 1965; Bernheimer, 1966). Extinctions of suspensions of protoplasts and spheroplasts were recorded continuously with a Cary spectrophotometer at 500 nm. for 30 min. at about 20°, with or without subtilysin. Substantial decrease in extinction was interpreted as evidence of lysis; estimates of lytic activity were made from the quantity of subtilysin needed to cause half-maximum decrease in extinction. The results (Table 4) show that protoplasts of a variety of Gram-positive bacteria were lysed, whereas among Gram-negative bacteria spheroplasts of *Escherichia coli* were lysed while those of *Vibrio comma* were not. The concentration of subtilysin required to disrupt protoplasts of the more sensitive bacterial species was about the same as that needed to lyse rabbit red cells.

Antibiotic activity was assayed by the tube dilution method. Doubling dilutions of

subtilysin were prepared in trypticase soy broth for all organisms with the exception of *Streptococcus pyogenes*; for the streptococcus, Todd Hewitt broth was used. Each assay tube containing subtilysin in 2 ml. sterile broth was inoculated with a 5 mm. loopful of a 20 hr culture of test bacterium, and incubated at 37° for 3 days. Growth was estimated visually and scored at c to 4+. Growth of *S. pyogenes* was completely inhibited by subtilysin in a concentration of 0·6 haemolysin units/ml. (about 6 μg./ml.). *Corynebacterium diphtheriae* and *Bacillus megaterium* were completely inhibited by 1 and 2 haemolysin units/ml., respectively. *Sarcina lutea* and *Micrococcus lysodeikticus* were partially inhibited by 15 haemolysin units/ml., whereas *Escherichia coli* was not affected by this concentration, the highest tested. Similar results were obtained in experiments with subtilysin-containing paper discs on agar plates, but *S. lutea* was more sensitive and *B. megaterium* less sensitive than would have been predicted from the data derived from the broth assays. No inhibition of growth was obtained with *Staphylococcus aureus*, *Gaffkya tetragena*, *Streptococcus faecalis*, *Bacillus cereus*, *B. subtilis*, *Lactobacillus casei*, *E. coli* or *Pseudomonas aeruginosa*.

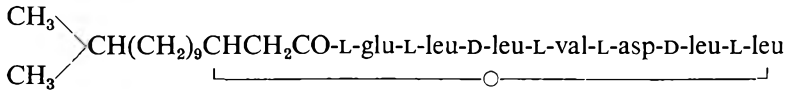
Table 4. *Lysis of bacterial protoplasts and spheroplasts by subtilysin*

	Approximate concentration producing half-maximal reduction in turbidity (μg./ml.)
Bacillus megaterium KM protoplasts (lysozyme)	5
Streptococcus pyogenes C203S protoplasts (phage-associated enzyme)	7
S. pyogenes GL8 protoplasts (phage-associated enzyme)	10
Escherichia coli K12 spheroplasts (lysozyme)	10
E. coli K12 spheroplasts (penicillin)	11
S. faecalis ATCC9790 protoplasts (lysozyme)	20
Sarcina lutea protoplasts (lysozyme)	65
Vibrio comma spheroplasts (penicillin)	N*

* N means no lysis at 100 μg./ml.

DISCUSSION

The agent responsible for the haemolytic activity of *Bacillus subtilis* was present in cultures in relatively large amounts, and it seems likely that it would have been seen before. The haemolytic moeity examined by Williams (1957) was probably subtilysin, and the properties of a substance termed serolysin by Aida, Koyama & Uemura (1964) suggest that it may be the same as the material we have studied. While the present work was in progress there came to our attention a paper by Arima, Kakinuma & Tamura (1968) describing the isolation and characterization of a *B. subtilis* product that is a potent inhibitor of blood clotting. The clotting inhibitor, named surfactin, is a peptidelipid whose structure has been elucidated (Kakinuma *et al.* 1969*a*, *b*, *c*) as



Comparison of the properties of subtilysin and surfactin showed that they possess many features in common, not least being a peptide of the same amino acid composi-

tion. A sample of surfactin supplied by Dr Kakinuma was found to have the same specific haemolytic activity for rabbit red cells as subtilysin. Moreover, the two substances showed a similar time-course of haemolysis (Fig. 4). A sample of subtilysin sent to Tokyo was examined for inhibition of the thrombin–fibrinogen reaction by Dr Kakinuma who provided the plot reproduced as Fig. 5. The clotting system consisted of 0·5 % fibrinogen (Armour Bovine Fraction I containing about 30 % clottable protein), subtilysin as indicated, thrombin 5 units/ml. and 0·01 M-tris + 0·073 M-NaCl (pH 7·4). Clotting time, the interval from thrombin addition to clot formation, was measured at 37°. The combined results left no doubt about the identity of subtilysin with surfactin; it would therefore seem appropriate to abandon the former designation.

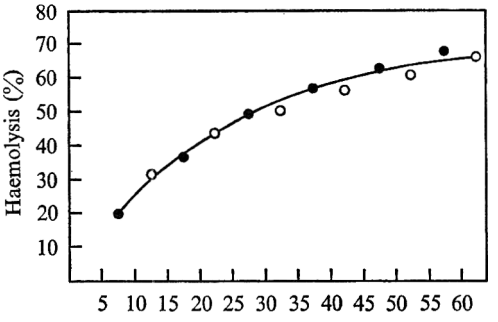


Fig. 4

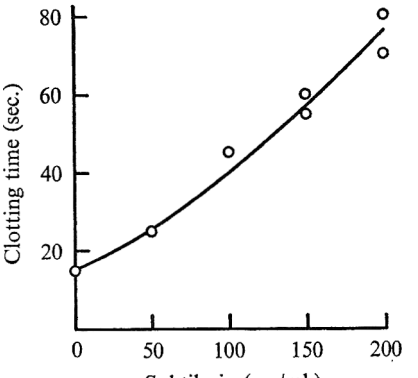


Fig. 5

Fig. 4. Course of haemolysis by subtilysin, 9 μg./ml. (open circles), and by surfactin, 10 μg./ml. (solid circles).

Fig. 5. Inhibition of clotting by subtilysin.

Surfactin possesses certain features common to at least two other agents of bacterial origin: staphylococcal δ-toxin and streptolysin S. As extracellular products of growth all are lytic, not only for mammalian erythrocytes but also for protoplasts and spheroplasts of certain bacteria. The lytic effects of all three are inhibited by phospholipids, and presumably phospholipids are the constituents of plasma membranes with which they interact to produce cell lysis. The biological activity of surfactin appears to depend upon the amphipathic nature of the molecule. One can speculate that the same may prove true of staphylococcal δ-toxin. It is noteworthy that surfactin and streptolysin S both contain peptides: neither of them is antigenic nor is δ-toxin.

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The Electron Transport System of Kappa Particles from *Paramecium aurelia* Stock 51

By C. KUNG*

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.

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SUMMARY

NADH oxidase activity demonstrated spectrophotometrically in kappa of *Paramecium aurelia* stock 51, was inhibited by KCN, antimycin A and HOQNO. Using a split beam spectrophotometer we obtained low temperature reduced minus oxidized spectra of whole paramecia and of the mitochondrial fraction of paramecia. Two absorption maxima at 588 and 556 nm. were revealed in addition to the peaks of the known *a* and *c* cytochromes. From the reduced minus oxidized difference spectrum, the carbon monoxide difference spectrum and other studies, we concluded that kappa has a cytochrome system very different from that of its host but very much like that of bacteria in the families of Brucellaceae and Enterobacteriaceae. The slight contamination in the preparations could not be responsible for the spectra obtained. These findings strongly support the recent belief that kappas and other symbionts in *P. aurelia* are procaryotic in nature and origin.

INTRODUCTION

Purified kappa particles from 'killer' stock 51 of *Paramecium aurelia* respire aerobically and have the activities of the enzymes of the catabolic pathways (Kung, 1968, 1970). A study of the electron transport system of these particles is reported here. Since most procaryotes have cytochrome systems quite different from the 'typical' cytochrome system of yeast and mammalian mitochondria (Smith, 1961), the present study is very helpful in testing the recent belief that kappas and their 'relatives' are bacterial in nature and origin.

METHODS

Sensitive (kappa-free) and killer (kappa-bearing) *Paramecium aurelia* of stock 51, syngen 4 were cultured in buffered Scotch grass infusion pre-inoculated with *Klebsiella aerogenes*. *Bacillus subtilis* was used in two cultures of killers used in obtaining the difference spectra of kappa. Kappa particles were purified using a column of epichlorohydrin triethanolamine cellulose (ECTEOLA) (Mueller, 1963). The kappa preparation was frozen and thawed three times for the study of NADH oxidase activity. Methods of culturing paramecia, and of purifying and counting kappas have been described in detail in Kung, 1968.

NADH oxidase activity was measured by following the decrease in optical density with time at 340 nm. at room temperature using a Beckman DU monochromator with a Gilford model 2000 multiple sample absorbance recorder attachment. Reactions were carried out in a phosphate buffer (0·1 M, pH 7), in a final volume of 2 ml. After

* Present address: Zoology Department, Indiana University, Bloomington, Indiana 47401, U.S.A.

the addition of pre-counted cell-free kappas, obtained by freezing and thawing three times, the reaction was started with NADH at a final concentration of $2{\cdot}05 \times 10^{-4}$ M. The inhibitors used were KCN, antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) supplied by Sigma Chemical Company. Antimycin A and HOQNO were dissolved in ethanol, and control runs with ethanol were made for the assays of these inhibitors.

A split beam spectrophotometer (Chance, 1954) was used in obtaining the difference absorption spectra. For study at the temperature of liquid nitrogen, Estabrook's method with an unsilvered Dewar flask (Estabrook, 1956) was used. The samples were frozen in the low temperature absorption cells with 2 or 3 mm. optical paths designed by Bonner (1961).

To obtain the spectra of paramecia, 2 ml. packed sensitive animals of stock 51 containing no kappa were diluted with 7 ml. of 0·01 M-phosphate buffer, pH 6·8. The thick suspension of paramecia was homogenized with a cream homogenizer before use. The mitochondrial fractions of the sensitive paramecia were obtained by conventional methods of differential centrifugation at 500*g* and 5000*g* using a raffinose medium (Preer & Preer, 1959) and 0·25 M-sucrose. Both the purified kappa preparations and the control bacterial fractions were used directly in obtaining the spectra.

Control bacterial mixtures, which simulated qualitatively and exaggerated quantitatively the contaminants in the kappa preparations, were collected by centrifugation (14,000*g*, 15 min.) from the culture fluid in which the stock 51 killer paramecia were collected.

RESULTS

NADH oxidase activity. NADH oxidase activity of cell-free kappa in 0·1 M-phosphate buffer, pH 7, at room temperature was measured to be $10{\cdot}6 \pm 1{\cdot}1 \times 10^{-9}$ mole/min./mg. dry weight (mean ± standard error). Antimycin A, HOQNO and KCN were found to inhibit this NADH oxidase activity effectively. Typical experiments showed that 10^{-3} M-KCN inhibited 70·6 and 71·7 % of the NADH oxidase activity: $9{\cdot}2 \times 10^{-6}$ M-antimycin A inhibited 72·2 and 73·1 % of the activity, whereas less than $2{\cdot}1 \times 10^{-5}$ M-HOQNO inhibited 84·9 and 86·5 %. Preliminary study showed no cytochrome *c* oxidase activity (direct oxidation of reduced cytochrome *c* from horse heart) in either cell-free kappa preparation or the homogenate of the sensitive paramecia.

Difference of spectra of homogenate from whole cell and the mitochondrial fractions of Paramecium. The enzymatically and chemically (dithionite) reduced minus oxidized difference spectra of the homogenate of paramecia were the same. One of these spectra is shown in Fig. 1, which was obtained at the temperature of liquid nitrogen. Peaks in the region at 609 and 550 nm. and a broad shoulder at 555 nm., as well as troughs at 579 and 535 nm., were seen. Considering the difference in methods, our result was in good agreement with the spectroscopic observations of Sato & Tamiya (1937) on *Paramecium caudatum*. Absorption peaks at 608 and 551 nm. occupied the position of the typical cytochromes *a* and *c* and were assigned to these cytochromes by Sato and Tamiya.

A large amount of haemoglobin exists in paramecia. The wide shoulder at 555 nm. and the troughs at 579 and 535 nm. in Fig. 1 coincide with the absorption maxima of haemoglobin and oxyhaemoglobin respectively (Smith, George & Preer, 1962). This raises the question of whether other cytochromes might also be present but concealed

in the spectrum of whole cell homogenate. Since haemoglobin has not been found in mitochondria where the cytochromes are located, we studied the mitochondrial fractions of these sensitive animals in order to test this possibility. The fraction had only a low endogenous respiratory rate which could be increased slightly by substrate but upon which neither ADP nor NADH had any obvious effect. Microscopic observation

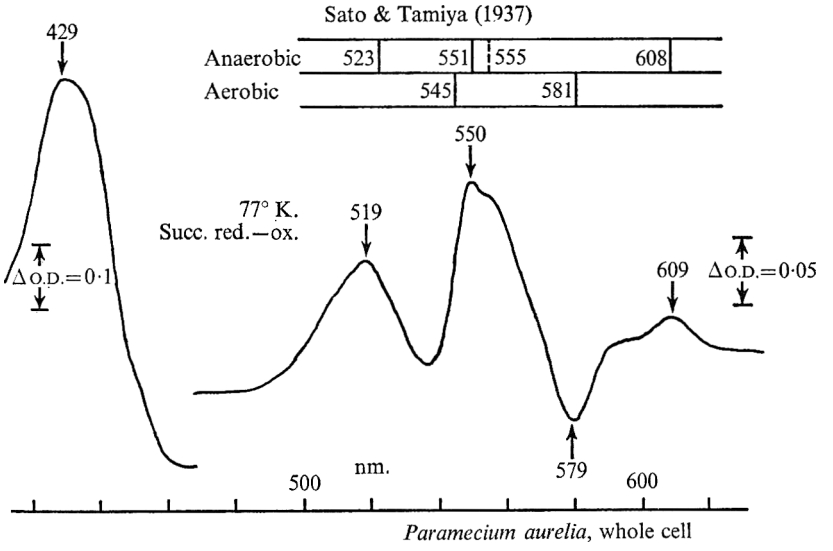

Fig. 1. Succinate reduced minus oxidized difference spectrum of *Paramecium aurelia*, stock 51, sensitive, at the temperature of liquid nitrogen. The spectroscopic observation of Sato & Tamiya (1937) is given here for comparison.

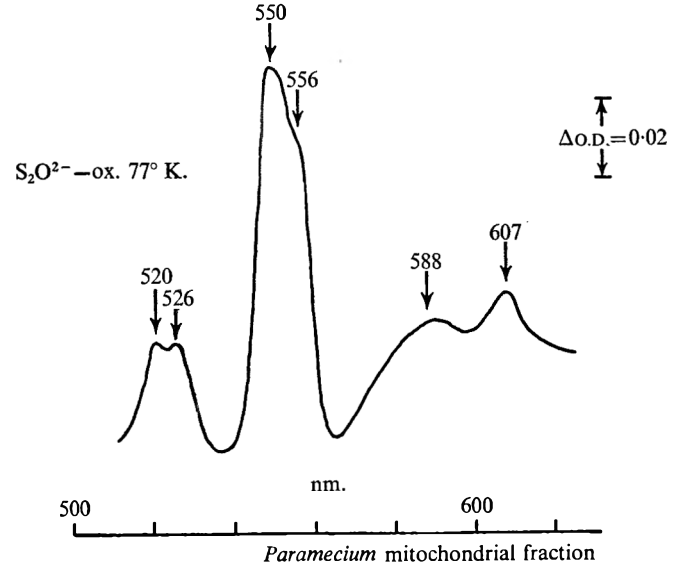

Fig. 2. Dithionite reduced minus oxidized difference spectrum of paramecium mitochondrial fraction at the temperature of liquid nitrogen.

showed that the mitochondrial fractions contained also trichocysts, cilia and some bacteria (see below and Fig. 4 for the discussion of bacterial contamination). These fractions gave consistent spectra, one of which is given in Fig. 2. The low temperature reduced minus oxidized difference spectra of this type resemble that of the whole animal in their peaks at 550 and 607 nm. The absence of haemoglobin is noted by the loss of the broad shoulder near 555 nm. and the trough at 579 nm. A new shoulder at 556 nm. is found to slope steeply toward a trough at 565 nm. Another small peak is also revealed at 588 nm. A 30,000*g* supernatant of the mitochondrial fraction treated with cetyltrimethyl ammonium bromide (CTAB) (Obayashi *et al.* 1966) gives an α peak at 548 nm. in low temperature absolute spectrum which is not accompanied by any shoulder (Kung, 1968).

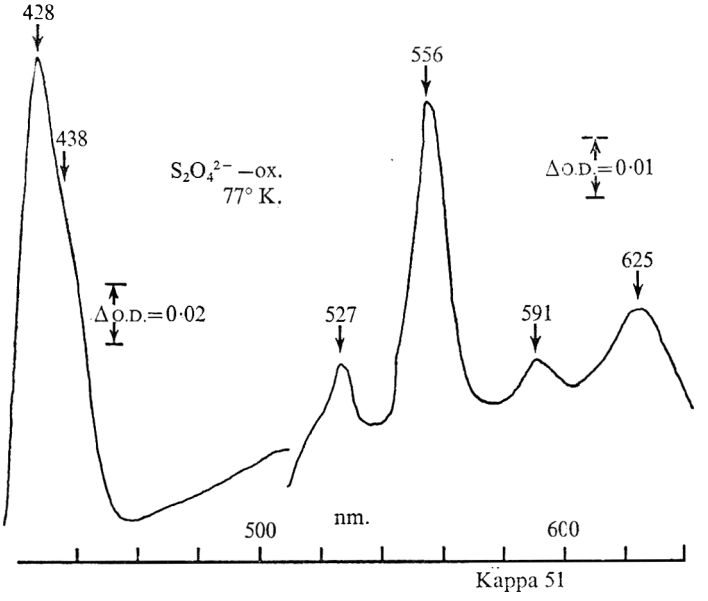

Fig. 3. Dithionite reduced minus oxidized difference spectrum of kappa 51 at the temperature of liquid nitrogen.

Reduced minus oxidized difference spectra of kappa and control bacteria. In 0·01 M-phosphate buffer, pH 6·8, purified kappas in the order of 10^{10}/ml. gave consistent spectra. Fig. 3 is one of the spectra obtained from nine kappa preparations of different concentrations reduced in various ways. This dithionite reduced minus oxidized difference spectrum of purified kappa at the temperature of liquid nitrogen is very different from that of whole paramecia (Fig. 1) and that of the paramecium mitochondria (Fig. 2). There are two α peaks at 625 and 591 nm. in the *a* region but only one sharp peak at 556 nm. in the region usually occupied by *b*- or c-type cytochromes in the classical systems, similar to the situation in many coliform bacteria (Smith, 1961).

The preparations of kappa came from killer paramecia grown in killer culture started on a food source of either *Klebsiella aerogenes* or *Bacillus subtilis*. Besides the species inoculated, however, many other airborne micro-organisms had invaded the cultures before the killers were harvested. These bacteria are apparently the major

source of contamination. As was described before (Kung, 1968, 1970), the purified kappa preparations were slightly contaminated (from 3000:1 up to 300:1 in kappa:bacteria ratio) and the above spectrum of kappa is much like the spectra of bacteria in the families of Brucellaceae and Enterobacteriaceae. Critical examination of the possible bacterial contribution to these spectra was made.

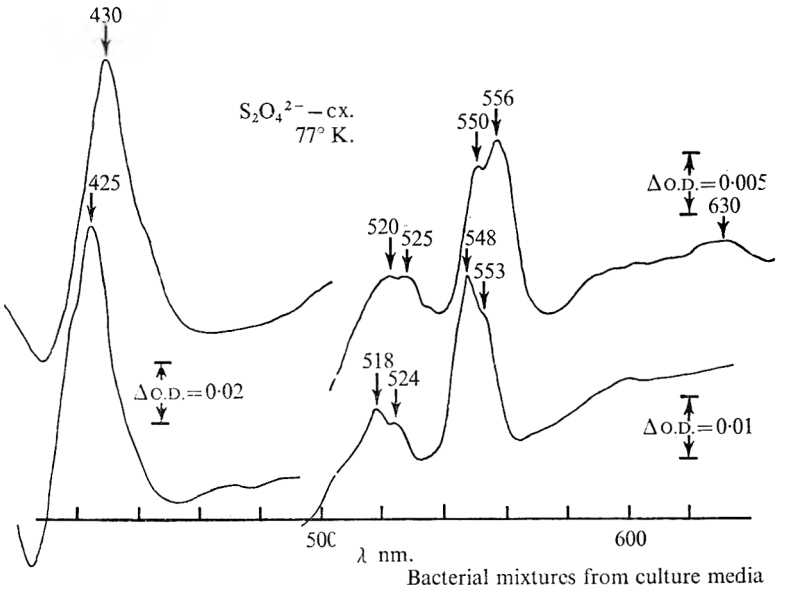

Fig. 4. Dithionite reduced minus oxidized difference spectra of bacterial mixtures collected from killer cultures. These spectra were obtained at the temperature of liquid nitrogen.

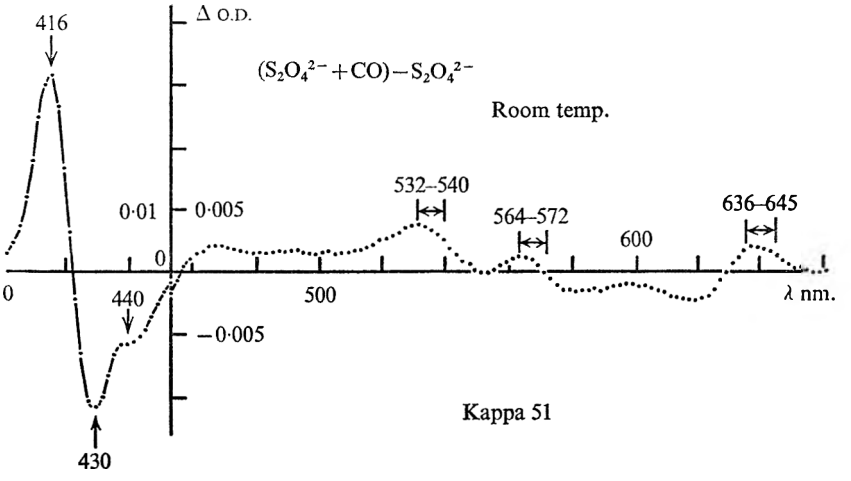

Fig. 5. Carbon monoxide treated minus reduced difference spectrum of kappa 51 at room temperature.

Bacteria in the order of 10^7 to 10^8/ml. from the cultures of killers whose kappas were used in spectral studies gave only the baseline in spectral analysis. Spectra of these bacterial mixtures in the order of 10^{10}/ml. are shown in Fig. 4. Not only did these

spectra differ from that of kappa, but they also differed from each other and from the spectra of any single bacterial species reported. Thus neither were the contaminants quantitatively sufficient to produce the spectrum of kappa, nor were their spectral characteristics qualitatively similar to those of kappa.

CO difference spectra of kappa. Figure 5 is one of the carbon monoxide difference spectra of purified kappa preparation, obtained at room temperature and corrected for the baseline. Spectra of this kind showed a peak at 415 to 417 nm., a trough around 430 nm. and plateau near 440 nm. in the γ region. The peaks in the α and β regions were not prominent even with the magnification produced by low temperature. The two α peaks at room temperature ranged from 636 to 645 nm. and 564 to 572 nm. in several studies. The peak at 415 to 417 nm. was thought to belong to cytochrome *o*, a cytochrome peculiar to procaryotes. That this peak was produced by an active cytochrome oxidase is confirmed by the photochemical action spectrum in a preliminary study using the technique of Hyde (1967) (Kung, 1968).

DISCUSSION

The cytochrome systems of paramecium have not been extensively studied before. Unmasking the haemoglobin showed the existence of more cytochromes than just *a* and *c*, as reported by Sato & Tamiya (1937). While cytochrome oxidase activity was indirectly demonstrated in paramecia (Simonson & van Wagtendonk, 1952; Boell, 1945; Preer & Preer, 1959), none of the methods used involved direct reduction of cytochrome *c*. From our findings we conclude that there are oxidases in kappa but they do not oxidize the 'typical' cytochrome *c* of mammals. The hump revealed at 588 nm. might correspond to the 580 to 590 nm. diffuse band spectroscopically observed by Ryley (1952) in *Tetrahymena pyriformis* which he considered to be the band of cytochrome a_1 or oxyhaemogoblin. Since we observed this 588 nm. peak consistently in the difference spectra of the mitochondrial preparations from *Paramecium aurelia*, it is not likely that it could be the absorption peak of oxyhaemoglobin or bacterial contamination, which varied from time to time (Fig. 4). Thus we tentatively assign this peak to cytochrome a_1 of paramecium. The identity of the 556 nm. shoulder is uncertain, although this spectral position at low temperature speaks against the possibility of many *c*-type cytochromes. When a *c*-type cytochrome is extracted by CTAB, this shoulder is not seen to accompany it. Tests on the stability to heat and acidity changes such as solubility test upon acetone-HCl treatment will help to ascertain the nature of this cytochrome.

The similarity between the difference spectrum of kappa and that of *Klebsiella aerogenes* and other coliform bacteria (Smith, 1961; Kung, 1968) is of great help in identifying the cytochromes in kappa particles. The two low peaks at the *a* regions, from 621 to 626 nm. and from 585 to 592 nm. in the reduced minus oxidized spectra of kappa, agree with the α bands of a_2 and a_1 respectively in these bacteria. The peaks in the CO difference spectrum (Fig. 5) also support this assignment. The existence of cytochrome *o* is clearly demonstrated by the CO difference spectrum, for the γ peak at 416 nm. is unique for CO-compound of this cytochrome oxidase. Our preliminary study of the CO action spectrum also revealed the same peak. In the reduced minus oxidized difference spectrum of kappa (Fig. 3) the region for the α bands of *b*- and *c*-type cytochromes is occupied by a single sharp peak, the position of which agrees

with the peak of cytochrome b_1 in coliform bacteria (Smith, 1961; Kung, 1968). HOQNO difference spectra (see Kung, 1968) also bear this out.

The KCN, CO and HOQNO inhibition of the aerobic respiration of kappa particles (Kung, 1970) as well as the presence and inhibitory characteristics of the NADH oxidase indicate the existence of an electron transport system in kappa. Perhaps the most significant finding is that the cytochrome system of kappa particles as revealed in this study is completely different from that of their hosts, the paramecia. The kappa system is strikingly procaryotic in character and is almost identical with the system of bacteria in the families of Brucellaceae and Enterobacteriaceae, but differs from those in Azotobacteriaceae, Lactobacteriaceae, Pseudomonadaceae, Bacillaceae and Micrococcaceae. Control experiments with bacteria had shown that the slight amount of contamination in the kappa preparations was not significant. That kappa has spectra like those of *Klebsiella aerogenes* is purely coincidental. Like *Klebsiella* (*Aerobactor*) *aerogenes*, *Escherichia coli* and *Proteus vulgaris* (Smith, 1954*a*, *b*, 1961) kappa seems to have a similar system of cytochrome a_1, a_2, b_1 and *o*. Having more than one, in this case three, cytochrome oxidases is not the case in a 'typical' eucaryotic system. Cytochrome *o*, in particular, is strictly an oxidase of bacteria and blue-green algae (Smith, 1961; Webster & Hackett, 1966). None of the absorption peaks of the 'typical' cytochrome system of yeast and mammalian mitochondria is seen in the spectra of kappa preparations. In fact, a trough appears at 340 nm. in the carbon monoxide difference spectrum of kappa where the major peak of cytochrome a_3–CO complex should be.

These findings justify the belief that kappas and their 'relatives' are bacterial in origin and have retained a large number of procaryotic characteristics.

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Methods for the Study of Nuclear and Cytoplasmic Variation in Respiratory Activity of *Neurospora crassa*, and the Discovery of Three New Genes

By O. J. GILLIE
National Institute for Medical Research, Mill Hill, London, N.W.7

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SUMMARY

Three methods of detecting respiratory variation in *Neurospora crassa* have been investigated. They involved the addition of dyes (pontamine sky blue and eosin yellowish) tellurite or tetrazolium to a complete medium containing ethionine. The latter prevented conidiation and enabled the colours of the colonies to be seen clearly. These methods were used to distinguish nuclear and cytoplasmic cytochrome mutants (*cyt-1*, *cyt-2*, *mi-1*, *mi-3*, *SG-3* and *SG*) from wild-type colonies on the basis of colour. Tetrazolium violet (2·5-diphenyl-3-(1-napthyl)-tetrazolium chloride) incorporated into modified Nagai medium with ethionine is recommended for distinguishing cytoplasmic mutants of Neurospora from wild-type. Certain other nuclear mutants were also distinguishable from wild-type in some of these tests. These were *ac-1*, *ac-2*, *acr-3*, *ad-4*, *leu-5*, *nit-1*, *oxD-1*. Thirty-two wild-type strains in reciprocal crosses were tested on these media to find whether these tests would distinguish any nuclear or cytoplasmic variation. No new cytoplasmic variants were found. Three new nuclear genes, B^m (mauve on dye media), *Tet-R* (ability to reduce tetrazolium) and su^- (suppressor of *mi-3*) are described and have been mapped. All three loci were linked to the mating type locus on chromosome I. *Tet-R* was found in only certain wild-type strains of mating type *a* (e.g. 74-OR8-1*a*), and *Tet-W* (unable to reduce tetrazolium) was found in all wild-type strains of mating type *A* (e.g. 74-OR23-1*A*) and certain wild-types of mating type *a*; *f*, a suppressor of *mi-1*, also suppressed *SG-3* and *SG*, but not *mi-3*; su^- suppressed *mi-3*, but none of the other cytoplasmic mutants.

INTRODUCTION

The existence of cytoplasmic mutants of *Neurospora crassa* which have abnormal cytochromes has been known for almost as long as cytoplasmic *petites* in yeast have been known (Mitchell & Mitchell, 1952; Mitchell, Mitchell & Tissières, 1953; Ephrussi, 1953). However, it has never been possible to obtain such mutants of Neurospora with ease, partly as a result of lack of specific tests. Nevertheless, about 14 different, independently obtained cytoplasmic mutants of *N. crassa* have been described in the literature. They have various different phenotypes and are recognized by various different criteria of cytoplasmic inheritance (see Table 1). Twelve are recognized by their slow or abnormal growth, and the other two are primarily recognized by ultraviolet sensitivity and morphological abnormality. At least 11 are believed to have abnormal cytochromes, which suggests that they are mitochondrial mutants.

More recent studies of cytoplasmic inheritance in Neurospora (Reich & Luck, 1966)

Table 1. *Known examples of cytoplasmic inheritance in Neurospora crassa*

Name	Mutagenic origin *	Criteria of cytoplasmic inheritance†	Abnormal cytochromes	Phenotype	Reference
abn-1	SP	2, 3, 4, 5?	Yes	Low frequency and viability of conidia, female sterile, slow and irregular growth, cultures often die. Growth best on potato dextrose agar	Diacumakos, Garnjobst & Tatum (1965); Garnjobst, Wilson & & Tatum (1965)
abn-2	SP	2, 3, 4, 5?	Yes		
AC-7	ACR	1	Yes	Slow and irregular growth	Srb (1963)
Breakdown	SP	2, 5	Not known	Low frequency and viability of conidia, female sterile, slow growth, cultures often produce brown pigment and die	Fitzgerald (1963)
Degenerate	UV	2	Not known	Slow linear growth rate, cultures often die	Lindegren (1956)
mi-1 (*poky*)	SP	1, 3, 4	Yes	Slow linear growth rate	Mitchell & Mitchell (1952); Mitchell, Mitchell & Tissières (1953)
mi-3	SP	1, 3	Yes	Slow linear growth rate	Gowdridge (1956); Mitchell & Mitchell (1952)
mi-4	SP	1, 3, 4	Yes	Low viability of conidia, female sterile, slow and irregular growth rates, cultures, often die	Pittenger (1956)
SG-3 (*mi-9*)	—	1	Yes	Slow growth	A. M. Srb, personal communication
SG	ACR	1, 3	Yes	Slow germination of spores	Srb (1963)
Stopper (*stp*)	UV	2, 3, 5	Yes	Female sterile, irregular growth, brown exudate on aerial hyphae	McDougall & Pittenger (1966)
stp-1	SP	2, 3	Yes	Female sterile, irregular growth	Bertrand & Pittenger (1964) Bertrand, McDougall & Pittenger (1968)
stp-2	SP	2	Yes	Female sterile, irregular growth	
UVS-2	SP	2	—	Ultraviolet-sensitive, female sterile.	Chang & Tuveson (1967)

* Origin of cytoplasmic variants: ACR, acriflavin-induced mutant; SP, spontaneous mutant; UV, ultraviolet-induced mutant.

† Criteria of cytoplasmic inheritance: 1, the variant shows maternal inheritance; 2, the variant shows non-Mendelian segregations; 3, the variant shows transmission in heterokaryon and related tests; 4, the variant shows 'infective' spreading through a culture; 5, the variant shows variation in phenotype on subculturing.

have claimed that the mitochondrial DNA as identified in caesium chloride gradients, shows maternal inheritance and that the mitochondrial structural proteins from *mi-1* and *mi-3* cytoplasmic mutant strains differ from the wild-type mitochondrial structural protein by one or two amino acid substitutions (Woodward & Munkres, 1966).

This paper describes specific tests for reductive activity which have been developed in order to recognize partial defects or alterations in the mitochondrial respiratory activity of colonies of *Neurospora crassa* growing on plates. Although Neurospora is an obligate aerobe (Denny, 1933), variation in ability of colonies to reduce dyes, tetrazolium and sodium tellurite has been found here. These tests have been used to survey a wide selection of well-known laboratory mutants of Neurospora and also a wide selection of wild-type strains of *N. crassa* to standardize the tests and to seek further respiratory variation. Three new spontaneous nuclear mutants which affect the expression of phenotypes in these tests are described. Despite recent very impressive progress in the study of yeast mitochondrial inheritance (Thomas & Wilkie, 1968), it still seems desirable to study mitochondrial mutants in an obligate aerobe such as Neurospora which would be expected to yield mutants with more restricted, and so possibly more readily defined, defects.

METHODS

Strains. Most wild-type and mutant strains were obtained from the Fungal Genetics Stock Center, Hanover, New Hampshire, U.S.A. Information about these mutants may be obtained from *Neurospora Newsletter* no. 13 (1968) or from Bachmann & Strickland (1965). The Oak Ridge wild-types 74-OR8-1*a* and 74-OR23-1*A* were used as standard wild-types. I am greatly indebted to Dr A. M. Srb for supplying cytoplasmic mutants *SG-3* and *SG* and many wild-type strains. The cytoplasmic mutants of Neurospora *mi-1* (*poky*), *mi-3*, *SG-3* and *SG* were chosen for detailed investigation since they have readily recognized phenotypes. All the cytoplasmic mutants of *Neurospora crassa* described in the literature are listed in Table 1. Of these, *breakdown*, *degenerate*, *mi-4* and possibly *AC-7* have been lost; *abn-1*, *abn-2*, *stp*, *stp-1* and *stp-2* are female sterile and so the criterion of maternal inheritance cannot be used, and *uvs-2* and the *stp* strains have a comparatively awkward phenotype to score. Cytoplasmic mutants not listed in Table 1 (namely, *mi-2R1*, *mi-4R1*, *mi-5R1*, *mi-6R1*, *m-7R1* and *mi-8R1*) were isolated at the same time as *mi-3* by Mitchell *et al.* (1953) and may be identical with *mi-3*, although *mi-8R1* was isolated from a different cross (Mary Mitchell, personal communication). The nuclei of all these strains, including *mi-8R1*, were alike in containing the B^m gene described below.

A number of cytoplasmic mutant strains obtained from the Fungal Genetics Stock Center and another laboratory were found after testing to have reverted. *mi-1* was recovered from a [*mi-1*]*f* stock, which may have been prevented from further reversion by the *f* (fast growth) gene which it already contained. *mi-3* was recovered from a reverted stock as described below. Similar difficulties with reversion of cytoplasmic mutants have been described by Grindle & Woodward (1968).

As suggested by Barratt (1968), cytoplasmic mutants are designated by a symbol enclosed in square brackets when it is necessary to make a distinction between nuclear and cytoplasmic mutants.

The phenotype of the *nit* mutants was recognized by the production of a yellow or reddish colour on minimal slants containing 1 % KNO_3 and 25 ml./l. bromocresol purple. *ac-1*, *ac-2*, *cyt-1*, *cyt-2* and *ty-1* were recognized by their comparatively poor growth or conidiation on minimal slants. *oxD-1* was identified as a methionine requirer and *oxD-8* by its comparatively poor growth on sulphur-free minimal medium plus D-methionine (Ohnishi, Macleod & Horowitz, 1962)

Crosses. Crosses were always made on plates of cornmeal agar at 25°. The female parent was inoculated first and when abundant protoperithecia had formed, after about 5 to 7 days, a suspension of conidia of the male parent was poured over the plate and left for several hours before pouring off. In describing crosses, the female or protoperithecial parent is always written first, according to the usual convention. Unless otherwise stated, plating tests always used ascospores spread in 0·01 to 0·5 ml. runny agar.

Media. Since anaerobically growing yeast has nutritional requirements additional to those of aerobically growing yeast (Andreasen & Stier, 1953), it seemed desirable to use a complete medium to aid the growth of mutant strains of *Neurospora crassa* with partial respiratory defects. Therefore all experiments employed a complete medium modified after Nagai (1963*a*), which consisted of peptone 0·15 %, dehydrated yeast extract 0·15 %, KH_2PO_4 0·15 %, $(NH_4)_2SO_4$ 0·15 %, $MgSO_4$ 0·1 %, agar (New Zealand) 1·5 %, sorbose 2 % (added to induce colonial growth). Biotin, 5 mg./l., was routinely added but can be omitted from dye media and probably also from the other media without making any difference. The pH of the medium was about 5·2. The medium was sterilized by steaming for 75 min. in Pyrex flasks and for subsequent use re-melted by steaming for 90 min. Before pouring plates, 2·5 g./l. glucose and 100 mg./l. DL-ethionine (obtained from British Drug Houses and other suppliers) were added to the medium. The ethionine was added to produce small nonconidiating colonies whose colour could be clearly seen. To prepare standard dye medium, 30 mg./l. each of pontamine sky blue (Gurr) and eosin yellowish (Gurr) were also added. For standard tellurite medium, 30 mg./l. Na tellurite were added. The glucose must be well mixed into the medium before the tellurite is added, or tellurite may be reduced directly by the glucose, turning the medium black. For tetrazolium medium, 100 mg./l. tetrazolium violet was added. Tetrazolium could also be used as an overlay. This consisted of 0·9 % New Zealand agar containing 2·0 mg./ml. red tetrazolium. In later experiments, 0·25 mg./ml. tetrazolium violet was used with better results. The overlay agar was cooled to about 45° before pouring. Soda-glass bottles and Petri dishes should be avoided when using eosin yellowish pontamine sky blue media, as the dye may be decolorized in an unpredictable way on the surface of the glass. The medium was prepared in Pyrex conical flasks, and could be stored in these for weeks or possibly months. Autoclaved medium was darker than steam-sterilized medium and did not give such good colour distinction between B^m and B^+ colonies. Dye solutions were kept in the refrigerator for several months without losing activity and plastic Petri plates containing the medium could be kept at room temperature for several days (so long as they were not in direct sunlight) without deteriorating. Different batches of dye have been used and no difference in the behaviour of the medium has been noted as a result of this.

Four different types of tetrazolium obtained from British Drug Houses were used: red tetrazolium, or 2,3,5-triphenyl-tetrazolium chloride; blue tetrazolium, or 3,3[1]-

diamisole-bis-4,4[1]-(3,5-diphenyl)-tetrazolium chloride; tetrazolium violet, or 2-5-diphenyl-3-(1-napthyl)-tetrazolium chloride; INP tetrazolium, or 2-(p-iodophenyl)-3-(p-nitrophenyl-5-phenyl)-tetrazolium chloride.

RESULTS

Media

Dye media. Nagai (1963*a*, *b*, 1965*a*) found that addition of certain dyes to the medium resulted in the coloration of respiratory competent yeast colonies but not of respiratory deficient (*petite*) colonies. Experiments were conducted to see if dyes could be used to distinguish wild-type Neurospora from *mi-3* and other maternally inherited mutants with abnormal cytochromes.

In preliminary experiments with dyes added to modified Nagai medium, colonies at the time of scoring were often covered with powdery conidia which prevented the true colour being observed from above, and colony colour could not be observed satisfactorily from below. However, the addition of DL-ethionine to the medium totally inhibited conidiation and enabled the true colours to be observed from above by reflected light. The 'anticonidial' effect of ethionine in liquid medium has been described by Strauss (1958).

Of a number of dyes that were tested, pontamine sky blue with eosin yellowish gave best results, staining wild-type colonies (74OR8-1*a* or 74OR23-1*A*) bright blue, whereas *mi-3* colonies were pale. The mycelium of *mi-3* colonies was stained in some places with purplish flecks of dye. The morphology also of the *mi-3* colonies was abnormal compared with the wild-type; *mi-3* colonies were very flat but were of more or less the same diameter as the wild-type.

Other dyes which distinguished between *mi-3* and wild-type were aniline blue (Gurr), azorubin S (Gurr) and Magdala red (Gurr). Little or no distinction between *mi-3* and wild-type was possible with azur *A*, malachite green, nile blue, phloxine, rose bengal and trypan blue from Gurr, and Evans blue, erythrosin B and thymol blue from British Drug Houses.

Other cytoplasmic mutants also could be distinguished from the wild-type by means of standard dye media; they all appeared thin and colourless on the first and second day, but eventually stained blue as the wild-type did. By the third day after plating, *SG* and [*mi-1*]*f* were of the same size and colour as 2-day-old wild-type colonies. *mi-3* and *SG-3* took 4 to 5 days to reach the same size and colour, and *mi-1* in the absence of the *f* (fast growth) nuclear gene took 5 or more days. *mi-2R1*, *mi-4R1*, *mi-5R1*, *mi-6R1*, *mi-7R1* and *mi-8R1* all behaved in exactly the same way as *mi-3*, with which they may be identical. The nuclear cytochrome-defective mutants *cyt-1* and *cyt-2* gave very characteristic thin, pale colonies on standard dye media that closely resembled those of *mi-3*, although *cyt-2* colonies were scarcely visible to the naked eye after two days at 32°.

Since Horne & Wilkie (1966) showed that the magdala red method described by Nagai gave the same colour reaction for respiratory deficient colonies of yeast as for certain respiratory-competent auxotrophs, it was necessary to test the specificity of the method. Twenty-seven mutants tested gave blue colonies on dye media; these included 16 different auxotrophic loci and 11 other loci which included albino, morphological, nitrate non-utilizing and acriflavin-resistant loci. The following mutant

stocks gave non-blue colonies, *ac-1*: *ac-2*, *acr-3*, *ad-4*, *leu-5*, *nit-1* and *oxD-1*. At least 20 colonies (and as many as 100 of *leu-5* and *ad-4*) were tested. The results showed that the non-blue phenotype was exclusively associated with the mutant type. The colour distinctions between *mi-3*, B^{+} (blue colony), and B^{m} (mauve colony) could be observed at 22°, 25°, 32°, and 35°. Thirty-two degrees was used for all experiments unless otherwise stated.

The pH of the medium could be changed from 4·8 to 5·6 without affecting these colour distinctions. At higher pHs B^{m} colonies tended to become blue, and at lower pHs viability of colonies was reduced. Addition of certain substances to the medium at pH 5·2 caused blue wild-type colonies to turn pink, red or pale. Substances which had this effect included: metallic salts of Zn, Cu, Cd, Hg and Co; Na azide and 2,4-dinitrophenol (uncoupling agents); malonate and fluoroacetate (Krebs cycle inhibitors); cycloheximide, 5-fluorouracil, thiamine; and glucose or sucrose in a total concentration of 1 g./l. or more. Presumably these additions caused greater reduction of the blue dye.

Experiments with the dyes *in vitro* showed that they existed in a fully oxidized form and that they could be reduced to a completely colourless form by addition of sodium borohydride. They did not act as pH indicators in the physiological range (pH 4·2 to 8·0). However, N/10NaOH rapidly decolorized eosin yellowish, and pontamine sky blue was decolorized when in solution with eosin yellowish in N/10NaOH, but not when in solution alone. Eosin yellowish was precipitated by N/10HCl, and pontamine sky blue would coprecipitate with it but did not precipitate alone. This interaction of the two dyes could be important *in vivo*.

Tellurite media. Nagai (1965*b*) showed that respiratory deficient and respiratory competent yeasts could be distinguished by their ability to reduce sodium tellurite in the medium to metallic tellurium. Wild-type yeast colonies reduced tellurite and turned black, whereas respiratory-deficient (*petite*) colonies did not. When tellurite was added to modified Nagai medium at a concentration of 30 mg./l. or to Schopfer medium 'C' (Nagai, 1965*b*), normal wild-type Neurospora colonies grown on the medium turned grey on the second day after plating, and black on the third. Two wild-type strains, *S-2* 'bisexual' (which behaved only as *A* mating type in my hands) and *79a*, were unable to reduce tellurite but they also gave colonies which were morphologically abnormal. Nuclear genes B^{+}, B^{m}, *Tet-R* and *Tet-W*, which affect colour on dyes and tetrazolium, all gave black colonies on tellurite.

The cytoplasmic mutants and nuclear cytochrome mutants (*cyt-1* and *cyt-2*) on tellurite media gave white or pale colonies until the fifth day after plating, when *mi-3*, *SG-3* and *SG* colonies turned slightly grey. *mi-1*, [*mi-1*]*f* and *cyt-1* colonies were all still white on the fifth day. Even when compared at equivalent colony sizes (rather than ages), the cytoplasmic mutants were markedly paler than the wild-type.

Twenty-three auxotrophic and other mutants on tellurite media gave normal black colonies. However, specific supplement had to be added to am^{1}, *leu-2* and *tryp-3* to give the fully wild-type phenotype. *nit-1* gave normal-sized, paler colonies which appeared to reduce tellurite more slowly, but *nit-3* gave normal black colonies. *ox D-1* gave white colonies which became greyer, resembling wild-type, if methionine (0·2 mg./ml.) was added to the medium but not if Na_2SO_3 (0·2 mg./ml.) was added to the medium. Both compounds when added to minimal medium are known to alleviate the nutritional requirement of *oxD-1* (Ohnishi *et al.* 1962). *oxD-8* gave normal black

colonies, *ad-4* and *ac-2* rather pale colonies, and *ac-1* minute thin pale colonies on tellurite media.

When sodium hydrogen selenite was added to modified Nagai medium at a concentration of 30 mg./l., wild-type ascospores developed into pink colonies after three days at 32° due to reduction of selenite, although the viability of the spores was rather reduced. The reduction of selenite by Neurospora when added to liquid medium has previously been described (Zalokar, 1953). Bismuth sulphite (5 mg./l.) in modified Nagai medium was not reduced by Neurospora colonies after 3 days. although this is readily reduced to black bismuth sulphite by *Candida albicans* (Nickerson, 1953).

Tetrazolium media. Reduction of tetrazolium has been observed in a wide variety of micro-organisms (British Drug Houses *Handbook*) and has been used to detect respiratory deficiency in yeast (Ogur, St John & Nagai, 1957). It therefore seemed, in principle, a very good test to use to detect respiratory variation in Neurospora, although there was a belief that filamentous fungi do not reduce tetrazolium (Brock, 1958).

The tetrazolium overlay method of Ogur *et al.* (1957) worked well when used with modified Nagai medium in the following way. Ascospores were spread on the medium and overlaid after at least 26 hr. at 32° with red tetrazolium at an optimal concentration of 2 mg./ml. in 9 % agar. If the colonies were overlaid before 26 hr, results were poor and variable. After overlaying it was necessary to wait at least 24 hr before the colour developed. By this method two genes were identified in the Oak Ridge wild-types which affect the colour produced. *Tet-R* readily reduces red tetrazolium, whereas *Tet-W* does not. The *Tet-R* colonies stopped growing for 2 or 3 days after overlaying, so were smaller than *Tet-W* colonies at the time of scoring. The genetics of these genes is described below.

Experiments were conducted to see whether other tetrazolium compounds could also be reduced by *Tet-R* and *Tet-W* strains of Neurospora when used in agar overlay. Blue tetrazolium and INP tetrazolium were reduced by both *Tet-W* and *Tet-R* colonies, although blue tetrazolium gave only a very weak colour that developed after several days. *Tet-W* colonies reduced tetrazolium violet at high concentrations (2 mg./ml.) but not noticeably at lower concentrations at which *Tet-W* colonies readily reduced this compound and became bright violet-red. The colour of colonies given by the reduction of tetrazolium violet at 0·25 mg./ml. in the overlay was much more intense than that given by the reduction of red tetrazolium at 2 mg./ml. Because the cost of using tetrazolium violet was only slightly greater, but the results very much better, this compound was adopted for routine studies. Good results could be obtained at 25 or 35° as well as 32° if overlaying was done when the colonies had reached the right size (at least 1·5 mm). At 22° INP tetrazolium and tetrazolium violet worked as at other temperatures, but red and blue tetrazolium gave very poor results.

If overlaid with tetrazolium at 26 hr the cytoplasmic mutants developed little or no colour whereas the wild-type developed excellent colour. However, wild-type colonies were larger than the mutant colonies at this age and younger, smaller wild-type colonies did not reduce tetrazolium. For this reason, colonies of cytoplasmic mutants were overlaid with tetrazolium when about the same size (at least 2 mm. in diameter by about 1 mm. high) as the wild types are at 26 hr. The response of four different cytoplasmic mutants to tetrazolium overlay was tested in this way in the presence of the *Tet-R* nuclear gene. Although all reduced the four different tetrazolium compounds, they did so to different degrees, and on the whole gave colonies paler than those of the

wild-type. Experiments with tetrazolium violet at 32° and overplating at daily intervals for 6 days (scoring after 24 hr) showed that *SG* could be distinguished from wild-type only when overplated on the first day (26 hr after inoculation); [*mi-1*]*f* gave reddest colonies when overlaid on the fifth day after inoculation; *mi-3* and *SG-3* colonies never became very red, and *mi-1* colonies were always very pale but became pink at the edges when overlaid after four days.

Since growth of some fungi is completely inhibited by quite low concentrations of tetrazolium (Brock, 1958), and since tetrazolium itself can induce the formation of 'petites' in yeast (Laskowski, 1954), it is usually thought necessary that tetrazolium be used as an overlay. However, tetrazolium violet or INP tetrazolium could be put into the medium itself at appropriate concentrations without preventing the growth of Neurospora colonies or markedly decreasing the viability of ascospores. This method is much simpler than the overlay method and using tetrazolium violet at 32° gave excellent distinction between *Tet-R* and *Tet-W* colonies after 24 to 48 hr INP tetrazolium stained both *Tet-R* and *Tet-W* colonies after 24 hr Red and blue tetrazolium gave rather poor colour which did not develop well until after 5 to 6 days at any of the temperatures used.

This method was also much superior for distinguishing between cytoplasmic mutants and wild-type and was also cheaper. All the cytoplasmic mutants tested (in the presence of *Tet-R* nuclear gene) were easily distinguishable from wild-type on tetrazolium violet medium at 32° for at least 4 days after plating. By the fifth and sixth days *SG*, [*mi-1*]*f*, and *SG-3* appeared much more like wild-type, but *mi-1* and *mi-3* still appeared lightly coloured. With INP tetrazolium the cytoplasmic mutants began to colour earlier and closely resembled the wild type by the fourth day. At 22° and 25° the wild-types stain most readily with INP tetrazolium, and the cytoplasmic mutants grow relatively poorly, staining very little with INP tetrazolium and not at all with tetrazolium violet. The responses at 35° of wild-type and cytoplasmic mutants were the same as at 32° only slightly faster.

The cytoplasmic mutants tended to grow at first as very flat colonies (whether on dye, tellurite or tetrazolium media), closely apposed to the surface of the medium. Later the edges of the colony tended to thicken and stain with the tetrazolium whether it was in the medium or in an overlay, giving the colony the appearance of a plate with a red border. Still later, the colonies of the cytoplasmic mutants thicken in the middle but always remain slightly concave, unlike the wild-type colonies which are always convex. Curiously, *cyt-1* colonies are raised in the centre and flat at the edges (hat-shaped), and it is the raised central portion of the colony which first becomes tetrazolium-positive.

Twenty-five auxotrophic and other mutants were tested as before to see if the mutant block interfered with reduction of tetrazolium *Tet-R* colonies. Mutants which gave abnormal pale colonies were: *ac-1*, *ac-2*, *ad-4* and *nit-1*.

Genetics of B^m, *Tet-R and* su^-

When *mi-3 A* (2343, Fungal Genetics Stock Center no. 383) was crossed to wild-type, blue, mauve and pale colonies were obtained when it was the female parent, but only blue and mauve colonies when it was the male parent. Ten colonies of each type from each of the reciprocal crosses were picked and crossed reciprocally to wild-type, and more than 300 reciprocal crosses made amongst themselves.

The results could be accounted for by the action of two nuclear genes, B^m which gave a mauve colony colour, and su^- which suppressed the *mi-3* phenotype. The pale colonies obtained when *mi-3* was the maternal parent were the unsuppressed *mi-3* phenotype, and the suppressed *mi-3* phenotype appeared as blue or mauve depending on whether B^+ or B^m was present in addition to su^-. These suppressed *mi-3* colonies were indistinguishable from blue or mauve colonies with the wild-type cytoplasm. Both B^m and su^- showed linkage to the mating type locus (Table 2) but they showed no significant linkage to each other.

Table 2. *Analysis of linkage of B^m and su^- to mating type and to each other*

In crosses 1 and 2 an arbitrary number of pale, blue or mauve colonies were picked and tested for mating-type. In crosses 3 to 6 the numbers of pale, blue and mauve colonies are those obtained in counts from platings of random ascospores.

	Phenotype and genotype					
	Blue		Mauve		Pale (B^m or B^+)	
Cross						
1. $B^+ su^+ a \times B^m su^- A$, [*mi-3*]	$B^+ A$ 20	$B^+ a$ 58	$B^m A$ 52	$B^m a$ 30		
2. $B^m su^- A$, [*mi-3*] $\times B^+ su^+ a$	$B^+ su^- A$, [*mi-3*] 27	$B^+ su^- a$, [*mi-3*] 43	$B^m su^- A$, [*mi-3*] 53	$B^m su^- a$, [*mi-3*] 17	$su^+ A$, [*mi-3*] 57	$su^+ a$ [*mi-3*] 94
3. $B^m su^- A$, [*mi-3*] $\times B^+ su^+ A$, [*mi-3*]	$B^+ su^-$, [*mi-3*] 585		$B^m su^-$, [*mi-3*] 504		su^+, [*mi-3*] 1087	
4. $B^+ su^+ a$, [*mi-3*] $\times B^m su^- A$ [*mi-3*]	345		354		548	
5. $B^m su^+ A$, [*mi-3*] $\times B^+ su^- a$, [*mi-3*]	122		71		177	
6. $B^+ su^- a$, [*mi-3*] $\times B^m su^+ A$, [*mi-3*]	745		603		1054	

Analysis of linkage relationships

		recombinants	recombination
Cross 1	Recombination between *B* and *A*	50/160	31·25
Cross 2	Recombination between *B* and *A**	44/140	31·4
Cross 2	Recombination between *su* and *A*	117/291	40·2
Crosses 3 to 6	Recombination between *su* and *B*	585/1089, 345/699, 71/193, 603/1348	45 to 54†

* Calculated assuming no linkage between su^+ and B^+, which is justified by independent measurement of this in crosses 3 to 6, and since the same value for linkage between B^+ and *A* is obtained as in cross 1.

† Test for heterogeneity of crosses 3 to 6 using the index of dispersion χ^2_3 showed heterogeneity, so the results of these crosses cannot be pooled. However, the results show that recombination is between 45 and 54%, ignoring cross 5, which was rather infertile and so may have given biased results.

Amongst the 30 single colonies isolated (10 mauve, 10 blue and 10 pale) from the original cross in which *mi-3* was the maternal parent, three exceptional isolates were found which in crosses to the wild-type or to other isolates did not give pale colonies and therefore could not contain the *mi-3* cytoplasm. These all proved to be su^+, and two were B^m and one was B^+. In crosses of su^+, [*mi-3*] with wild-type, occasional blue or mauve colonies were obtained when all the progeny would be expected to be pale colonies. An experiment was designed to discover whether such colonies originated from the supposed male parent forming occasional perithecia which were fertilized

by the supposed female parent, or arose from comparatively rare transfer of male cytoplasm, or were perhaps contaminants. It seemed reasonable that comparatively rare transfer of male cytoplasm might occur since paternal inheritance of *SG* has been described by Srb (1963).

The method used was to isolate a whole ripe perithecium and disperse its contents in 1 ml. of runny agar in a bijou bottle. The ascospores were activated in the bijou bottle, and then as much agar as possible was shaken out of the bottle onto a single plate, thus making a sample of the contents of a single perithecium readily identifiable. Only two wild-type blue colonies were found out of 9300 colonies examined from 234 perithecia taken from four different crosses of an *mi-3* female crossed with a wild-type male. These two colonies came from the same perithecium, which also gave 220 *mi-3* colonies. It was shown by crossing that they did not result from mutations at suppressor loci. These two colonies could have resulted from either back mutation of a *mi-3* determinant or from rare transfer of determinants from the male. A control cross of an *mi-3* female crossed with an *mi-3* male produced 6656 pale (*mi-3*) colonies from 78 perithecia and no wild-type colonies. It may be concluded from these results that formation of perithecia by the 'male' parent happened rarely, if at all (no instances in 234 perithecia analysed); transfer of *mi-3* male cytoplasm occurred rarely, if at all (not more than 2/9300 ascospores); spontaneous back, mutation of the *mi-3* determinant occurred rarely, if at all (not more than 2/15,956 ascospores); and that spontaneous mutation at suppressor loci was rare (0/15,956 ascospores). The more frequent occurrence of the wild-type colonies in the original *mi-3* cross-analysed above may have been due to back-mutated particles having become established in the cytoplasm of the strains analysed.

Thirteen laboratory 'wild-type strains' of Neurospora (Emerson, Lindegren, St Lawrence & Yale) were tested and all gave normal blue colonies on standard dye media except two which gave mauve colonies. It may be significant that these two strains had at one period been revived from very old subcultures kept on slants. Seventeen other wild types from different parts of the world were tested on dye media, and six of these were found to give blue colonies, five to give mauve colonies, and six to give intermediate mauvish blue colonies. These wild-types were tested in crosses with each other and with standard Oak Ridge wild-types which were made reciprocally with respect to maternal parent. No differences between reciprocal crosses (maternal inheritance) were observed.

Crosses of the two Oak Ridge wild-types (74-OR8-1*a* and 74-OR23-1*A*) with each other gave both red and white colonies on tetrazolium media. In several crosses of such red and white colonies an overall proportion of 3146 red colonies to 3197 white colonies to 61 ambiguous or sectored colonies was obtained. The gene determining red colonies was called *Tet-R*, and its allele determining white colonies was called *Tet-W*. The locus concerned was closely linked to mating-type. Wild-type 74-OR8-1*a* contained the *Tet-R* gene and wild type 74-OR23-1*A* contained *Tet-W* gene. Of 239 progeny of a cross of *Tet-R* × *Tet-W* scored for mating type and colour on tetrazolium, 14 putative recombinants between *Tet* and the mating-type locus were found. The 14 putative recombinants were tested by crossing to confirm their genotypes, and four of these were found to have been misclassified or to have been assigned phenotypes which differed from their genotypes. However, of 29 non-recombinant colonies whose genotype was tested by crossing, all were correctly classified. It therefore seems justi-

fiable to assume that misclassified colonies were of high frequency amongst putative recombinants only because misclassification would almost always have led to a colony being classified as a recombinant rather than a parental type. This experiment shows that the *Tet-R* locus was closely linked to the mating type locus, with only 4·2 % (10/239) recombination occurring between them.

Of the recombinants obtained, seven were *Tet-Wa* and only three *Tet-RA*. By means of back-crosses it was shown that these phenotypes were determined by genes allelic with the parental ones (i.e. they were not new mutants). Reciprocal crosses of all *Tet-Wa* recombinants with a *Tet-RA* tester showed that 1:1 segregations occurred whichever parent was the maternal parent, and therefore all the seven *Tet-W* recombinants were genuine and mutation of a cytoplasmic particle was not involved. The small number of ambiguous or sectored colonies obtained (61/6404 = 0·95 %) occurred at rather too high a frequency to be accounted for entirely by non-disjunction of the mating-type chromosome leading to transient aneuploids (Martin, 1959) but may also have been accounted for by mixed and phenotypically variable colonies.

It was surprising that the Oak Ridge wild-types (74-OR8-1*a* and 74-OR23-1*A*) differed in the presence of the *Tet-R* or *Tet-W* gene, since they had been selected after extensive backcrossing in various laboratories from the original Lindegren isolates. This might have been expected to make the strains homozygous. A number of these laboratory strains of Neurospora and other wild-types from different parts of the world were analysed to see if they contained the *Tet-R* or *Tet-W* allele. The *Tet-R* allele was found in some of the mating type *a*, and never in the mating type *A*. All the common laboratory wild-type stocks of the *a* mating type (Emerson *a*, Lindegren *a*, St Lawrence *a*) contained the *Tet-R* gene except for SY4*f*8*a* which gave white colonies. The Yale strain SY4*f*8*a* had been backcrossed for eight generations, and so no doubt the *Tet-W* gene was introduced into these stocks in the course of these crosses. Of 16 other wild types from different parts of the world, only Puerto Rico 18*a* gave red colonies with tetrazolium, and crosses to a *Tet-RA* tester strain showed that the gene involved was an allele of *Tet-R*. Puerto Rico 15*A* gave white colonies in crosses with *Tet-R*, and so obeyed the rule that strains of *A* mating type are always *Tet-W*.

All the other wild-types of both mating types gave only white colonies in crosses with tester strains. By making crosses reciprocal with respect to male or female parent with *Tet-R* and *Tet-W* tester strains, it was possible to show for 27 laboratory and other wild-types that the white or red phenotype was determined by alleles of *Tet-R* or *Tet-W*. Reciprocal crosses between wild-types showed that there were no cytoplasmic effects on the expression of *Tet-R* or *Tet-W*. The majority of mutant strains tested of the *a* mating type contained the *Tet-R* allele and the majority of mutant strains of *A* mating type tested contained the *Tet-W* allele. Exceptions to this rule involved mutants on chromosome 1 (on which *Tet* and the mating-type locus are located) which presumably have been subjected to more recombination and selection involving the *Tet* and mating-type loci. Further investigations of the linkage relationships of *Tet-R* (see Table 3) were consistent with this locus being on the left arm of chromosome 1, very close to the *ad-3B* locus and probably located between the *ad-3B* and the mating-type locus. *Tet-R* also showed very close linkage to *acr-3* (resistant to 50 mg./l. acriflavin), which may also be to the right of the mating-type locus; this arrangement is consistent with the linkage data of Hsu for the *acr-3* locus (Hsu, 1965).

Mutant *su*$^-$ was found to be on the right arm of chromosome 1, located between

al-2 and *nit-1* (Table 3); *su*⁻ showed 40 % recombination with *Tet-R*. B^m was located on the left arm of chromosome 1 to the left of *nit-2* and showing 29·6 % recombination with it (Table 3).

Table 3. *Linkage data on random segregants from crosses involving the genes Tet-R, su*⁻, *[mi-3] and* B^m.

In the data for each cross, the top number for each pair represents the progeny class which contains the top marker on the left.

Zygote genotype and percentage recombination	Parental combinations	Recombinations: Singles Region I	Recombinations: Singles Region II	Recombinations: Doubles Regions I & II	Total isolates tested	Marker isolation numbers (Cf. first column left to right)
A *Tet-W* *ad-3B*	30	3	1	0	73	m.t.
a *Tet-R* +	36	3	0	0		No number
8·2 1·4						2-017-0128
A *acr-3*ʳ *Tet-W*	54	2	1	0	119	m.t.
a *acr-3*ˢ *Tet-R*	56	5	0	1		r(KH14)
6·7 1·7						No number
+ *a* *Tet-R*	77	10	4	1	170	K31
nit-2 *A* *Tet-W*	64	7	6	1		m.t.
11·2 7·1						No number
A *su*⁻ +	65	45	2	2	201	m.t.
a *su*⁺ *al-2*	51	31	3	2		No number
39·8 4·5						15,300
♀ parent [*mi-3*]						
A + *su*⁻*2*	56	32	13	3	197	m.t.
a *nit-1* *su*⁺	51	24	14	4		34,547
32·0 17·3						No number
♀ parent [*mi-3*]						
B^m *A* +	12	12	16	11	100	No number
+ *a* *al-2*	13	11	13	12		m.t.
46 52						15,300
+ *A* *ad-3B*	55	28	12	5	200	No number
B^m *a* +	57	32	11	0		m.t.
32·5 14						2-017-0128
B^m + *a*	55	27	24	0	206	No number
+ *nit-2* *A*	49	30	17	4		K31
29·6 21·8						m.t.

Table 4. *Action of suppressors f and su*⁻ *on cytoplasmic mutants*

Suppression of the cytoplasmic phenotype is indicated by +. No suppression is indicated by −. Suppressed cytoplasmic mutants were recognized by clearly increased colony size in crosses of the cytoplasmic mutant with *f* or *su*⁻, scored after 2 or 3 days growth on Nagai medium with dyes at 32°.

Cytoplasmic mutant	Nuclear suppressor: *f*	Nuclear suppressor: *su*⁻
mi-1	+	−
mi-3	−	+
SG-3	+	−
SG	+	−

A gene called *f* (fast growth), which suppresses (or partially suppresses) the *mi-1* phenotype, was described by Mitchell & Mitchell (1956), and a suppressor of *mi-3*, called *su*$^-$, is described here. Since the suppressed and unsuppressed colonies could be very clearly distinguished on dye or other indicator media, tests were made to see if either *f* or *su*$^-$ affected cytoplasmic characters other than the one in which the suppressor was isolated. The results of these tests are given in Table 4 and show that the suppressors had different complementary actions. *su*$^-$ would only suppress *mi-3*; and *f* suppressed *mi-1*, *SG-3* and *SG* but not *mi-3*.

DISCUSSION

Three basic methods used previously to detect respiratory deficiency in yeast have been adapted to detect respiratory variation in *Neurospora crassa*. The four cytoplasmic mutants *mi-1*, *mi-3*, *SG-3* and *SG* could be readily distinguished from wild-type on medium containing dyes, tellurite or tetrazolium. These mutants, on all three media, began with very thin growth, but could eventually form colonies almost indistinguishable in size and shape from wild-type.

All colonies of Neurospora (whether wild-type or the cytoplasmic mutant) were colourless to begin with and developed colour over a period of time on the various indicator media. The wild-type developed colour within 2 days, whereas the nuclear and cytoplasmic cytochrome mutants took longer, and on tellurite and tetrazolium media always had a paler colour than the wild-type for a given age or colony size.

All the cytoplasmic mutants examined could be distinguished on these media from the wild-type by size alone for the first 2 or 3 days, but the addition of tellurite or tetrazolium violet made it easier, particularly in the case of *SG*, which grew faster than the other cytoplasmic mutants.

Wild-type Neurospora behaved differently from wild-type yeast on dye media. Whereas wild-type yeast is colourless and respiratory-deficient yeast is purple to violet, wild-type Neurospora was blue and partially respiratory-deficient Neurospora was pale, turning blue later.

Eosin yellowish was normally reduced by Neurospora B^+ colonies but not by B^m colonies. Pontamine sky blue was presumably reduced in the brilliant pink colonies produced when thiamine or additional sugar was added to the medium. It has been suggested that addition of thiamine to cultures of the fungus *Rhizopus nigricans* forces the anaerobic breakdown of glucose (Foster & Goldman, 1948); this might also occur when the sugar concentration in the medium is high and might, because of changes in the organization of electron transport, cause colonies to reduce the blue dye and so become pink in colour.

None of the methods for detecting respiratory variation was entirely specific in that certain nuclear mutants which do not, as far as we know, have cytochrome abnormalities could nevertheless be recognized by their appearance on the medium. *ac-2*, *ad-4* and *nit-1* gave large, pale-coloured colonies on all three media. This response is difficult to interpret, since a paler colour on dyes should, on the simplest interpretation, indicate a stronger reductive activity, and a paler colour on tellurite or tetrazolium a weaker reductive activity. *ac-2* is an acetate-requiring mutant, but unfortunately little more information seems to be generally available (Perkins, Glassey & Bloom, 1962). *ad-4*, an adenine-requiring auxotroph, lacks the enzyme adenylo-

succinase and accumulates, amongst other substances, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide in the mycelium (Bernstein, 1961). This substance is colourless but becomes red on exposure to air so might well interfere with the normal reduction of the indicator substances. *ad-3B*, which gives normal colonies on the three media, is blocked at an earlier point in the enzyme sequence and does not accumulate this substance (Bernstein, 1961). *nit-1* lacks nitrate reductase, but so does *nit-3*, which has wild-type phenotype on these media. The differences between these two nitrate non-utilizing mutants are not yet fully understood, although there seems to be a close functional relationship, if not an identity, between nitrate reductase and cytochrome *c* reductase in Neurospora (Sorger, 1963).

Mutants *acr-3* (acriflavin resistance) and *leu-5* (leucine auxotroph) gave pale mauvish and pale purple colonies respectively on dye media but reduced tellurite and tetrazolium normally. *oxD-1* gave mauve colours on dyes and did not reduce tellurite very well; this mutant lacks D-amino acid oxidase and is also unable to utilize sulphate, probably because it cannot reduce this compound (Ohnishi *et al.* 1962). *oxD-8* also lacks D-amino acid oxidase, but is not deficient in reduction of sulphate or abnormal in its appearance on dye or tellurite media; this suggests that the abnormal phenotype of *oxD-1* on these media results from its defective reduction of sulphates. B^m, which was a poor reducer of dyes, reduced tellurite and tetrazolium normally, while *Tet-W*, which was a poor reducer of tetrazolium, reduced tellurite and dyes normally.

The most specific tests for respiratory variation were found to be those using tellurite or tetrazolium violet in the medium. These tests, particularly the latter, showed differences in colour betweeen cytoplasmic mutants and wild-types not only at the same age but also when colonies of the same size were compared. Details of the mechanism of tellurite reduction do not appear to be known (Nagai, 1965*b*), but it is believed that red tetrazolium may be reduced by the action of succinic dehydrogenase (Nagai, Yanagishima & Nagai, 1961). The ease with which tellurite and the various tetrazolium salts were reduced by Neurospora colonies did not seem to be simply related to the redox potential of the substance (redox potentials are given in Pearce, 1960) since red tetrazolium and blue tetrazolium were least easily reduced by Neurospora colonies and have redox potentials in between that of the tellurite and INP tetrazolium which were both very readily reduced by Neurospora colonies under the conditions described.

The discovery of the *Tet-W–Tet-R* gene difference between the supposedly isogenic Oak Ridge wild-type strains (74-OR8-1*a* and 74-OR23-1*A*) is very interesting, since this genetic difference can be traced through the various laboratory wild-types and has also been discovered in the Puerto Rican wild-types. Because the real function of this gene in nature is unknown, it is impossible to speculate constructively about whether or not this genetic difference at the mating-type locus is of some mutual advantage to the two mating-types in the wild. However, the existence of this genetic difference near the mating-type locus must make us extra cautious about attributing such phenomena as poor growth of heterokaryons comprising nuclei of opposite mating-types (Gross, 1950) to the action of the mating-type locus alone. It is interesting that Hsu (1965) has discovered a spontaneous difference in acriflavin resistance between wild-type strains 74*A* and 73*a* (closely related to the Oak Ridge strains), due to nuclear gene *acr-1* closely linked to the mating type locus in chromosome I and therefore closely linked to *Tet-R*. This is further evidence that the extensive backcrossing to which the laboratory wild-

type strains of Neurospora have been subjected (Barratt, 1962; Case, Brockman & de Serres, 1965) have failed to make them isogenic in the region of the mating type locus. There may therefore be other gene differences closely linked to the mating-type locus which are concerned with other functions such as the actual process of mating and sexuality.

The usefulness of the media described is undoubtedly limited by the presence of sorbose to give colonial growth which must impose abnormal adjustments on the energy pathways that are indirectly involved in the processes we wish to investigate (Crocken & Tatum, 1968). Use of colonial mutants instead of sorbose to induce colonial growth might involve similar difficulties since Brody & Tatum (1966) have shown that one colonial mutant *col-2* of *Neurospora crassa* has a partially defective glucose-6-phosphate dehydrogenase which must have widespread pleiotropic effects on energy metabolism. Ethionine has been shown to be incorporated into the protein of Neurospora (Kappy & Metzenberg, 1965), but it has also been suggested that the inhibitory action of ethionine in yeast and other organisms results from its trapping of ATP (Stekol, 1963; Spence, Parks & Shapiro, 1967). The anticonidial effect of ethionine might result from limited quantities of ATP being produced, which the organism reserves for essential growth and maintenance functions rather than for sporulation.

The observations of suppressor activity on cytoplasmic mutants first made by Mitchell & Mitchell (1956) have been extended here to one other suppressor and two other cytoplasmic mutants. The results show that *f* is specific in suppressing *mi-1*, *SG-3* and *SG*; and *su*$^-$ is specific in suppressing only *mi-3*. This shows that *mi-1*, *SG3* and *SG* must have related defects, although all three have readily distinguishable growth rates on plates, and *SG-3* is most similar in colony growth rate to *mi-3*. It may be significant that Griffiths, Bertrand & Pittenger (1968) have observed that the cytochrome spectra of *mi-1* and *SG-3* are similar to each other but different from wild-type and that the cytochrome spectrum of *mi-3* differs from that of *mi-1*, *SG-3* and wild-type.

During the course of the investigations described here, preliminary reports have appeared describing the differential staining of wild-type and *poky* colonies by means of a red tetrazolium overlay (Wilson, 1967), and 'respiratory-deficient' strains of *Aspergillus nidulans* have been isolated by a tetrazolium overlay method (Houghton, 1967).

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Mutants of *Streptomyces coelicolor* Defective in Sporulation

By D. A. HOPWOOD, H. WILDERMUTH AND HELEN M. PALMER
John Innes Institute, Norwich NOR 70F

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SUMMARY

Sporulation-defective mutants were isolated on the basis of altered colonial coloration. The mutants were classified into a number of morphological classes by means of phase-contrast and electron microscopy. Some mutants lacking sporulation septa, the special septa that lead to spore delimitation and separation in the wild-type, or differing from the wild-type in the spacing of such septa, were subjected to genetic analysis. This has so far revealed two, and possibly three, genes concerned with the formation and spacing of sporulation septa.

INTRODUCTION

Mutants defective in any complex biological process are likely to aid its understanding by dissection of the total phenomenon into unit processes, and recognition of their interrelationships. A familiar example is provided by the use of auxotrophic mutants in the description of biosynthetic pathways. In the analysis of morphogenesis, this approach has proved most valuable in one of the simplest systems, T4 bacteriophage (reviewed by Levine, 1969), where many genes controlling the assembly of different components of the virus have been identified and part of their sequence of operation has been determined.

In a more complex system, the endospore of the protokaryotic *Bacillus subtilis*, some progress has been made in the isolation and characterization of mutants with a defective sporulation ability (reviews: Balassa, 1969; Schaeffer, 1969). The prerequisites for such a study, well-developed experimental genetics combined with suitably complex morphological processes, are currently found in only one other protokaryote, *Streptomyces coelicolor*; it therefore seemed worth while to attempt an analysis of a morphogenetic sequence in this organism to provide a comparison with studies on the *B. subtilis* system, as well as with those on the more numerous eukaryotic systems.

The organization of *Streptomyces coelicolor* colonies is complex (Hopwood, 1960; Wildermuth, 1970). On an agar medium a spore germinates to give rise to a 'substrate' mycelium consisting of much-branched, septate hyphae, and branches of these hyphae later initiate the 'aerial' mycelium in which the spores develop. Several phases of colonial development might be analysed by mean of mutants: spore germination, branching and septation of the substrate mycelium, development of the aerial mycelium, spore delimitation and maturation. An advantage of working with mutants defective in some aspect of the aerial mycelium is that the whole of this phase of the colony is dispensable: colonies totally lacking aerial mycelium can be propagated by sub-culturing substrate mycelium. Hence any mutants with specific defects in the

aerial mycelium should be viable. We chose spore delimitation for a first study because this process is the most regular part of the life cycle of the wild-type colony; hence description of the lesions in particular mutants, by comparison of their phenotype with that of the wild-type, would be simplified. In this paper we describe the isolation of such mutants and some information on their phenotypes and genetics. A preliminary report on this work has appeared (Hopwood, Wildermuth & Palmer, 1969).

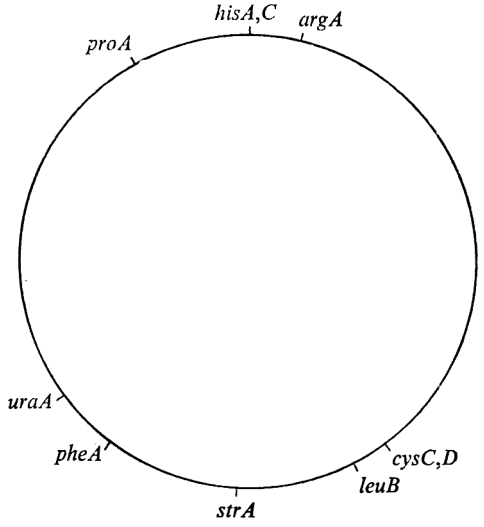


Fig. 1. Linkage map showing the locations of markers referred to in the paper.

Table 1. *Characteristics of strains*

Strain no.	Phenotype in respect of these markers									
	cysC3	*cysD18*	*leuB5*	*strA1*	*pheA1*	*uraA1*	*proA1*	*hisA1*	*hisC9*	*argA1*
A3(2)	+	+	+	S	+	+	+	+	+	+
876	−	+	+	R	−	+	−	+	−	−
1077	+	−	+	R	+	−	−	+	+	−
1107	+	−	−	R	+	+	+	−	+	+

METHODS

Organisms. The wild-type strain was *Streptomyces coelicolor* (*S. violaceoruber* sensu Kutzner & Waksman, 1959) strain A3(2) (Hopwood, 1959). The mutational and recombinational derivatives of this strain referred to in this paper are listed in Table 1. The locations of markers on the circular linkage map of the organism are indicated in Fig. 1. Markers of *S. coelicolor* have for some time been designated by three-letter symbols according to the recommendations of Demerec, Adelberg, Clark & Hartman (1966); accordingly the mutations described in this paper, leading to a *white* aerial mycelium, have been called *whi*.

Mutagenesis. Ultraviolet irradiation was carried out as described by Hopwood & Sermonti (1962). Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was performed according to the schedule of Delić, Hopwood & Friend (1970).

Media and general methods of culture and genetic analysis. These were as described

by Hopwood (1967). Stock cultures were maintained on slants of complete medium stored in the refrigerator after a week's incubation at 30°, or as lyophils prepared by the method of Hopwood & Ferguson (1969).

Light microscopy. Colonies were examined at low magnification (about ×25) with a Watson stereoscopic microscope using incident illumination from a 'Streamlite'. Impression preparations for phase-contrast microscopy were made by touching a coverslip on a colony and mounting in water.

Electron microscopy. Details of fixation, embedding, sectioning, negative staining and microscopy were those described by Wildermuth (1970).

RESULTS

Isolation of morphological mutants. The aerial phase of *Streptomyces coelicolor* wild-type colonies is weakly pigmented; young colonies appear white, but as sporulation occurs the colonies take on a pale fawn coloration. This coloration was found to be intense enough for the visual recognition of mutant colonies devoid of pigmentation, and therefore appearing white even after prolonged incubation. Some, at least, of such white colonies were expected to be morphological mutants defective in a process necessary for the production of mature spores, and this expectation was fulfilled.

In the isolation of white mutants, plates of minimal medium were sown with mutagen-treated spore suspensions of the wild-type strain, at a density yielding many hundreds of colonies per plate. After 4 days incubation at 30° the plates were scanned with the stereoscopic microscope for white colonies. These were picked off with a fine needle and purified once or twice by streaking, before being examined in the phase-contrast microscope.

Preliminary classification of mutants. The stages in the development of spores in the aerial mycelium of wild-type *Streptomyces coelicolor* A3(2) have been described by Wildermuth & Hopwood (1970). At first the aerial mycelium consisted of long aseptate hyphae, before cross-walls divided them into cells. At least the apical cells, and probably also the non-apical ones, were next subdivided by special 'sporulation septa' into spore-sized compartments, each of which was destined to give rise to a spore. When the process was complete, the mycelium fragmented into spores, typically some 1 to 1·5 μm long. The aerial mycelium occasionally fragmented into separate cells by splitting at the sites of cross-walls, even before the formation of sporulation septa. Such separated units were observed to be viable (Hopwood, 1960); this was presumably due to the cross-walls being double (Wildermuth & Hopwood, 1970), and splitting between the components of the double wall left the cell on either side with an intact end-wall. However, such fragmentation probably does not occur spontaneously, and even in the artificial conditions of impression preparations few short fragments were seen; most of the hyphae in such preparations were very long.

As explained in the Introduction, we sought mutants defective in the subdivision of the cells of the aerial mycelium into spore-sized units by means of the sporulation septa. Such mutants should have been recognizable by the fact that their aerial mycelium did not fragment at maturity into pieces (spores) some 1 to 1·5 μm long as in the wild-type, but either failed to fragment or fragmented into longer pieces. We therefore looked for strains with such phenotypes among our collection of white mutants by examining them in the phase-contrast microscope. Out of about 100 white mutants,

four showed regular fragmentation into pieces consistently longer than wild-type spores; two of these mutants, *whi-6* and *whi-13*, were chosen for further study. The majority of the others showed little spontaneous fragmentation of the aerial mycelium, and one mutant, *whi-46*, was chosen to represent this group. The remaining mutants fragmenting into units indistinguishable from wild-type spores were not studied further; some might have been expected merely to lack the pigment of the wild-type spores, if indeed their coloration was due to the presence of a pigment rather than to a structural colour, while others might turn out to have structural defects in spore maturation. As such, they could provide material for a separate study.

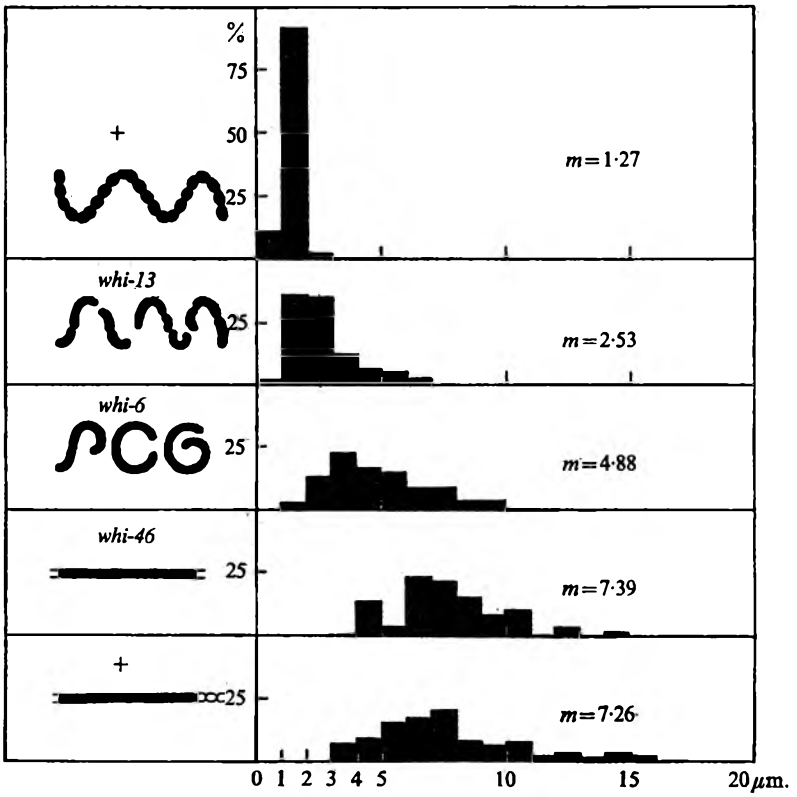


Fig. 2. Length distributions of samples of wild-type spores (+ : top), non-sporulating wild-type cells (+ : bottom), aerial mycelial fragments of mutants *whi-13* and *whi-6*, and aerial mycelial cells of mutant *whi-46*. Mean spore, fragment or cell lengths are indicated. Sample sizes were between 60 and 155. Measurements were made on electron micrographs of negatively stained impression preparations

Phenotypes of mutants defective in sporulation septation.

Phase-contrast photomicrographs of impression preparations of the three *whi* mutants and the wild-type are in Pl. 1, fig. 1 to 4. It is characteristic of *Streptomyces coelicolor*, and many other streptomycetes, that the aerial hyphae become helically coiled before spore-delimitation; vestiges of such coiling are still seen in spore chains approaching maturity (Pl. 1, fig. 1). The aerial hyphae of the three mutants also coiled. Mutants *whi-6* (Pl. 1, fig. 2) and *whi-13* (Pl. 1, fig. 3) differed from the wild-type in failing

to delimit spore-sized units; instead, the spiral hyphae fell apart into sausage-shaped pieces, those of *whi-6* being on average longer than those of *whi-13*. Mutant *whi-46* (Pl. 1, fig. 4) presented unfragmented helical hyphae at maturity.

More precise information on the lengths of the fragments, and of the cells of unfragmented hyphae, were obtained from electron micrographs of impression preparations (Wildermuth, 1970) stained negatively (Brenner & Horne, 1959). Randomly chosen fields of such preparations were photographed, and measurements were made on the prints, in which cross-walls, as well as fragment- or spore-ends were clearly visible. The lengths of curved objects were measured along the median line. For the wild-type, separate measurements were made of spores and of cells in the non-sporulating parts of sporulating hyphae. Typical specimens of wild-type spores (Pl. 2, fig. 5), *whi-6* (Pl. 2, fig. 6) and *whi-13* (Pl. 2, fig. 7) fragments and *whi-46* cells (Pl. 2, fig. 8) are shown. The results (Fig. 2) show that wild-type spores had a rather constant length, with a mean of 1·27 μm. (top panel of Fig. 2); cells not subdivided by sporulation septa had a mean length of 7·26 μm. (bottom panel of Fig. 2), suggesting that each cell was normally subdivided into some six spores. This conclusion might be invalidated if apical cells were consistently longer than sub-apical ones, a possibility that has not been excluded (Wildermuth & Hopwood, 1970). The data of Fig. 2 confirm that *whi-13* and *whi-6* fragmented into pieces of average length greater than wild-type spores, the length of *whi-13* fragments being twice that of wild-type spores, while that of *whi-6* fragments was some four times that length. The data also show that *whi-46* cells had the same length as those of the wild-type. Thus the lesion of *whi-46*, which represented a large class of mutants, could have been a simple disability in the production of sporulation septa. The lesions of *whi-6* and *whi-13*, which represented much smaller classes of mutants, may have been more complex and may have involved disturbance of the control of the spacing of sporulation septa rather than their absence; although no conclusive evidence of the production of such septa could be obtained from electron micrographs of thin sections (see below), the fact that the aerial hyphae of these mutants fragmented as readily as those of sporulating wild-type hyphae strongly suggests the operation of the fragmentation mechanism normally associated with the sporulation septa.

The fine structure of the aerial mycelium of each mutant in thin sections was next compared with that of the sporulating wild-type, which has already been described in detail (Wildermuth & Hopwood, 1970). In the wild-type, cells of the aerial hyphae bounded by double cross-walls (Pl. 3, fig. 9, *CW*) were subdivided by regularly spaced sporulation septa (*SS*) into spore-sized compartments. The developing sporulation septum had a characteristic appearance in thin sections; it was formed of two layers. the inner margin of the ingrowing annulus being clearly double (Pl. 3, fig. 10). When the annulus was complete, a double lamella therefore resulted, representing the end walls of adjacent spore compartments. At this stage each spore compartment was surrounded by a wall of constant thickness; the thickness of the normal hyphal wall (10 to 12 nm.). Subsequently, further layers of wall material were deposited, so that the wall of the mature spore was about 30 to 50 nm. thick (Pl. 3, fig. 11). Simultaneously, separation of adjacent spore compartments began marginally, by rupture of the originally continuous parent hyphal wall (and its overlying superficial fibrous sheath: Hopwood & Glauert, 1961) opposite the sporulation septa. Adjacent spores were finally attached only over a narrow central region (Pl. 3, fig. 11) before separating in

response to slight external forces. At maturity the internal details of the spore in thin sections had become ill-defined (Pl. 3, fig. 11), in comparison with those of earlier stages in sporulation and of vegetative hyphae, in which nuclear material, mesosomes and 'vacuoles' were clearly visible.

The fine structure of the aerial hyphal cells of the three mutants was not found to differ in any reproducible way from that of wild-type cells. In particular, cross-walls (*CW*) were observed in *whi-46* (Pl. 3, fig. 12), *whi-13* (Pl. 4, fig. 13) and *whi-6* (Pl. 5, fig. 17), and these closely resembled in appearance those of the wild-type (Pl. 3, fig. 9). The internal structure of the cells of the mutants was also similar to that of the wild-type.

Mutant *whi-46*, which showed no spontaneous fragmentation visible in the light microscope, revealed no evidence in thin sections of the formation of sporulation septa; it resembled the wild-type at a juvenile stage before the onset of subdivision of the aerial hyphal cells. Mutant *whi-13*, on the other hand, showed rounding-off of adjacent units at the sites of septation (Pl. 4, fig. 14), presumably leading to the fragmentation observed in this mutant. Whether such septation was initiated by the formation of typical sporulation septa is not clear; stages in the formation of sporulation septa were rarely seen even in the wild-type (Wildermuth & Hopwood, 1970), and it was difficult to obtain unequivocal evidence for their presence in the mutant although the almost complete septum in Pl. 4, fig. 15 may represent a sporulation septum. Most of the fragments produced by this mutant were surrounded by unthickened walls, but a small proportion had thickened walls (Pl. 4, fig. 16) and therefore resembled spores.

Thin sections provided little or no information on the process of fragmentation in *whi-6*; as for *whi-13*, a failure to observe stages in the formation of sporulation septa in sections could not be taken as evidence for their absence. Occasionally, structures that could be interpreted as abortive septa were observed as ingrowths of wall material on one side only of a hypha. Such structures were more easily recognized in negatively stained preparations, as finger-like ingrowths from the wall, usually associated with mesosomes (Pl. 5, fig. 19), but they were also seen in sections.

Genetics of the sporulation–septum mutants

Each of the three mutants was crossed with one or more genetically marked strains of wild-type morphology, and recombinants were selected to contain one marker from each parent (Hopwood, 1967). Samples of these recombinants were then classified in respect of the non-selected markers. For the *whi* versus *whi*$^+$ phenotype, classification was done on the basis of colony colour, and the phenotype of a proportion of the segregants was confirmed by phase-contrast microscopy.

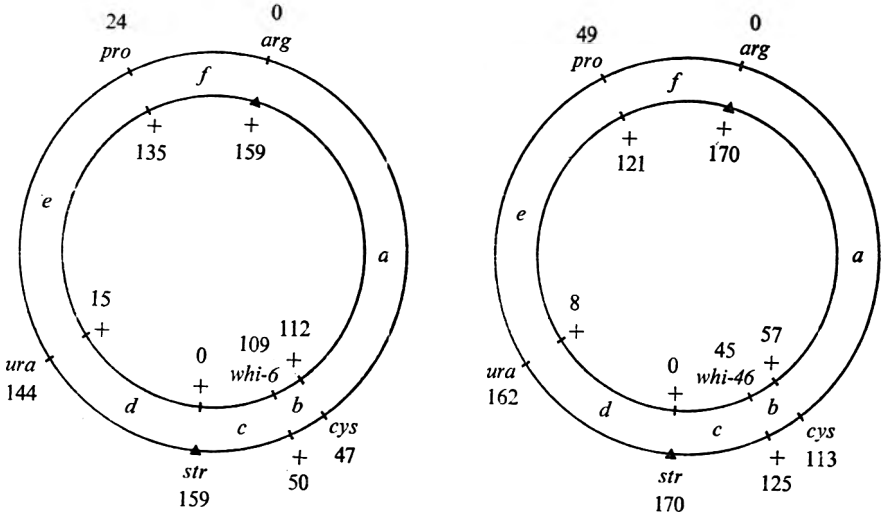
Each cross gave rise to a clear segregation of mutant versus wild-type morphology, providing presumptive evidence for a single gene mutation affecting morphology. This conclusion was strengthened by the finding of an approximate map-location for each mutation by analysis of the segregation of *whi* in relation to that of other non-selected markers.

Mutants *whi-6* and *whi-46*. Preliminary crosses of these mutants were made with strain 1077, selecting *argA1*$^+$ and *strA1*. The allele ratio at the *whi* locus in each cross (see Table 2) indicated its location either between *cysD* and *strA* or between *uraA* and *proA*. A choice between these two locations was easily made on the basis of the crossovers needed to explain the observed recombinant classes. Assuming a location

in the interval *cysD–strA* (indicated in the diagrams of Table 2), only five and three recombinants, respectively, in the two crosses required more than the minimum of two crossovers, whereas a position in interval *uraA–proA* would have necessitated 18 and 19 multiple crossovers.

Table 2. *Preliminary location of whi-6 and whi-46*

Each *whi* mutant (inner circle) was crossed with strain 1077 (outer circle) and *argA1*$^+$ and *strA1* (indicated by triangles) were selected. Numbers in the diagrams are allele frequencies.



Genotype*	Crossovers in intervals	Frequency in cross involving *whi-6*	Frequency in cross involving *whi-46*
cysD	a, d	2	3
cys D uraA	a, e	32	75
cysD uraA proA	a, f	9	32
—	b, d	1	3
uraA	b, e	5	8
uraA proA	b, f	0	4
whi	c, d	10	2
whi uraA	c, e	83	29
whi uraA proA	c, f	12	11
whi cysD uraA	a, b, c; e	2	1
whi cysD uraA proA	a, b, c; f	1	2
cysD proA	a; d, e, f	1	0
whi proA	c; d, e, f	1	0
	Totals	159	170

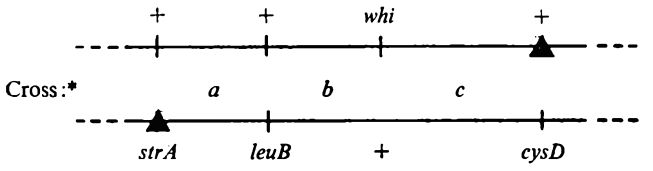
* Wild-type alleles omitted.

The interval *cysD–strA* contains several known loci (Hopwood, 1967). Two series of crosses were analysed in which *whi-6* or *whi-46* was crossed with strains bearing the markers *cysD* (or *cysC*, which is closely linked to it) and *strA* and each bearing a different marker in the interval between them. Recombinants were selected to contain *cysC*$^+$ (or *cysD*$^+$) and *strA*; thus recombination in the interval *cysC(cysD)–strA* was obligate. The segregation of *whi* in relation to the other non-selected marker (*leuB5*,

thiA1, *mthB2*, *hisD3* or *uvsB6*) indicated a location for both *whi-6* and *whi-46* anti-clockwise of each of the markers. Data for the crosses involving *leuB5*, the most anti-clockwise of the markers, are summarized in Table 3. Thus these two *whi* mutations were consigned to the comparatively short interval of the map between *cysC*(*cysD*) and *leuB*.

Table 3. *Location of whi-6 and whi-46 between cysD and leuB in crosses with strain 1107*

Segregation of *hisA1* is ignored.



Genotype	Crossovers in intervals	Number in cross involving *whi-6*	Number in cross involving *whi-46*
strA + *whi* +	a	175	134
strA leuB whi +	b	13	20
strA leuB + +	c	11	38
strA + + +	a, b, c,	1	6
	Totals	200	198

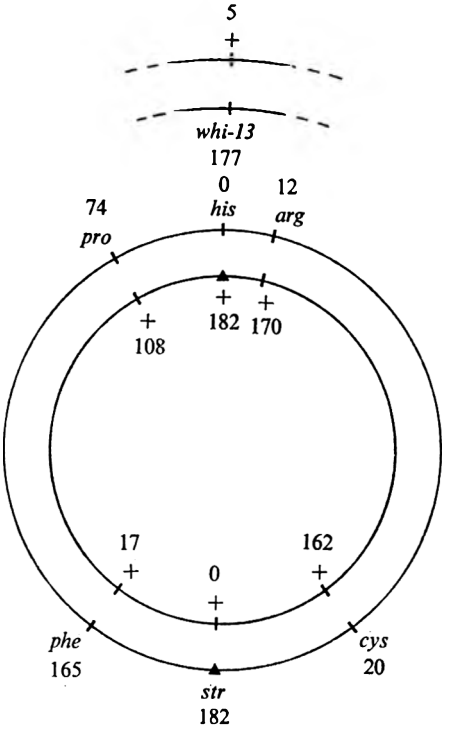
* Triangles indicate selected alleles.

Mutant *whi-13*. The results of a cross between mutant *whi-13* and strain 876 are tabulated and analysed in Table 4. The allele ratio at the *whi-13* locus indicated a position close to *hisC*. At first sight it should have been possible to choose a location for *whi-13* clockwise or anti-clockwise of *hisC* on the basis of the segregation of *whi-13* in relation to that of *argA* and *proA*; a clockwise position would have been indicated by the finding that all, or nearly all, *whi-13*$^+$ recombinants carried *arg*, while an anti-clockwise position would have followed from their carrying *pro*. In fact, three out of five carried *pro* but none carried *arg*, tending to favour the anti-clockwise position. However, further considerations show that this conclusion is not justified. The cross involves two fertility types (A. Vivian & D. A. Hopwood, unpublished results); the *whi-13* strain, being a mutant derivative of the wild-type strain A 3(2), is believed to be of the IF type, whereas 876 is of the NF type. In IF × NF crosses the IF strain contributes the complete chromosome to the majority, at least, of the zygotes, and the NF strain contributes a chromosome fragment; this fragment appears normally to include the 9 o'clock region of the genome (A. Vivian and D. A. Hopwood, unpublished results). Thus the majority, at least, of the zygotes able to give rise to selected progeny in the cross under consideration would have had the constitution shown in Fig. 3. Most of the segregants carrying the *arg* marker would therefore have required multiple cross-overs in the right-hand half of the map: in intervals f and g together with either a of b. Nine of the 12 *arg* segregants were manifestly members of multiple crossover classes since they did not carry *cys* and therefore arose by crossing over in f, g and b. It is therefore very likely that, if *whi-13* were located between *his* and *arg*, most of all or the segregants carrying *whi-13*$^+$ would also have arisen by multiple crossover patterns; therefore the finding that all five such segregants lacked *arg* by no means excludes such a location.

We thus conclude from these data only that *whi-13* is close to *hisC*. As such it clearly defined a different locus from *whi-6* and *whi-46* and the analysis was taken no further at this stage.

Table 4. *Preliminary location of whi-13*

Frequencies of different genotypes* of selected recombinants from a cross of mutant *whi13* (inner circle) and strain 876 (outer circle). *hisC9*$^+$ and *strA1*, indicated by triangles, were selected.



(i) *whi* classes			
pheA whi-13	80	*argA pheA proA whi-13*	3
pheA proA whi-13	57	*argA cysC pheA proA whi-13*	2
whi-13	11	*argA proA whi-13*	2
cysC pheA whi-13	10	*argA cysC pheA whi-13*	1
cysC pheA proA whi-13	5	*proA whi-13*	1
argA pheA whi-13	4	*cysC proA whi-13*	1
(ii) *whi*$^+$ classes			
pheA proA	1	*pheA*	1
cysC pheA proA	1	—	1
proA	1		

* Wild-type alleles omitted.

DISCUSSION

This study has shown that morphological mutants of *Streptomyces coelicolor* with defects in a particular stage of colonial development can be isolated by a comparatively efficient visual selection procedure.

In all probability the group of mutants discussed in this paper serves to identify

genes concerned with the synthesis or spacing of the sporulation septa that subdivide the aerial hyphae during sporulation. The three mutants examined here have revealed two such genes; *whi-13* is certainly genetically distinct from the other two mutants, whereas *whi-6* and *whi-46*, falling as they do in the same segment of the linkage map, could be allelic, in spite of their different phenotypes.

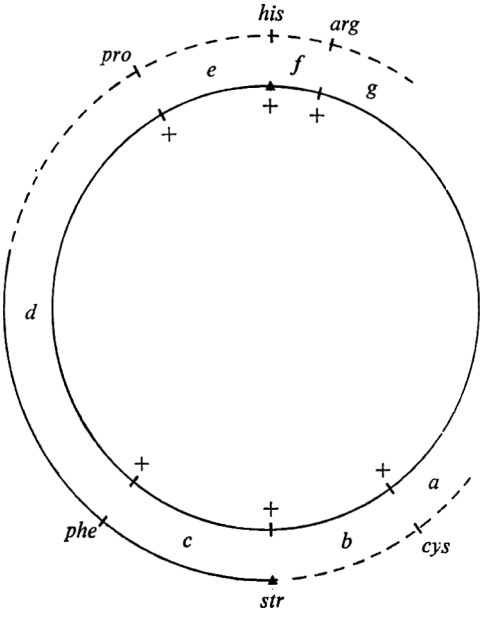


Fig. 3. Constitution of the majority of the zygotes in a cross of a prototrophic IF strain (inner circle) and marked strain 876 NF (outer arc). Continuous lines indicate sections of genome obligately included in all zygotes capable of yielding selected progeny, while dashed regions are variably included.

Although the three mutants discussed are the only ones to have been studied morphologically in any detail, many of the remaining *whi* mutants have been mapped approximately; a few of the mutants map in the region of *whi-13*, and the remainder in the region between *strA* and *cysD*. Thus, unless there is extensive clustering of genes, it would appear that mutation to the *whi* phenotype involves few genes. If so, the situation resembles that in the eukaryote fungus *Aspergillus nidulans*, where Clutterbuck (1969) found mutation in only two genes led to interruption in conidiophore development, a morphogenetic sequence roughly analogous with sporulation in *Streptomyces coelicolor*.

A contrasting result has emerged from studies in the other protokaryotic system, the endospore of *Bacillus subtilis*, where mutation in many genes leads to reduced or no spore production. Mapping studies have identified a minimum of 15 genes (Schaeffer, 1969), and an indirect argument leads to an estimate of hundreds of genes being involved (Balassa, 1969). At first sight this conclusion appears to conflict with the findings in *Streptomyces coelicolor*. However, the *B. subtilis* system is probably very much more complex than that in *S. coelicolor*. Very many attributes of the endospore, its metabolism as well as its morphological components, are probably controlled by sporulation-specific genes which are not involved in the life of the vegetative cell;

the spore is in many respects a separate 'organism'. In contrast, sporulation in *S. coelicolor* consists merely of segmenting a pre-existing hypha into separate units; mutants of the type described in this paper should contribute to an understanding of this simple morphogenetic sequence.

The finding that septation of the substrate and aerial mycelium by cross-walls appears to be normal, even in a mutant (*whi-46*) that totally lacks sporulation septa, tends to confirm the conclusion from morphological studies of the wild-type that the two kinds of septum are distinct (Wildermuth & Hopwood, 1970).

This work was begun in the Institute of Genetics, University of Glasgow, and we gratefully acknowledge the advice of Dr P. T. P. Oliver of that Institute during electron microscope studies of the wild-type and *whi-6*. We should like to thank Helen M. Ferguson for the isolation of some of the mutants and for skilled assistance with some of the genetic studies.

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EXPLANATION OF PLATES

PLATE 1

Phase-contrast photomicrographs of impression preparations. Magnification approx. × 1400.

Fig. 1. Wild-type. The preparation consists largely of isolated spores and chains of spores of varying maturity.

Fig. 2. Mutant *whi-6*. The preparation consists largely of separated fragments and short chains of fragments.

Fig. 3. Mutant *whi-13*. Separated fragments.

Fig. 4. Mutant *whi-46*. Helically coiled aerial hyphae.

PLATE 2

Electron micrographs of impression preparations negatively stained with potassium phosphotungstate. Magnification approx. × 16,500.

Fig. 5. Wild-type. Two isolated spores and a chain of younger spores.

Fig. 6. Mutant *whi-6*. Two fragments and part of a third. Note mesosomes.

Fig. 7. Mutant *whi-13*. Separated fragments and short chains of fragments.

Fig. 8. Mutant *whi-46*. Note two cross-walls delimiting one cell and parts of two adjacent cells.

PLATES 3, 4 AND 5

Electron micrographs of thin sections (Figs. 9 to 18) or of negatively stained preparations (Fig. 19).

Fig. 9. Wild-type. Non-sporulating hypha. Note cross-wall (*CW*), mesosomes, nuclear material. × 50,000.

Fig. 10. Wild-type. Sporulating aerial hypha at Stage 2 (Wildermuth & Hopwood, 1970). Note sporulation septa (*SS*). × 65,000.

Fig. 11. Wild-type. Part of a chain of mature spores. Note thickened spore walls surrounded by vestiges of fibrous sheath, and poorly defined spore contents. × 50,000.

Fig. 12. Mutant *whi-46*. Aerial hyphal cell with cross-wall (*CW*). × 50,000.

Fig. 13. Mutant *whi-13*. Aerial hyphal cell with cross-wall (*CW*). × 50,000.

Fig. 14. Mutant *whi-13*. Two adjacent aerial mycelial fragments separating. × 65,000.

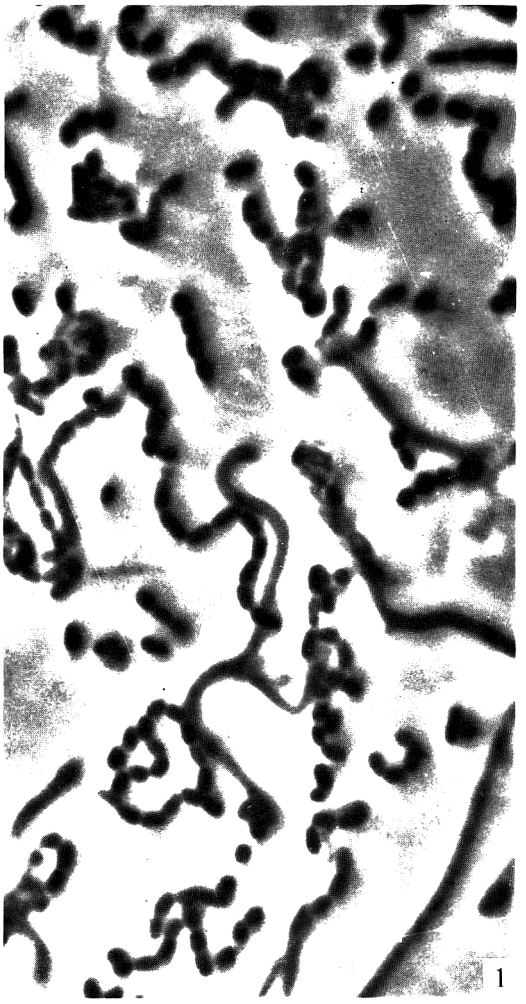
Fig. 15. Mutant *whi-13*. Possible sporulation septum dividing aerial mycelial cell. × 50,000.

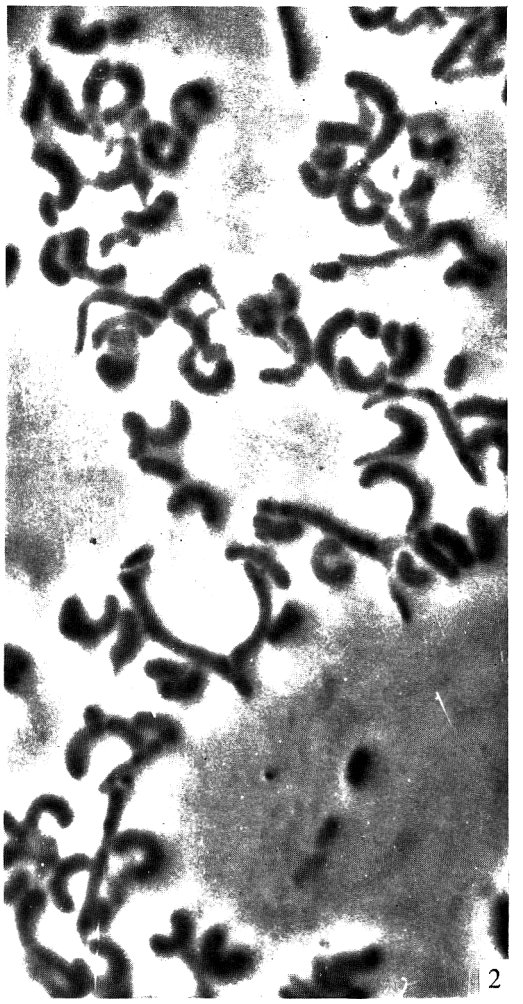
Fig. 16. Mutant *whi-13*. A fragment with thickened wall. × 50,000.

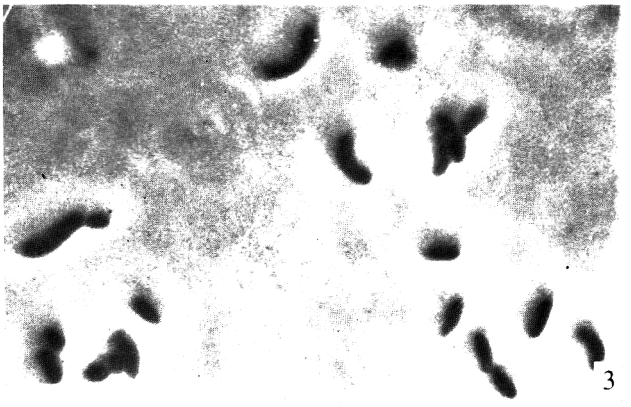
Fig. 17. Mutant *whi-6*. Aerial hyphal cell in a cross section of a helically coiled hypha (compare longitudinal section in Fig. 18) with cross-wall (*CW*). × 50,000.

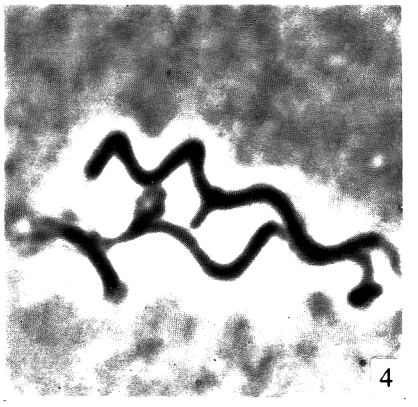
Fig. 18. Mutant *whi-6*. Longitudinal section of a helically coiled aerial hypha (compare cross section in Fig. 17). × 50,000.

Fig. 19. Mutant *whi-6*. Negatively stained aerial hypha with possible abortive sporulation septum as a finger-like ingrowth of the wall associated with a mesosome. × 65,000.









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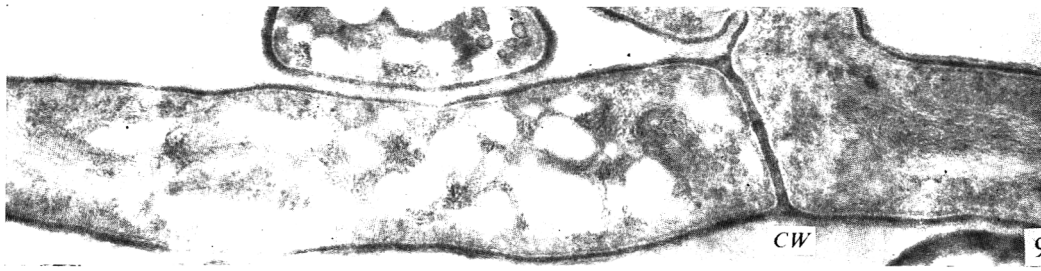
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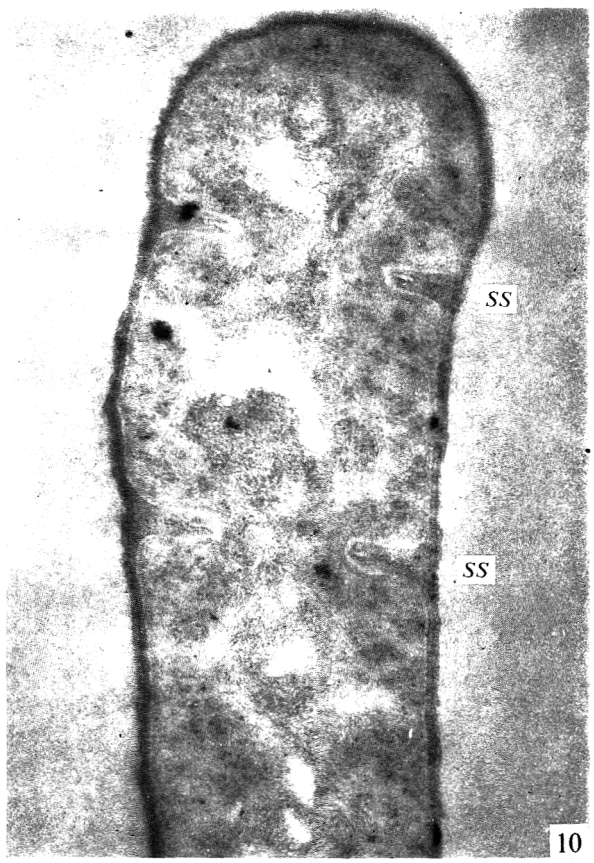
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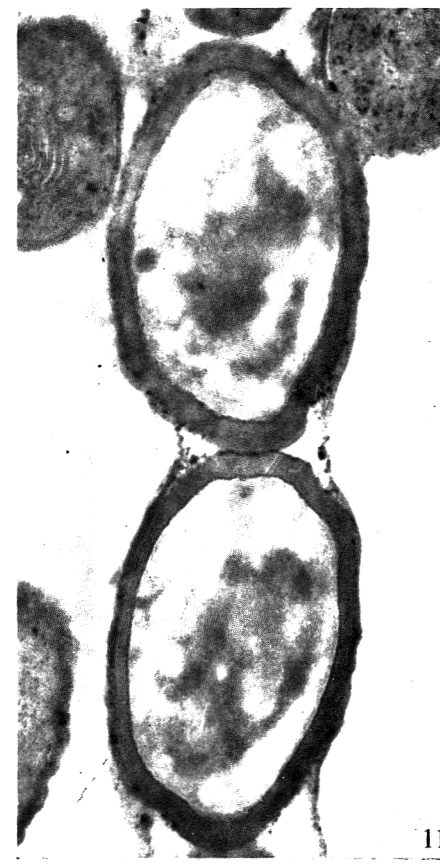
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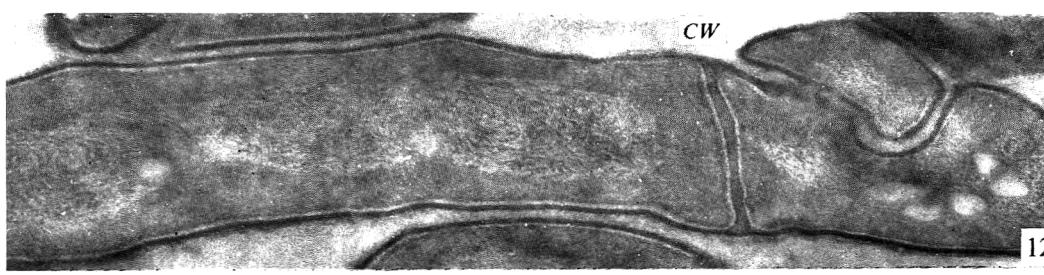
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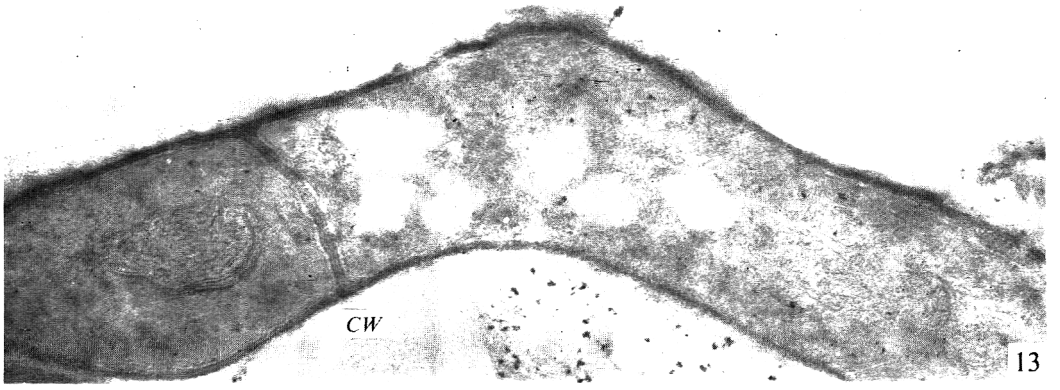

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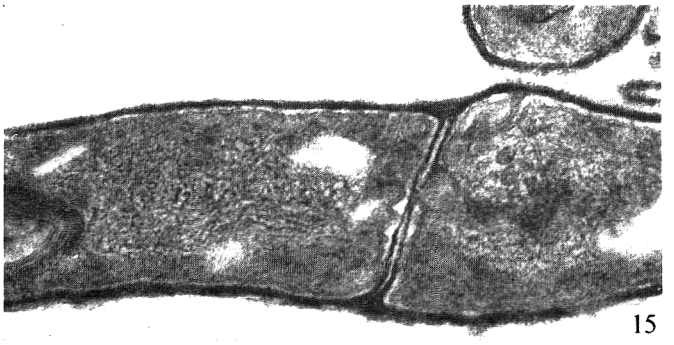

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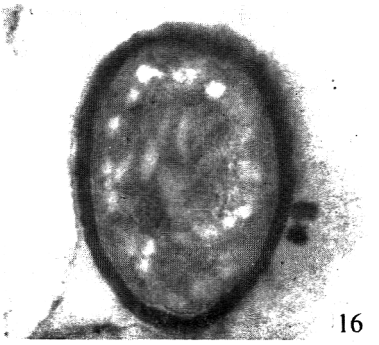

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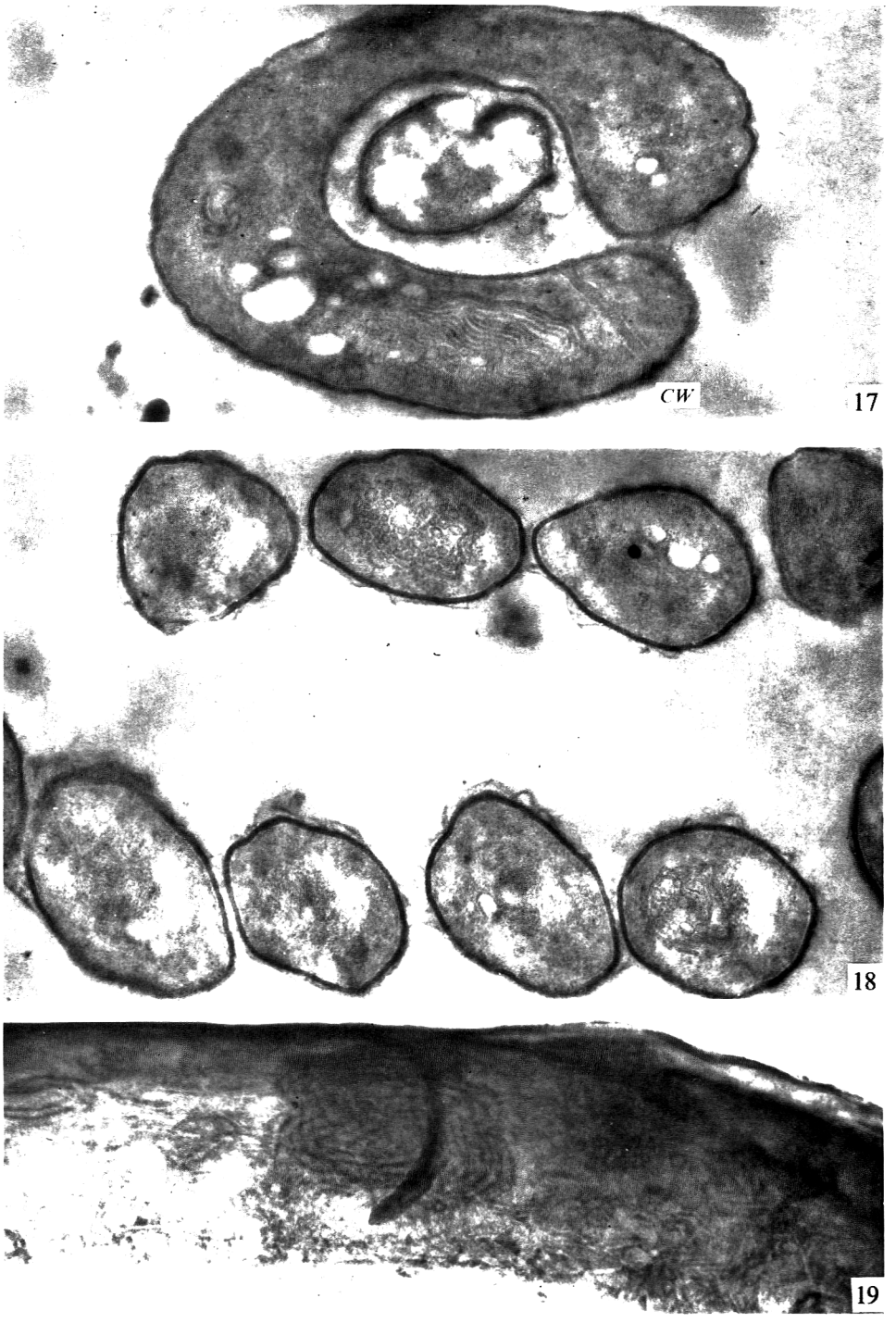

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Pisatin Production by Tissue Cultures of *Pisum sativum* L.

By J. A. BAILEY*
Department of Botany, University of Glasgow

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SUMMARY

Callus tissues of *Pisum sativum* produced the phytoalexin pisatin when grown in axenic culture. Coconut milk, which was in the culture media, induced pisatin formation in pea leaf discs. Cultures which had been established for over 18 months showed a marked reduction in the ability to produce pisatin. Pisatin inhibited the growth of pea callus at low concentrations (5 to 10 μg./ml.), and may control callus tissue initiation and cellular necrosis during host-fungus interactions.

INTRODUCTION

The resistance of plant cells to fungal invasion appears to be explained, at least in part, by production of fungitoxic compounds following infection. Two major problems in investigating this possibility are: (i) that mechanical barriers must often be overcome by artificial means, as when storage fruits are inoculated with spore suspensions, thereby altering the metabolism of the tissue; (ii) that the amount of infected tissue differs in resistant and susceptible reactions. Measurements obtained from extracted plant material may indicate that the concentration of fungitoxic compounds is greater in rotted tissue, where large amounts of tissue are affected, than in resistant tissue, where only a few cells are affected. Precise conclusions can be made only when the amounts of tissue affected by the invading fungus can be determined.

Tissue cultures provide large volumes of actively dividing cells growing in a controlled chemical and physiological environment, and can be exposed to fungi without prior treatment to remove mechanical barriers. Large numbers of cells can be brought into contact with the fungal inoculum, thus enabling the number of cells affected by a nonpathogen to be similar to the number affected by a pathogen.

It is not yet known whether it is valid to compare the reaction of intact plant cells with that of similar cells from tissue culture. Ingram & Robertson (1965) and Ingram (1967) showed that callus cultures from potato tubers exhibited the same R-gene resistance to *Phytophthora infestans* as the intact tubers from which they were derived. Robertson *et al.* (1968) demonstrated that this inhibition was due to phenolic acids produced after infection. However, Tomiyama *et al.* (1968) implicated the phytoalexin rishitin in R-gene resistance of potato tubers. Rishitin was not detected in tissue culture supernatants (Robertson *et al.* 1968). Saad & Boone (1966) reported that tissue cultures from the leaf and the petiole of apple shoots of cultivars either resistant or susceptible to *Venturia inaequalis* did not support the growth of this fungus.

Pisatin, a phytoalexin produced by various tissues of *Pisum sativum*, is believed to be

* Present address: Department of Botany, Imperial College of Science and Technology, London, S.W.7.

responsible for the resistance of *P. sativum* to nonpathogenic fungi (Cruickshank, 1963). In the present work, callus cultures were derived from the stem and root of *P. sativum*, with a view to investigating the production of pisatin by these tissues after inoculation with various fungi and the relationship of this to the subsequent disease production. However, it became clear from early experiments that pisatin was produced by these cultures without infection by fungi. Because an essential feature of the original phytoalexin concept of disease resistance is that the phytoalexin is not a normal metabolic product of plant tissues, this phenomenon was investigated more closely.

METHODS

Media. Cylindrocarpon heteronema was grown on Czapek Dox agar (Thom & Raper, 1945). Two media were used for tissue cultures. Medium I: mineral salts and micronutrients: $Ca(NO_3)_2.4H_2O$, 288 mg.; KNO_3, 80 mg.; KCl, 65 mg.; $NaH_2PO_4.2H_2O$, 21·5 mg.; $MnCl_2.4H_2O$, 6 mg.; $ZnSO_4.7H_2O$, 740 mg.; H_3BO_4, 1·5 mg.; H_2MoO_4, 0·0017 mg.; $CuSO_4.5H_2O$, 0·02 mg.; $FeCl_3$, 3·1 mg.; Na_2EDTA, 0·8 mg.; vitamins and amino acids: aneurin hydrochloride, 0·1 mg.; pyridoxine hydrochloride, 0·1 mg.; nicotinic acid, 0·5 mg.; glycine, 3·0 mg.; other additions: 2,4-dichlorophenoxyacetic acid, 6 mg.; coconut milk, 150 ml.; sucrose, 20 g.; agar, when required, 6 g.; glass distilled water to 1 litre. Medium II: as for medium I, plus yeast extract, 1 g. The stock cultures were maintained on 30 ml. of medium solidified with agar in 100 ml. Pyrex conical flasks at 26° in a growth room illuminated with a single 40 W tungsten lamp. Experiments using liquid media were carried out in conical flasks incubated at 26° in the dark in a shaking incubator rotating at 150 rev./min.

Establishment and maintenance of tissue cultures. Pea seeds were washed in 70 % (v/v) ethanol for 5 min. and then treated with 0·1 % (w/v) mercuric chloride for 10 min. They were rinsed in ten changes of sterile water and placed on moist cotton wool in 100 ml. conical flasks which had previously been sterilized by autoclaving at 121° for 20 min. The flasks were incubated at 15° in the dark. After 4 days the seedlings were dissected. The final $\frac{1}{4}$ in. of the root was discarded and the next $\frac{1}{4}$ in. was placed on medium II in order to initiate callus growth. Similar seedlings were grown for a further nine days. Segments of stem $\frac{1}{4}$ in. long were obtained and placed on medium I in order to initiate callus growth. The callus tissues which were produced after 4 weeks were transferred to fresh media. The root callus tissues were very variable and three visually distinct clones were distinguished and established. These isolates were maintained on medium II. The calluses produced from pea stem were more uniform, and the clone established was maintained on medium I.

The ability of several constituents of the tissue culture media to induce the formation of pisatin in intact plants was tested by floating 8 mm. leaf discs on drops of test solution maintained in plastic Petri dishes with lids lined with moist filter paper. The concentration of pisatin in the diffusates was measured after 48 hr.

Pisatin concentration was measured spectrophotometrically. The callus culture filtrates and leaf diffusates were extracted with redistilled light petroleum (B.P. 40° to 60°). The light petroleum fractions were evaporated to dryness at 40° under vacuum, the residue redissolved in absolute ethanol (5 ml.) and the extinction of the resulting solution measured between 200 and 380 nm. The concentration of pisatin was calculated from the extinction at 309 nm. (Cruickshank & Perrin, 1961).

Preparation and purification of pisatin. Drops of a culture filtrate of *Penicillium expansum* were placed on pea pod endocarps. The diffusates were collected after 72 hr and pisatin was obtained by extracting with light petroleum. The pod tissue was macerated in 90 % (v/v) ethanol, and after filtration the ethanol was removed under vacuum at 40° and the aqueous fraction extracted with light petroleum. The pisatin-containing fractions were bulked and the ether soluble material was chromatographed on 1 mm. silica gel plates developed in chloroform. A distinct band (R_F = 0·25) was detected with ceric sulphate reagent (ceric ammonium nitrate, 1 % (w/v) in 4-N-H_2SO_4) and corresponded to pisatin. This band was eluted with ether and the high purity of the pisatin obtained was confirmed by infrared and ultraviolet spectrophotometry (Bailey, 1968).

Table 1. *Pisatin concentration in the supernatant of stem callus growing in medium I*

Age of culture (days)	Vol. of supernatant (ml.)	Pisatin concentration* (μg./ml.)
	Pisatin concentration assayed in 10 ml. of supernatant	
0	150	0·00
2	150	0·40
4	150	0·40
6	140	0·90
8	140	0·80
10	140	1·70
12	130	3·00
14	130	6·60
16	130	7·50
18	120	6·80
20	120	7·40
22	120	6·50
24	110	6·80
26	110	8·30
28	110	6·10
38	100	5·50

* Concentration in supernatant at the time of assaying.

RESULTS

Production of pisatin by young stem callus

Callus was grown for 29 days on solid medium I. A complete callus from each flask was used as inoculum into 250 ml. Pyrex conical flasks containing 150 ml. of liquid medium I. Twelve flasks were divided into three samples each containing four replicates. The pisatin concentration in 10 ml. of supernatant removed from each replicate was measured alternatively every 6 days, i.e. one sample was assayed on days 0, 6, 12, 18, etc. The results are shown in Table 1. They demonstrate that sterile callus produced pisatin when grown in liquid medium. During the initial 8 days after the callus was transferred into liquid medium, only small traces of pisatin were detected in the supernatant. During the following 12 days pisatin was produced rapidly, and thereafter the pisatin detectable in the supernatant declined slowly.

To determine whether this production of pisatin was due to the presence of chemicals

in the nutrient medium which themselves could induce pisatin formation, various constituents of the medium were tested for ability to induce pisatin synthesis in pea leaf discs. The results are shown in Table 2. Coconut milk induced the formation of the greatest amounts of pisatin; the remainder did not induce pisatin levels appreciably above those produced in distilled water.

Table 2. *Pisatin concentration in diffusates obtained from pea leaf discs after 48 hr*

Pisatin was assayed in 5 ml. of diffusate.

Treatment	Pisatin concentration (μg./ml.)
Medium II	28·8
Coconut milk (15%, v/v)	24·5
2,4-D (6 mg./l.)	9·2
Vitamin solution*	9·2
Sucrose (2%, w/v)	9·2
Inorganic stock solution†	7·8
Distilled water	7·8

* The vitamin solution consisted of aneurin hydrochloride, 0·1 mg.; pyridoxine hydrochloride, 0·1 mg.; nicotinic acid, 0·5 mg.; and glycine, 3·0 mg. in 1 litre distilled water.

† The inorganic stock solution consisted of the 'mineral salts and micronutrients' listed under 'media' in 1 litre distilled water.

Table 3. *Pisatin production by four culture lines of pea tissue culture*

Results are the average of four flasks.

Culture line	Medium	Dry wt (g.)	Pisatin concentration of supernatant (μg./ml.)
Pea root A	II	0·0782 ± 0·0039	3·0
Pea root B	II	0·1113 ± 0·0035	8·3
Pea root C	II	0·0946 ± 0·0042	24·0
Pea stem	II	0·1313 ± 0·0049	4·1
Pea stem	I	0·0976 ± 0·0074	3·4

Pisatin production by various culture lines of Pisum sativum

The four culture lines of pea callus which had been established for 18 months were tested for ability to produce pisatin in liquid media. The root cultures were grown as callus on medium II for 28 days; the stem culture on medium I for 28 days. The entire calluses were transferred to 100 ml. Pyrex flasks containing 40 ml. of either medium I or medium II and incubated for 38 days. Pisatin was produced by all the callus cultures (Table 3); the amounts produced by these visually distinct culture lines differed markedly. Although yeast extract stimulated tissue growth, it did not affect the yield of pisatin.

The amount of pisatin produced by a given culture tended to decrease the longer the culture had been established: the pea stem culture shown in Table 3 had previously produced over 9 μg. pisatin/ml. Fresh isolates were made from sections of young pea stem placed on solid medium, when callus cells formed on their surfaces. When sufficient callus tissue had been produced, the sections were divided into two equal parts; one part was transferred to fresh solid medium, the other was used to determine capacity to produce pisatin. The callus which was grown on the solid medium was

divided after 35 days, and the process repeated. The decrease in pisatin production was confirmed. An average measurement of 16 flasks showed that callus which had undergone only one passage produced over 14 μg. pisatin/ml. after 35 days, but after eight passages the amount produced had fallen to only 6·5 μg./ml. This decrease in pisatin production was associated with a visual change in callus type. The callus cells produced initially were slow growing and darkly pigmented; subculturing selected a faster growing lightly pigmented strain. Pea root culture C (Table 3), which was darkly pigmented, also produced large amounts of pisatin.

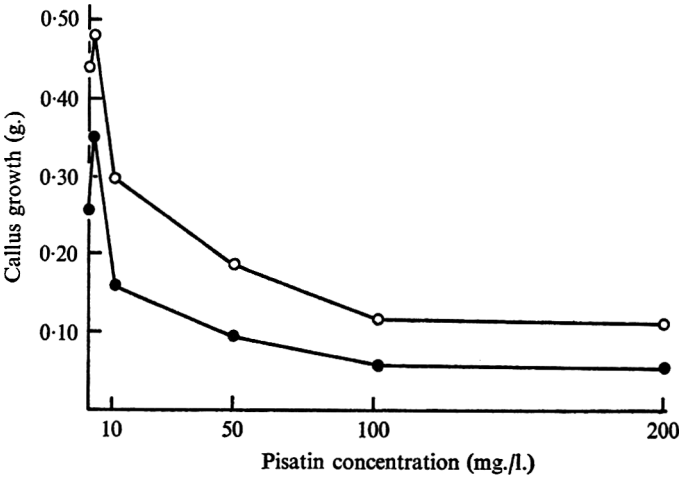

Fig. 1. The effect of pisatin on the growth of pea stem callus. O—O, Dry weight; ●—●, 10^{-1} × fresh weight. Callus was incubated for 20 days.

The effect of pisatin on the growth of stem callus in liquid medium

Pisatin was dissolved in ether, dispensed into flasks and the ether allowed to evaporate. Thirty ml. of liquid medium I was added to each flask. An additional flask containing neither pisatin nor ether was also set up. Stem callus, which had been established for 2 years and was producing less than 2·0 μg. pisatin/ml. after 35 days was transferred to each flask, and measurements of growth were made after 20 days. The results (Fig. 1) showed that pisatin caused a marked inhibition of callus growth at concentrations below 10·0 μg./ml. The calluses failed to fragment and were darkly pigmented, resembling newly established callus, whereas the unhibited calluses were finely divided and light brown in colour.

DISCUSSION

Pea callus cultures resembled pea leaf tissues. Callus cultures produced pisatin in axenic culture as a result of the presence of coconut milk, which, unlike most chemical agents known to induce pisatin synthesis, is non-toxic, and in fact was essential for growth of tissue cultures. After continued growth and the establishment of fast-growing callus, the capacity of pea callus to synthesize pisatin decreased. A similar phenomenon was reported by Robertson *et al.* (1968). Ingram (1967) showed that, when a suspension of potato tuber callus was infected with *Phytophthora infestans*

zoospores, the supernatant became increasingly toxic when tested against zoospore of *P. infestans*. In the initial work this toxicity was complete within 6 days. However, after continued growth of the callus for 3 years, toxicity was achieved only after 10 days (Robertson *et al.* 1968). By careful selection one culture (pea root C) was obtained which continued to produce pisatin at high concentrations. In other work designed to compare the reaction of callus tissue with similar tissue from which it was derived, both apple fruit tissue and callus derived from the mesocarp of mature fruits failed to support the growth of microconidia of *Cylindrocarpon heteronema*. However, attempts to show the presence of a fungitoxic effect were only partially successful. A complete inhibition of spore germination on apple callus and in juice expressed from this callus was demonstrated. Similar effects could not be found using apple mesocarp tissue (Bailey, 1968). The use of apple callus may facilitate the identification of inhibitors which, perhaps due to their instability and/or localization, have not yet been detected in apple fruits.

Pisatin inhibited the growth of pea callus. Steward, Caplin & Millar (1952) showed that explants of carrot grew more rapidly in liquid than on solid media. In the present work, frequent subculturing of the faster growing sectors resulted in the establishment of clones of faster growing cells. Newly established calluses appeared to grow more rapidly when transferred from solid to liquid media. Pisatin production by excised pea tissues probably restricts cellular growth so that, by repeatedly transferring the callus produced or by growing it in liquid media, callus tissue is selected which produces less pisatin; the concentration of pisatin in contact with the cells is thus reduced and rapid growth becomes possible.

During host-fungus interactions phytoalexin formation is associated with cellular necrosis (Cruickshank, 1963). Phaseollin production by *Phaseolus vulgaris* following inoculation with *Colletotrichum lindemuthianum* coincided with the occurrence of necrosis in both the hypersensitive reaction and in lesion formation (Rahe, Kuć, Chuang & Williams, 1969; Deverall & Bailey, 1969). Phytoalexin formation can occur without this associated necrosis, e.g. in the presence of coconut milk. However, in view of the phytotoxicity of pisatin shown in this work and by Cruickshank & Perrin (1961), it would appear that the possible regulatory role of phytoalexins in infected plants tissues should be further investigated.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-eighth General Meeting at the Imperial College of Science and Technology, London, S.W. 7, on Monday, Tuesday and Wednesday, 6, 7 and 8 April, 1970. The following communications were made.

ORIGINAL PAPERS

SYMPOSIUM ON ORGANIZATION AND CONTROL IN PROKARYOTIC AND EUKARYOTIC CELLS

Batch Culture and Computer Simulation of Growth of Bacterium NCIB 8250 Using Inhibitory Substrates. By C. A. FEWSON, A. BARCLAY and R. EASON (*Department of Biochemistry, University of Glasgow, Glasgow W. 2*)

Many of the aromatic compounds that serve as carbon+energy sources for bacterium NCIB 8250 are quite toxic and show a depression of growth rate at relatively low concentration (Kennedy, S. I. T. & Fewson, C. A. (1968), *Journal of General Microbiology* **53**, 259). Our studies on enzyme induction in the presence of these substances have therefore necessitated analyses of situations where growth is partially inhibited and frequently not exponential. One method adopted has been to simulate growth with the KDF-9 computer using the approach suggested by Andrews, J. F. ((1968), *Biotechnology and Bioengineering* **10**, 707). We have expanded his equations to include a molar maintenance coefficient since maintenance energy, or its algebraic equivalent, may be quite significant at low growth rates (cf. Pirt, S. J. (1965), *Proceedings of the Royal Society* **136 B**, 224). These expanded equations are only moderately successful in accounting for the kinetics of batch culture of bacterium NCIB 8250 on salicylate. This is partly because growth rate corresponds to the Haldane inhibition function only at low substrate concentrations. At salicylate concentrations less than 2·5-mM there is at least superficial agreement between the experimental and simulated growth curves. At higher substrate concentrations the model is inadequate since growth not only starts at a lower rate but also accelerates at lower cell yields than simulation studies predict. Andrews concluded that one of the main effects of substrate inhibition in batch culture is to produce a lag period. It is difficult to extend conventional definitions of lag to this type of non-exponential growth, and we have found that in both simulated and experimental cultures growth starts immediately after inoculation.

We conclude that this type of approach is potentially valuable in analysing complex growth curves and in indicating factors that affect growth.

A New Thermophilic Thiobacillus. By R. A. D. WILLIAMS and D. S. HOARE (*Department of Microbiology, University of Texas, Austin, Texas 78712, U.S.A.*)

A new type of thiobacillus was isolated from the hot, acid water of a spring in Yellowstone National Park. The short non-motile, Gram-positive rods had an optimum temperature of 50° and optimum pH of 5·6 in thiosulphate-mineral medium. No growth occurred at 60°, and no spores were observed. Threonine deaminase and rhodanese showed maximal activity at 45° in cell-free extracts. An AMP-independant sulphite oxidase was present.

The strain was facultatively autotrophic. The mean base composition of DNA was 66·5 % G+C (buoyant density analysis, M. Mandel) in cells grown autotrophically, mixotrophically or heterotrophically. No satellite bands of DNA were detected. Washed cell

suspensions oxidized thiosulphate and tetrathionate rapidly to sulphate. Sulphur and sulphite were oxidized slowly. The fixation of $^{14}CO_2$ was supported by thiosulphate, tetrathionate and sulphur, but not by acetate. Carboxydismutase was not repressed by acetate in the growth medium. Radioactive acetate was both incorporated and oxidized by cell suspensions. In the absence of thiosulphate approximately 20 % of utilized acetate was oxidized to carbon dioxide. In the presence thiosulphate acetate utilization was doubled, but less than 0·5 % was oxidized. Radioactive acetate was incorporated into cell components without restriction by growing mixotrophic cultures.

No α-ketoglutarate dehydrogenase was detected in autotrophic or mixotrophic cells, although it was readily measured in thiobacillus A_2 (Taylor, B. F. & Hoare, D. S. (1969), *Journal of Bacteriology* **100**, 487). Lack of this enzyme, together with NADH oxidase, was cited as the biochemical basis of obligate autotrophy (Smith, A. J., London, J. & Stanier, R. Y. (1967), *Journal of Bacteriology* **94**, 972). The present facultative strain has an inducible glyxoylate cycle by which unrestricted incorporation of acetate may occur. It is postulated that acetate oxidation may take place by the dicarboxylic acid cycle (Kornberg, H. L. & Sadler, J. R. (1961), *Biochemical Journal* **81**, 503).

A Serological Method for Distinguishing Different Sex Pili in the Electron Microscope and its Use in Sex Factor Genetics. By A. M. LAWN, ELINOR MEYNELL, MARILYN COOKE and ANNA FRYDMAN (*The Lister Institute of Preventive Medicine, London*)

We have now replaced our original method of investigating antigen–antibody reactions in the electron microscope (Lawn, A. M. (1967), *Nature, London* **214**, 1151) by filtering technique using minute membrane filters for washing the preparations. Only bound antibody remains which can be visualized by negative-contrast, as before. With the increased reliability, we can extend our investigation of the antigenic relationships between different sex pili (Lawn, A. M. *et al.* (1967), *Nature, London* **216**, 343) and distinguish four divisions of the F-like class (F and ColV; R 1; R 538–1; R 100–1, R 136 and R 192); and two divisions of the I-like class (R 64; R 144, R 163 and R 583-2).

The method has been particularly useful in studying the genetics of sex factor function. Donor-specific phages distinguish F-like from I-like pili but the most illuminating experiments often involve two sex factors of the same class. Cross-absorbed sera can distinguish their pili. For instance, the function of a sex factor ceasing to produce sex pili because of a genetic defect is sometimes restored by a second sex factor. We can see if both types of pili are then produced. Similarly, derepressed mutant sex factors may be separated into two classes; one fails to produce repressor, while the other is insensitive to its action. When a cell carries two repressor-minus factors both pilus antigens are present, but if one is repressor-insensitive it continues to synthesize repressor which prevents the appearance of the other pilus. An important finding is that when a cell contains two sex factors of the same class, neither of which is repressed or defective, almost all its sex pili appear to be mixed polymers of the two kinds of subunit. Similar pairs consisting of one F-like and one I-like factor produce separate F-like and I-like pili.

Dark Growth of Phototrophic Bacteria under Microaerophilic Conditions. By NORBERT PFENNIG (*Institut für Mikrobiologie der GSF, Göttingen, Germany*)

Eight species of the purple non-sulphur bacteria are able to grow either anaerobically in the light (photo-pigmented) or aerobically in the dark (colourless) while five species have originally been described as being strictly anaerobic and growing only in the light. More detailed studies on the latter species: *Rhodospirillum fulvum* (5 strains), *R. molischianum* (3 strains), *R. photometricum* (2 strains), *Rhodomicrobium vannielii* (6 strains) and *Rhodopseudomonas viridis* (3 strains) revealed that all strains are able to grow in the dark under microaerophilic to aerobic conditions. Growth is obtained in deep agar shake cultures and in stationary liquid cultures (the latter with 0·1 % ascorbate). The Rhodospirillum species grow only fully photopigmented 3 to 5 mm. below the agar surface, one strain of *R. viridis*

and 5 strains of *R. vannielii* grow pigmented below the surface and colourless up to the surface. It is supposed that the two different types of growth reflect the occurrence of two different oxidative energy-yielding mechanisms in the dark: (*a*) via part of the photosynthetic electron transport system (Rhodospirilla) also functioning under anaerobic conditions in the light and (*b*) via an oxidative electron transport system similar to that functioning in the facultatively aerobic species of the purple non-sulphur bacteria.

Among the purple sulphur bacteria, *Thiocapsa roseopersicina* (all nine strains tested) is exceptional in being able to grow under microaerophilic conditions in the dark (organotrophically); under aerobic conditions, photopigment formation is repressed and growth ceases when the cells become colourless.

Lysis and Protection of Erythrocytes by T-Mycoplasmas. By R. J. MANCHEE and D. TAYLOR-ROBINSON (*Clinical Research Centre, Harvard Hospital, Salisbury, Wiltshire*)

Shepard ((1967), *Annals of the New York Academy of Sciences* **143**, 505) reported that colonies of T-mycoplasmas isolated from man caused β-haemolysis of guinea-pig erythrocytes suspended in an agar overlay. Others have failed to observe this, possibly due to the small size of the colonies. The large colonies of T-mycoplasmas which we have obtained recently (Manchee, R. J. & Taylor-Robinson, D. (1969), *Journal of Bacteriology* **100**, 78) have enabled us to investigate with more certainty their haemolytic properties. Colonies of T-mycoplasmas isolated from a monkey, a dog, a bull and man lysed erythrocytes suspended in an agar overlay. Haemolysis was of the α'-type and was usually detectable only with the low power of the microscope. As compared with *Mycoplasma pneumoniae*, T-mycoplasma haemolysis occurred inconsistently in repeated tests. Crowding of colonies inhibited haemolysis. The addition of catalase also inhibited haemolysis indicating that the T-mycoplasma haemolysin is a peroxide. This haemolysin is probably produced in only small amounts.

During the course of this study we also observed protection of erythrocytes against lysis in the close proximity of colonies of T-mycoplasmas and *Mycoplasma pneumoniae*. This phenomenon occurred as frequently as haemolysis, and may have contributed to the difficulty experienced in detecting weak haemolysis. It too was inhibited by crowding of colonies. The protective effect was not due to catalase production and we suggest that a peroxidase might be responsible.

Auxotrophic Mutants of *Mycobacterium smegmatis*. By K. T. HOLLAND and C. RATLEDGE (*Department of Biochemistry, The University, Hull, HU6 7RX*)

To further our current interest in the elucidation of the biosynthesis of salicylate and mycobactin in species of *Mycobacteria* (Ratledge, C. (1969), *Biochemica et Biophysica Acta* **192**, 148). the isolation of auxotrophs requiring these compounds would be of great assistance. Mycobacteria usually grow slowly as a crusty pellicle and, therefore, the major problems were to ensure dispersed growth of cells for mutagen treatment, the subsequent segregation of deficient mutants and, finally, the production of colonies which were discrete and easily replicated. Addition of Tween 80 at the recommended concentration of 0·05 % (Konickova-Radochova, M. & Malek, I. (1969), *Folia microbiologica* (*Praha*) **14**, 201) was inadequate but at 1 %, in all fluid and solid media tested, the desired growth was achieved. All liquid cultures had to be constantly shaken to keep cells dispersed; a suitable agitation rate was 225 rev./min.

The organism was grown in a complex medium, pH 7·0, to a cell density of 10^8 cells/ml. These cells were incubated with continuous shaking for 1 hr at 37° in a minimal medium, pH 6·3 (which supported very slow growth), and contained N-methyl-N-nitroso-N′-nitroguanidine (NTG) at 1 mg./ml. Cells were then washed free of NTG on a membrane filter with 0·2 M phosphate buffer, pH 6·3, and transferred to a complete medium at 37° for 24 hr to allow for phenotypic expression. Cells were suitably diluted in 0·9 % saline–0·05 % Tween so that subsequent plating on a complete medium gave up to 300 colonies per plate. These master plates were incubated at 37° for 3 days then replicated on various minimal media.

By this technique over 700 mutants have been isolated requiring amino acids, vitamins,

purines, pyrimidines or a combination of these compounds. Only about 6% have so far reverted. For the selection of specific auxotrophs, an improvement on this method was to take mutated cells immediately after their incubation for phenotypic expression, and treat them with isoniazid (100 μg./ml.) for 24 hr with constant shaking in a medium capable of supporting growth of all types except the required mutants. To date auxotrophs requiring the aromatic amino acids or shikimic acid have been obtained using this modified method. The relevance of these and other mutants to the study of mycobactin biosynthesis and function will be discussed.

The Wellcome Trust is thanked for a grant which has enabled this work to be carried out.

An Electron Microscopic Study of Germination in *Botrytis fabae* Conidia. By D. V. RICHMOND and R. J. PRING (*Long Ashton Research Station, University of Bristol*)

The fine structure of *Botrytis fabae* conidia has been studied using both chemically fixed sections and freeze-etched replicas. Thin sections were fixed in permanganate, embedded in Vestopal, and stained with lead citrate.

The spore wall lacks conspicuous ornamentation and consists of microfibrils embedded in a granular matrix. The microfibrils tend to a longitudinal orientation on the conidium and are arranged circularly around the plugged central pore in the end-wall. The two distinct wall layers seen in chemically-fixed sections cannot be detected in freeze-etched replicas.

In young germinants the conidial wall is swollen at the base of the germ tube and new wall material, which appears to be continuous with the newly formed septal wall, is laid down in the centre of the old wall.

An apical corpuscle similar to that of *Mucor rouxii* is present in the hyphal tip of incipient germ tubes. Apical corpuscles have previously on been reported from the Phycomycetes (Bartnicki-Garcia, S. (1969), *Phytopathology* **59**, 1065).

In resting conidia the outer surface of the plasmalemma is completely covered with a ramifying network of invaginations. As the conidium swells the network becomes reduced until the invaginations resemble those in yeast cells (Moor, H. & Mühlethaler, K. (1963), *Journal of Cell Biology* **17**, 609) and *Penicillium* conidia (Sassen, M. M. A., Remsen, C. C. & Hess, W. M. (1967), *Protoplasma* **64**, 75). The invaginations disappear when germination commences. The formation of lomasomes by the passage of vesicles through the plasmalemma will be demonstrated in freeze-etched preparations.

Nuclei, vacuoles, and other organelles always appear smoothly rounded in freeze-etched replicas. The small strands of endoplasmic reticulum close to the cell wall of resting conidia become, on germination, multiple sheets surrounding the nuclei.

The Cultivation of the Rumen Ciliate *Polyplastron multivesiculum*. By G. S. COLEMAN (*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

The purpose of this communication is to report the successful cultivation of the rumen ciliate *Polyplastron multivesiculatum*. This organism which is one of the largest Entodiniomorphid protozoa found in the ovine rumen was isolated by removing individual protozoa from crude rumen contents with a micromanipulator. Attempts to grow *Polyplastron multivesiculatum* in the media used to culture rumen entodinia (Coleman, G. S. (1969), *Journal of General Microbiology* **57**, 81) were unsuccessful unless the rumen ciliate *Epidinium ecaudatum caudatum* was also present. For the initial isolation the live epidinia could not be replaced by live entodinia or dead epidinia. These epidinia were taken up by the *Polyplastron multivesiculatum* organisms each of which engulfed or otherwise destroyed up to 10 epidinia/day. Under these conditions *Polyplastron multivesiculatum* divided at least every 24 hr and has been maintained *in vitro* for over 6 months at the time of writing.

Although the initial isolation was made in a medium containing salts, bicarbonate/CO_2 buffer, cysteine, fresh rumen fluid (from which the protozoa had been removed), dried grass and wholemeal flour, it was subsequently found that a medium containing only salts (Coleman,

G. S. (1958), *Nature, London* **182**, 1104), cysteine, dried grass and wholemeal flour and equilibrated with 95 % N_2 + 5 % CO_2 supported higher population densities, e.g. 70 protozoa/ml. compared with 15/ml. In the early stages after the initial isolation it was essential to add approximately 10 epidinia/ml. each day to the culture or the *Polyplastron multivesiculatum* died in a few days. However, after several months in culture the epidinia could be omitted and the *Polyplastron multivesiculatum* continued to grow although the population densities were usually lower (30 to 60 organisms/ml.) and the protozoa were smaller, e.g. 160 μm. long compared with 230 μm.

n-Propanal and *iso*-Butanol from *Clostridium acetobutylicum*. By J. A. WARD and W. M. FOGARTY (*Department of Industrial Microbiology, University College, Dublin 4*)

In a study on the effects of peat extracts on *Clostridium acetobutylicum*, NCIB 8049, neutral volatile products other than *n*-butanol, acetone and ethanol were reported (Fogarty, W. M. & Ward, J. A. (1970), *Plant and Soil* **32**, 534). These compounds have now been identified as *n*-propanol and *iso*-butanol. In addition, it has been demonstrated that the corresponding three- and four-carbon fatty acids, namely *n*-propionic and *iso*-butyric acids, are also present. The detection of these four compounds was made by gas–liquid chromatography and identification confirmed by comparison of their retention times with retention time data for standards, on different columns.

In alcoholic fermentation by yeast, *n*-propanol and *iso*-butanol are present in the fusel oil and may be synthesized from both amino acid and carbohydrate sources. Levels of the alcohols are increased both by aeration and by supplementation of particular amino acids—threonine provides the carbon skeleton for *n*-propanol and valine behaves similarly for *iso*-butanol. When threonine was added to glucose–peptone–salts broth inoculated with *Clostridium acetobutylicum*, *n*-propanol was synthesized. At higher concentrations of threonine both *n*-propanol and *iso*-butanol were produced. Addition of valine was shown to lead to the synthesis of *n*-propanol and *iso*-butanol. Similarly, both these alcohols were detected in broth cultures of *C. acetobutylicum* through which oxygen was bubbled in the early phase of growth and which were then incubated anaerobically.

Cooked meat extract and thioglycollic acid are known to enhance conditions for the growth of anaerobic bacteria by lowering the oxidation-reduction potential. When broth cultures contained cooked meat extract, both *n*-propanol and *iso*-butanol were synthesized. This may be explained by the probable presence of amino acids in these extracts which provide the necessary carbon skeletons. The addition of thioglycollic acid to broth cultures, however, led to a retardation of growth but rather surprisingly the alcohols were present.

Clostridium acetobutylicum, therefore, has been shown to possess the capacity to synthesize *n*-propanol, *n*-propionic acid, *iso*-butanol and *iso*-butyric acid. It responds to amino acid addition and to oxygenation in a manner similar to yeast but this response was not expected from an anaerobic bacterium.

Observations on the Synchronization of *Bacillus subtilis* by a 'Stationary-phase Method'. By R. J. L. PAULTON (*Department of Bacteriology, University of Saskatchewan, Saskatoon, Canada*)

Synchronous growth of *Bacillus subtilis* has been obtained by harvesting cultures just prior to their entry into the maximum stationary phase of growth, followed by reincubation in fresh medium. A similar technique has been applied to *Escherichia coli* and *Proteus vulgaris* when it was assumed that the generally unfavourable growth conditions, prevalent in cultures of high cell density, preferentially inhibited cells at a particular stage in their division cycle (Cutler, R. G. & Evans, J. E. (1966), *Journal of Bacteriology* **91**, 469).

The synchronous cultures obtained demonstrated little deterioration in the degree of phasing during the first three to four cycles. Although the growth rate was that of the parent culture, a cell wall/cell septa stain revealed that a change in the division control mechanism had occurred. Before and immediately after transfer to fresh medium, most of the cells were found to contain three septa, although cells with seven septa were also evident. This situation

demonstrated an involvement in three successive divisions. However, a subsequent period of division, often resulting in a fourfold increase in cell numbers, reduced every cell to the single septate condition. During the following synchronous cycles, this condition was maintained, excepting periods just before and during cell division when triseptate cells were observed. Consequently, in comparison with the parent culture, a reduction had occurred in both the average number of septa/cell and in the time between their appearance and involvement in cell separation. Whilst the synchronous cultures were derived from a system in which preferential inhibition of cells at division had occurred, the method also resulted in cells with a lower degree of complexity.

A New Staphylococcal Plasmid? By ROSEMARY TAYLOR and ANNA MAYR-HARTING (*Department of Bacteriology, University of Bristol*)

In 1959, Parker & Simmons (*Journal of General Microbiology* **21**, 457) described the production by staphylococci of phage-type 71 of a bactericidal agent active on many Corynebacteria and, to a lesser extent, on other staphylococci. This property was frequently lost in culture and this loss was associated with the acquisition of sensitivity to phage 55, another of the group II phages.

We collected a number of these strains, freshly isolated from human-pathological material, and five of these were studied more extensively. Another strain, L, gave equally large inhibition zones but was untypable and coagulase-negative.

Four of the type 71 strains acquired sensitivity to phage 55 after loss of the bactericidal agent; the fifth, whether it still produced the agent or not, was killed by that phage without showing any plaques on agar. Adsorption to and killing of organisms that were producers of the agent was also marked with two of the other strains.

We consider the agent to be a bacteriocin although it has a wider range of activity than the bacteriocins produced by Gram-negative organisms. Its production in the five type 71 strains is frequently lost spontaneously which suggests a plasmid as responsible for its production. The probability of its loss is enhanced by some of the substances which eliminate other staphylococcal plasmids. In strain L, by contrast, the bacteriocin is very stable; its loss, spontaneous or after treatment of the culture, has never been observed.

Repression of β-Galactosidase by Raffinose in *Escherichia coli*. By A. G. ROBERTSON and W. H. HOLMS (*Department of Biochemistry, University of Glasgow*)

Escherichia coli ML 308 is derepressed in the *lac* operon ($i^- z^+ y^+ a^+$) but, in simple salts medium, is still sensitive to catabolite repression (Holms, W. H. (1966), *Journal of General Microbiology* **45**, ii). In media, e.g. glycerol/salts, which support high levels of both β-galactosidase and permease, the addition of raffinose (1 to 2 mM) markedly reduces the growth rate of the culture. We have suggested that raffinose, which is not catabolized by this strain, is tranported by the *lac* permease and the consequent energy drain is the prime cause of the slower growth rate (Holms, W. H. (1968), *Biochemical Journal* **106**, 31 P). This paper describes the effect of raffinose on β-galactosidase synthesis.

Addition of raffinose (1 mM) to cultures growing aerobically in glycerol/salts medium reduces the specific growth rate from 0·72 to 0·46 and the differential rate of β-galactosidase synthesis (units of enzyme/mg. total protein synthesized) from 9·10 to 5·53. Raffinose causes catabolite repression by deranging glycerol metabolism. At the same time, the intracellular pool of adenosinetriphosphate (ATP-pool) is drastically reduced. Choice of conditions (e.g. raffinose concentrations) yields cultures in which the ATP-pool varies within the range 7 to 23 nmoles ATP/mg. protein. Under these circumstances the differential rate of β-galactosidase synthesis is directly proportional to the ATP-pool.

Addition of cyclic 3′, 5′-adenosine monophosphate (cyclic AMP) to raffinose inhibited cultures increases the rate of enzyme synthesis from 5·53 to 7·07 without increasing growth rate or ATP-pool.

Thus we have confirmed, in an independent system, the conclusion of Perlman, R. L. & Pastan, I. ((1968), *Journal of Biological Chemistry* **243**, 5420), that the level of cyclic AMP controls the differential rate of β-galactosidase synthesis. The precursor of cyclic AMP is

ATP and the ATP-pool determines the level of cyclic AMP in the cell. Raffinose produces catabolite repression by reducing the ATP-pool and thence the intracellular level of cyclic AMP.

Extracellular Proteins and Lipopolysaccharides Accumulated by Growing Cultures of *E. coli* 12408. By J. JANDA* & ELIZABETH WORK (*Biochemistry Department, Imperial College, London, S.W. 7*)

The lysine auxotroph *Escherichia coli* 12408 shows biphasic growth when limited in lysine and accumulates an extracellular 'complex' of lipopolysaccharide (LPS), protein and phospholipid; this is probably derived from the outer layers of the cell walls and can be isolated in bulk from culture filtrates by flocculation with chloroform (Knox, K. W., Cullen, J. & Work, E. (1967), *Biochemical Journal* **103**, 192).

Such isolates, obtained during the first stationary phase of growth, differed from the cumulative isolates harvested at the end of secondary growth both in composition and in behaviour on density gradient centrifugation. The former product had more protein, less organic P and a lower content of a certain residue of 2-keto-3-deoxy-octonic acid (KDO) which differed from the rest of the KDO in giving a thiobarbituric acid colour reaction (Osborn, M. J. (1963), *Biochemistry* **50**, 499) without the usual preliminary acid hydrolysis, provided it had been already treated with 1 N-NaOH for 30 min. at 18°.

Passage of lysine-limited culture filtrates through columns of Biogel A 0·5 M showed the presence of two types of proteins; one was a high mol.wt protein (A) eluting as a symmetrical peak with most of the LPS; the other (lower mol.wt proteins B, C, D) emerged in at least three overlapping peaks. Proteins B and C increased during both stationary and secondary growth phases; D was produced during stationary phase, and decreased during secondary growth; A increased mainly during stationary phase and less during secondary growth. The cumulative LPS-containing isolate prepared by chloroform flocculation contained proteins A and D.

Cultures grown with adequate lysine, 10 % sucrose and 0·1 % silicone antifoam accumulated no extracellular LPS or proteins A, D, although B, C were produced. With 1·0 % silicone, culture filtrates contained some LPS and the four proteins resembling A, B, C, D of lysine-limited cultures.

* On leave from Institute of Antibiotics, Roztoky, Nr Prague, Czechoslovakia.

Synthesis of Glutamic Acid in Some Prokaryotic and Eukaryotic Organisms. By C. M. BROWN, *J. L. MEERS and *D. W. TEMPEST (*Department of Microbiology, Medical School, University of Newcastle upon Tyne and *Microbiological Research Establishment, Porton, Nr Salisbury, Wiltshire*)

In order to grow in a simple salts medium in which ammonia provides the sole nitrogen source, organisms must possess some mechanism for the synthesis of amino acids from ammonia and intermediary metabolites. In many species of bacteria and yeasts, net amino acid synthesis is effected by amino acid dehydrogenases (principally glutamate dehydrogenase: EC 1.4.1.4) but recently we found (Meers, J. L., Tempest, D. W. & Brown, C. M. (1970), in press) that *Aerobacter aerogenes* possessed an alternative pathway of glutamic acid synthesis which functioned under conditions where the intracellular ammonia level was low. This latter pathway involved first the amidation of glutamic acid to glutamine and then the reductive transfer of the glutamine amide-nitrogen to 2-oxoglutarate, and was found to have a low K_m value for ammonia (< 1 mM).

Using a chemostat to grow organisms in environments containing, alternately, high and low concentrations of ammonia we have determined quantitatively the distribution of glutamate dehydrogenase and glutamine-amide: 2-oxoglutarate amino transferase (oxido-reductase) in several species of bacteria (*Escherichia coli, Erwinia carotovora, Pseudomonas fluorescens, Bacillus subtilis* var. *niger, B. megaterium, B. polymyxa*) and yeasts (*Candida utilis, Saccharomyces cerevisiae*). Also we have examined their distribution in various tissues (liver, brain) from rat, mouse and guinea pig.

Whereas some glutamate dehydrogenase was present in all organisms, except in *Erwinia carotovora*, the glutamine pathway of glutamic acid synthesis was only detected in prokaryotic organisms; invariably, but not exclusively, in ammonia-limited bacteria. In this connexion, it is significant that the glutamate dehydrogenase present in prokaryotic organisms invariably had a K_m for ammonia $\geqslant 3 \times 10^{-3}$ M; well above the level of ammonia present in the ammonia-limited organisms. On the other hand, the glutamate dehydrogenase present in the yeasts studied had a K_m for ammonia *c.* 1×10^{-3} M below the level of ammonia (*c.* $2{\cdot}5 \times 10^{-3}$ M) present in ammonia-limited yeast organisms.

The Synthesis of Serine in Wild Type and Serine-requiring Mutants of Pseudomonas AM 1. By W. HARDER and J. R. QUAYLE (*Department of Microbiology, University of Sheffield*)

Two alternative pathways for serine metabolism in Pseudomonas AM 1 have been proposed (Heptinstall, J. & Quayle, J. R. (1970), *Biochemical Journal* in press). Evidence was presented that one of these, a pathway involving non-phosphorylated intermediates and converting serine to phosphoglycerate, is a major route for carbon assimilation during growth on C_1 compounds. To account for biosynthesis of serine during growth on compounds such as succinate and lactate, a pathway converting phosphoglycerate to serine via phosphorylated intermediates was postulated, similar to that operating in *Escherichia coli* (Pizer, L. I (1963), *Journal of Biological Chemistry* **238**, 3934).

The physiological significance of this latter pathway is being studied in Pseudomonas AM 1 using mutants which require serine for growth on succinate. Such mutants have been isolated by treatment of wild type cells with NNMG and subsequent penicillin enrichment of the desired phenotype. Two mutants 20S and 73S are able to grow on methanol and formate, but require serine for growth on succinate, lactate or ethanol. Assays of the enzymes involved in serine biosynthesis showed that both mutants lack phosphoserine phosphohydrolase (EC 3.1.3.3). Revertants of mutant 20S which had regained the ability to grow on succinate minimal medium had also regained the ability to synthesize phosphoserine hydrolase. These results indicate that during growth on carbon sources other than C_1 compounds the phosphorylated pathway is the major route for serine synthesis in Pseudomonas AM 1.

Either glycine or methanol can replace serine as a supplement for growth on succinate, and the growth response of both mutants is proportional to the initial concentration of serine, glycine or methanol. The relative molar amounts of these respective supplements allowing a similar growth response on succinate are in the ratio 1:2:2. This suggests that enzymes are present in Pseudomonas AM 1 which are able to catalyze the following reactions:

$$2 \text{ glycine} \rightarrow \text{serine}; \quad 2 \text{ methanol} + CO_2 \rightarrow \text{serine}.$$

A Defective F-*lac* Episome with *fi*$^+$ Repressor Activity from a Wild Strain of Klebsiella. By E. C. R. REEVE and J. A. BRAITHWAITE (*Institute of Animal Genetics, West Mains Road, Edinburgh*)

A strain of *Klebsiella* sp. (probably *aerogenes*) has been found to harbour an F-*lac* episome with unusual properties. The host strain was isolated from vole faeces from a forest in central Scotland, and is resistant to Tetracycline and Ampicillin, but cannot transfer either resistance unaided. However, after infection with either an F-type or an I-type sex factor, Tetracycline resistance is transferred at rather low frequency to strains of *Escherichia coli* K 12, showing that this resistance depends on a plasmid gene. In these matings it was noticed that Lac$^+$ was sometimes transferred form the Klebsiella to K 12, independently of antibiotic resistance.

Tests with F-primes and derepressed mutants of the *fi*$^+$ R-factor R 1 (Meynell, E. & Cooke, M. (1969). *Genetical Research* **14**, 309) show that the Lac$^+$ particle (F_K-*lac*) has the following properties:

(1) F_K-*lac* shows mutual exclusion with F-primes in *E. coli*—when a Lac$^-$ strain of K 12 carrying this particle is infected with F-*his* or F-*gal*, the Lac$^+$ particle is lost. Such mutual exclusion apparently does not occur in the Klebsiella host strain.

(2) F_K-*lac* is unable to promote conjugation in *E. coli* or Klebsiella.

(3) F_K-*lac* possesses an active sex-factor repressor gene of *fi*$^+$ type, as shown by its interaction with derepressed mutants of R 1 in both Klebsiella and *E. coli*: it represses the sex-factor activity of the *i*$^-$) mutant R 1 *drd* 19, but not of the (*o*^{c}) mutant R 1 *drd* 16. The Lac$^+$ particle represses sex-factor activity of an F-prime in Klebsiella but excludes it in *E. coli*.

The Lac$^+$ particle is not associated with a colicin factor, and we conclude that it is essentially an episome of F-prime type, which also carries the *fi*$^+$ repressor gene but is defective in some of the genes responsible for conjugation.

The Ferredoxin-dependent Formation of 2-Carbon Units from Pyruvate by Extracts of the Blue-green Alga, *Anabaena variabilis*. By C. K. LEACH and N. G. CARR (*Department of Biochemistry, University of Liverpool, Liverpool 3*)

The elimination of CO_2 from [1 − ^{14}C]pyruvate is readily detected in a washed suspension of blue-green algae, but the enzymic steps of pyruvate catabolism have not been elucidated. Cox, R. M. & Fay, P. (1969, *Proceedings of the Royal Society* B **172**, 376) using intact *Anabaena cylindrica*, have suggested that this nitrogen fixing blue-green alga metabolizes pyruvate to acetyl phosphate by a mechanism similar to that found in *Clostridia* sp. This communication reports the absence of pyruvate oxidase (EC 1.2.3.3) and pyruvate dehydrogenase (EC 1.2.41) in extracts of a non-nitrogen fixing strain of *A. variabilis* and the detection of a ferredoxin-dependent fission of pyruvate to a 2-carbon unit, assayed as acetohydroxymate.

When a cell-free extract of *Anabaena variabilis* was incubated with pyruvate, in the presence of hydroxylamine and ATP, two manometric changes were observed, CO_2, was evolved (0·3–0·38 μmole/hr/mg. protein) and O_2 was absorbed (0·15 to 0·25 μmole/hr/mg. protein), acetohydroxymate was formed concomitantly (0·15 to 0·23 μmole/hr/mg. protein). After treatment of the cell-free extract with DEAE-cellulose it was necessary to add back ferredoxin, Mg^{2+}, CoA and ATP for the restoration of acetohydroxymate formation. Thiamine pyrophosphate was without effect but benzyl viologen could replace ferredoxin. No hydrogenase activity was detected but when ferredoxin:NADP$^+$ diaphorase was prepared from *A. variabilis* and added to the incubation mixtures, under anaerobic conditions, the reduction of NADP$^+$ (0·024 to 0·04 μmole/hr/mg. protein) was observed in the presence of pyruvate. Inclusion of acetate in the reaction mixture did not dilute the isotopic content of [2 − ^{14}C]-acetohydroxymate formed from [2 − ^{14}C]pyruvate. When the concentration of ATP was varied, the rates of acetohydroxymate formation did not permit unequivocal identification of sigmoidal or hyperbolic kinetics, but the suggestion that the role of ATP was as an activator, rather than as a reactant, in this reaction is consistent with knowledge of the dissimilation of pyruvate in other systems. The relevance of this reaction to the synthesis of lipid from CO_2 by photosynthetic organism will be discussed.

Regulation of *Iso*citrate Dehydrogenase Activity in *Escherichia coli* by the Operation of the Glyoxylate Cycle. By P. M. BENNETT and W. H. HOLMS (*Department of Biochemistry, University of Glasgow*)

After aerobic growth on limited glucose in a simple salts medium, cells of *Escherichia coli* ML 308 lose approximately 80 % of their *iso*citrate dehydrogenase activity which subsequently recovers to a large degree (Bennett, P. M. & Holms, W. H. (1969), *Journal of General Microbiology* **58**, iv). Acetate accumulates during growth on glucose but is utilized when the sugar is exhausted. During acetate utilization, the activity of *iso*citrate dehydrogenase falls and that of *iso*citrate lyase rises. When the acetate is exhausted, *iso*citrate lyase activity remains constant but *iso*citrate dehydrogenase activity is restored almost to its original level. Inhibitors of protein synthesis added at the end of growth on glucose prevent both synthesis of *iso*-citrate lyase and loss of *iso*citrate dehydrogenase activity, although acetate utilization continues slowly.

Addition of acetate to cultures which have undergone the cycle of loss and recovery of *iso*citrate dehydrogenase again causes a very fast decay of enzyme activity which subsequently recovers. In this system, inhibitors of protein synthesis have little or no effect, suggesting that restoration of activity is not due to *de novo* protein synthesis.

Cells growing on acetate (30 mM) display only 25 % of the *iso*citrate dehydrogenase activity obtained during growth on glucose. Pyruvate (1·0 mM) added to cultures growing on acetate causes an immediate fourfold increase in *iso*citrate dehydrogenase activity and this stimulation is insensitive to inhibitors of protein synthesis and is, therefore, not due to synthesis of new enzyme protein.

These results suggest that some or all of the operations of the glyoxylate cycle inactivate *iso*citrate dehydrogenase in this organism. If induction of enzymes of the glyoxylate cycle is prevented, inactivation does not occur. If the cycle stops through exhaustion of substrate or because its operation is made redundant by addition of other carbon sources, the *iso*citrate dehydrogenase activity is restored.

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