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Current Problems in Studies of Streptococci

Second Griffith Memorial Lecture

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A brief synopsis of the full lecture.

'To bacteriologists Dr Fred Griffith is universally known for his fundamental work of type transformation in pneumococci and for his studies on the classification of the haemolytic streptococci (Griffith, 1928). He early developed a new method of slide agglutination which he used with success on pathogenic streptococci isolated from infections in man. In 1934 he published a study of the first 27 types (Griffith, 1934). Precipitin reactions with the M antigen were the basis of studies in my laboratory of the serological types of haemolytic streptococci (Lancefield, 1928). Dr Griffith and I exchanged strains and sera and found that with very few exceptions we agreed on the typing results. The number of types has now risen to 55. In this second Griffith Memorial Lecture some of the significant advances in streptococcal research since 1941 are reported.

'It was found that a second antigen, designated T, stimulated antibodies which reacted in slide-agglutination tests (Lancefield, 1940). The specificity of the T antigen did not always agree with that of the M antigen (Lancefield, 1954). Some strains isolated from impetigo were classified by slide agglutination into the three main T-antigen patterns by Parker and others in 1955 (Parker, Tomlinson & Williams, 1955; Barrow, 1955). However, these strains gave no recognizable M-precipitin reaction. Identification of these strains was important because impetigo is often associated with acute glomerulonephritis. Search for previously unrecognized M-types among pyoderma strains uncovered at least four new types, two of these from acute glomerulonephritis (Anthony, Perlman & Wannamaker, 1967). It is now known that certain M-type infections with some strains are followed by epidemic glomerulonephritis, and infections with other strains of the same type occur in streptococcal epidemics unassociated with nephritis. Five or six types are now generally accepted as including nephritogenic strains (Johnson, Baskin, Beachey & Stollerman, 1968). Attempts to isolate a toxin, or other substances of significance in glomerulonephritis, from nephritogenic strains have been unsuccessful.'

The Lecturer next discussed a few selected examples of immunological studies of the antigenic composition of group A streptococci, representing the trend of streptococcal research in recent years. The isolation and analysis of the streptococcal cell wall (Salton, 1952) and the localization of many antigenic components were stressed (McCarty, 1964). These studies showed that the mucopeptide is the only structural component of the rigid streptococcal cell wall left after removal of all other substances.

Active groupings of the cell-wall polysaccharides which determine the immunological specificities of many of the serological groups of streptococci have been established. This work has been greatly aided by specifically induced enzymes from soil bacilli.

Renewed interest in purifying the type-specific protein M-antigen of group A streptococci has been stimulated by the development of new biochemical methods for purification. This may make active immunization of rheumatic fever subjects possible, instead of protecting them from streptococcal infections by the use of chemoprophylaxis, as is the current practice. By using purified M preparations, Fox and colleagues have obtained bactericidal antibodies in infants with few or no side reactions (Fox, Wittner & Dorfman, 1966). It is not known, however, whether this anti-M immune response in the absence of an infection is a reliable index of type-specific immunity.

The possibility of danger to the patients from immunological cross-reactions between the streptococcal products injected and cardiac tissues of rheumatic-fever subjects has received much consideration due to studies of such cross-reactions by Kaplan (1963), by Zabriskie (1967), by Goldstein, Halpern & Robert (1967). In contrast to the possible dangers of active immunization is the fact that so far there is no indication that any of these cross-reactive antibodies actually cause cardiac lesions, despite many previous attempts to immunize man with a variety of streptococcal products.

Of the many important extracellular products of group-A streptococci, brief mention was made only of a few. The early studies of Dr Edgar W. Todd on streptolysin-O have been followed by the universal use of his test for the presence of anti-streptolysin-O, as indicative of a preceding streptococcal infection (Todd, 1938). Todd also introduced to streptococcal work the bactericidal test used today almost exclusively to measure in man the anti-M antibodies upon which type-specific immunity depends (Todd, 1927).

One of the most interesting and well-studied streptococcal systems is the proteinase and its precursor. This enzyme and its zymogen have been crystallized and extensively studied by Elliott (1950) and by other biochemical colleagues. These enzymes are of special interest to enzyme chemists because both zymogen and active enzyme contain only one-half cystine residue per molecule (Liu *et al.* 1963, 1965).

Another especially interesting extracellular product is the erythrogenic toxin. Zabriskie (1964) has shown that only strains infected with temperate bacteriophage are toxin producers.

In reviewing examples of the known array of streptococcal antigens, too many to be discussed here, it seems apparent that very few bacterial species have as much known about their chemical composition and immunochemical properties as the streptococci, which have been studied by so many different investigators.

REFERENCES

ANTHONY, B. F., PERLMAN, L. V. & WANNAMAKER, L. W. (1967). Skin infections and acute nephritis in American Indian children. *Pediatrics, Springfield.* **39,** 263.

BARROW, G. I. (1955). Clinical and bacteriologic aspects of impetigo contagiosa. *J. Hyg., Camb.* **53,** 495.

ELLIOTT, S. D. (1950). The crystallization and serological differentiation of a streptococcal proteinase and its precursor. *J. exp. Med.* **92,** 201.

FOX, E. N., WITTNER, M. K. & DORFMAN, A. (1966). Antigenicity of the M proteins of group A, streptococci. III. Antibody responses and cutaneous hypersensitivity in humans. *J. exp. Med.* **124**, 1135.

GOLDSTEIN, I., HALPERN, B. & ROBERT, L. (1967). Immunological relationship between streptococcus A polysaccharide and the structural glycoprotein of heart valve. *Nature, Lond.* **213**, 44.

GRIFFITH, F. (1928). The significance of pneumococcal types. *J. Hyg., Camb.* **27**, 113.

GRIFFITH, F. (1934). The serological classification of *Streptococcus pyogenes*. *J. Hyg., Camb.* **34**, 542.

JOHNSON, J. C., BASKIN, R. C., BEACHEY, E. H. & STOLLERMAN, G. H. (1968). Virulence of skin strains of nephritogenic group A streptococci: new M protein serotypes. *J. Immun.* **101**, 187.

KAPLAN, M. H. (1963). Immunologic reaction of streptococcal and tissue antigens. I. Properties of an antigen in certain strains of group A streptococci, exhibiting an immunologic cross reaction with human heart tissue. *J. Immun.* **90**, 595.

LANCEFIELD, R. C. (1928). The antigenic complex of *Streptococcus haemolyticus*. I. Demonstration of a type-specific substance in extracts of *Streptococcus haemolyticus*. *J. exp. Med.* **47**, 91.

LANCEFIELD, R. C. (1940). Type-specific antigens, M and T, of matt and glossy variants of group A haemolytic streptococci. *J. exp. Med.* **71**, 521.

LANCEFIELD, R. C. (1954). Cellular constituents of Group A streptococci concerned in antigenicity and virulence. In *Streptococcal Infections*. Ed. by M. McCarty. Chap. 1, p. 3. Columbia University Press.

LIU, T. Y., STEIN, W. H., MOORE, S. & ELLIOTT, S. D. (1965). The sequence of amino acid residues around the sulfhydryl group at the active site of streptococcal proteinase. *J. biol. Chem.* **240**, 1143.

LIU, T. Y., NEUMANN, N. P., ELLIOTT, S. D., MOORE, S. & STEIN, W. H. (1963). Chemical properties of streptococcal proteinase and its zymogen. *J. biol. Chem.* **238**, 251.

MCCARTY, M. (1964). The streptococcal cell wall and its biologic significance. In *The Streptococcus Rheumatic Fever and Glomerulonephritis*. Baltimore: Williams and Wilkins Company.

PARKER, M. T., TOMLINSON, A. J. H. & WILLIAMS, R. E. O. (1955). Impetigo contagiosa: the association of certain types of *Staphylococcus aureus* and of *Streptococcus pyogenes* with superficial skin infections. *J. Hyg., Camb.* **53**, 458.

SALTON, M. R. J. (1952). The nature of the cell walls of some Gram-positive and Gram-negative bacteria. *Biochim. biophys. Acta* **9**, 334.

TODD, E. W. (1938). The differentiation of two distinct serological varieties of streptolysin, streptolysin O and streptolysin S. *J. Path. Bact.* **47**, 423.

TODD, E. W. (1927). A method of measuring the increase or decrease of the population of haemolytic streptococci in blood. *Br. J. exp. Path.* **8**, 1.

ZABRISKIE, J. B. (1964). The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. *J. exp. Med.* **119**, 761.

ZABRISKIE, J. B. (1967). Mimetic relationship between Group A streptococci and mammalian tissues. *Adv. Immun.* **7**, 147.

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Pigment Formation in L-forms of *Serratia marcescens*

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SUMMARY

L-forms of *Serratia marcescens* were produced and serially transferred in osmotically stabilized agar with penicillin. The bacterial form was pigmented, while the L-form colony was not. Lack of colour in the L-form colony was not due to pigment diffusion into the agar; an extract of agar with L-form growth did not show an absorption spectrum for prodigiosin.

INTRODUCTION

Although considerable information has accumulated about the chemical steps in the synthesis of the red pigment 'prodigiosin' by *Serratia marcescens*, little is known of the site of synthesis. Purkayastha & Williams (1960) found that acetylhexosamine and pigment content of various fractions of the organism paralleled each other and concluded that this was evidence that the pigment was contained in the cell 'envelope'. From their results, the authors were unable to determine whether the pigment was located in the plasma membrane or wall portion of the cell envelope. However, since lysozyme treatment resulted in the release of a clear supernatant fluid containing hexosamine, they suggested that the 'pigment might be located in the cell or plasma membrane'. L-forms are microbial forms with part or all of their cell wall missing and would, therefore, be a suitable tool for determining the importance of cell wall in pigment production and/or localization. The present studies were designed to prepare L-forms of *S. marcescens* in order to determine whether they could produce pigment.

METHODS

Bacteria. A pigmented strain of *Serratia marcescens*, originally obtained from a throat culture of a patient, was used throughout.

Media. Several different osmotically stabilized media were tried for L-form production and propagation. The best was brain heart infusion agar (Difco) + 1·8 % sodium chloride + 0·001 % $MgSO_4 . 7H_2O$, inactivated (56° for 30 min.) 20 % (v/v) agamma horse serum (Hyland), and 10 % (w/v) yeast extract (Robbins and Fleischmann were both satisfactory). The bacterial form was routinely maintained on brain heart infusion agar. The bacterial form was grown on the medium used for growing the L-form for experiments involving pigment production.

Production of L-forms. Bacteria were inoculated as either a pour or streak plate on media containing various amounts of penicillin and incubated aerobically. The plates were examined daily; when L-forms were noted after several days, serial transfers

were made as follows: approximately one-quarter of the agar plate with L-form growth was mashed in 9 ml. liquid medium with the same composition as the agar in a Tri-R Homogenizer (Tri-R Co.), and 1 and 0·1 ml. samples used to inoculate fresh medium for pour and streak plates. Because L-forms grew better in pour plates, routine serial transfer was done with pour plates. The streak plates were used to examine colony morphology with low power microscopy.

Although 500 units penicillin/ml. was sufficient for initial production of L-forms, the penicillin concentration had to be increased to 30,000 units/ml. to prevent reversion to bacterial form during subsequent propagation. All incubations were aerobic and, as a compromise between conditions for pigmentation and for L-form growth, at 30°. The L-forms have now been serially transferred a total of 61 times at approximately twice-weekly intervals, without any visual evidence of reversion.

Extract of pigment. A modification of the method of Hubbard & Rimington (1950) was used. To obtain a large quantity of L-form growth of characteristic morphology, these forms were grown on the surface of nutrient agar in Kolle flasks. The bacterial form was grown in plastic Petri dishes (Falcon). For extraction of pigment, the nutrient agar with bacteria was homogenized with 1 ml. liquid medium for each 2 ml. nutrient agar in a Tri-R Homogenizer. Because of the large volumes involved, the L-form growth and agar were homogenized in a Waring blender with liquid medium in the same proportion. The volume of homogenate was measured and 10 g. solid NaOH dissolved in each 100 ml. of homogenate. The mixture was allowed to stand for 2 hr. An equal volume of absolute ethanol was mixed with the homogenate, followed by an equal volume of light petroleum. The mixture was shaken and separated in a separatory funnel. The petroleum phase was saved, and the aqueous phase re-extracted twice more with the same volumes of light petroleum. The combined petroleum fractions were reduced in volume over low heat and allowed to dry at room temperature (20°). The residue was dissolved in 3 ml. absolute ethanol and 0·03 ml. concentrated hydrochloric acid added to ensure that the prodigiosin was in acidic form. Suitable dilutions of the bacterial extract were made in absolute ethanol (no dilution was necessary for the L-form extract). The spectrum of both extracts was taken on a Beckman DU spectrophotometer.

RESULTS

It was noted from the beginning that L-form colonies appeared to be colourless. Although visual evidence suggested that the L-forms were not producing pigment, the possibility existed that they were, but that because of lack of an intact cell wall, the pigment was diffusing into the agar and thus was not noticed. Accordingly, extraction of nutrient agar in which L-forms were growing was done to determine whether pigment was present. To obtain approximately equal numbers of L-forms and bacteria, eight Kolle flasks were heavily streaked with L-form mash, a Petri dish streaked with the bacterial form, and all were incubated for 8 days. The results of the extraction procedure are shown in Table 1. The absorption spectrum of L-form extract did not at all resemble that of the bacterial form extract, having no peak at 540 mμ as had the bacterial form extract. Thus, it was unlikely that L-form mash contained any prodigiosin. However, even if the very small absorption at 540 mμ (no peak) did represent prodigiosin, it could not have been more than 1 % of that produced by a comparable number of bacterial forms. The question arose whether the amount of

potassium and citrate in buffered penicillin could in some way inhibit pigment production. Accordingly, 60 m-equiv. K^+/l. and 3·4 m-mole trisodium citrate/l. (amounts present in 30,000 units penicillin/ml.) were added to the salt + horse serum + yeast agar. When the bacterial form of *Serratia marcescens* was streaked on the surface, the colonies grew uniformly pigmented.

Table 1. *Determination of the amount of prodigiosin produced by bacterial and L-forms of Serratia marcescens*

	L-forms	Bacteria
Total number of colony-forming units extracted	$2{\cdot}88 \times 10^{10}$	$2{\cdot}98 \times 10^{10}$
Volume of ethanol in which extract was dissolved for A_{540} reading	3·03	75·8
A_{540} obtained	0·193	0·850
Total nanomoles prodigiosin in extract*	5·32	585

* Using Hubbard & Rimington's (1950) value of the molar extinction coefficient of the acidic form of prodigiosin 110,000 at 540 mμ.

DISCUSSION

The location of the metabolic block in L-forms of *Serratia marcescens* leading to lack of pigment production is not known. The sequence of chemical events leading to pigment production has recently been summarized by Morrison (1966). The current schema for late steps in prodigiosin production is that there is synthesized a volatile monopyrrole precursor, 2-methyl-3-*n*-amyl-pyrrole (MAP). In a separate sequence, a bipyrrole precursor, 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC), is synthesized. The final step is the coupling of the mono- and bipyrrole precursors.

The failure of L-forms to produce pigment may be that: (1) L-forms were originally selected by penicillin from non-pigment forming mutants. This is probably negated by the fact that, when penicillin is removed, reversion is to uniformly pigmented forms. (2) A metabolic block may prevent synthesis of the mono- or dipyrrole precursors. (3) The coupling enzyme is not synthesized or cannot act. (4) The cell wall is necessary as a physical site for one or more of the above (Monk, 1957). It is *only* the latter postulate that is in accord with the known action of penicillin, i.e. inhibition of cell wall synthesis. Otherwise, another action of penicillin would need to be postulated.

REFERENCES

HUBBARD, R. & RIMINGTON, C. (1950). The biosynthesis of prodigiosin, the tripyrrylmethene pigment from *Bacillus prodigiosus* (*Serratia marcescens*). *Biochem. J.* **46**, 220.

MONK, G. W. (1957). Spectral absorption of prodigiosin in intact cells. *J. Bact.* **74**, 71.

MORRISON, D. A. (1966). Prodigiosin synthesis in mutants of *Serratia marcescens*. *J. Bact.* **91**, 1599.

PURKAYASTHA, M. & WILLIAMS, R. P. (1960). Association of pigment with the cell envelope of *Serratia marcescens* (*Chromobacterium prodigiosum*). *Nature, Lond.* **187**, 349.

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The Effects of Inositol-deficiency on the Chemical Composition of the Yeast Cell Wall

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SUMMARY

Growth of a strain of *Saccharomyces cerevisiae* in an inositol-deficient chemically defined medium resulted in marked changes in the composition of the cell wall. Organisms grown in the complete absence of inositol possessed a weakened cell wall which contained more glucan and hexosamine but less mannan, phosphorus and protein than normal walls. The amino acid contents of the two types of cell wall were quite different but no significant change occurred in either the amount or the fatty acid composition of cell wall lipid. Treatment with ethylenediamine facilitated the isolation of three fractions, A, B, and C from normal and from deficient cell walls. The mannan and protein content of fractions A were strikingly similar. Deficient fractions B and C however contained considerably less mannan and protein but more glucan and hexosamine than the corresponding normal fractions.

INTRODUCTION

The yeast cell wall is composed mainly of glucan, mannan, protein and lipid (Northcote & Horne, 1952; Roelofsen, 1953). Several workers have also reported the presence of small amounts (1–3 %) of chitin (Roelofsen & Hoette, 1951; Houwink & Kreger, 1953; Falcone & Nickerson, 1956; Eddy, 1958) but Korn & Northcote (1960) estimated that not more than 9 % of the cell wall glucosamine of bakers' yeast could be present as chitin. Although its chemical composition is well established, comparatively little is known about the fine-structure of the yeast cell wall. However, the fundamental importance of polysaccharide-protein complexes has been demonstrated (Falcone & Nickerson, 1956; Kessler & Nickerson, 1959; Eddy, 1958; Korn & Northcote, 1960).

Inositol-deficiency results in various morphological changes in *Saccharomyces cerevisiae* W.S. including the formation of a cell wall markedly different in appearance under the electron microscope from that of the normal yeast (Challinor, Daniels & Hall, 1958). This finding prompted a study of the structure of the cell walls and preliminary investigations (Challinor, Power & Tonge, 1964) revealed changes in the chemical composition. In the present work, cell walls were fractionated by using ethylenediamine (Korn & Northcote, 1960) and the isolated polysaccharide-protein complexes analysed.

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METHODS

Saccharomyces cerevisiae W.S. isolated from a top fermentation brewery pitching yeast was grown in chemically defined medium containing per 100 ml.: glucose, 6 g., KH_2PO_4, 0·2 g., $(NH_4)_2HPO_4$, 0·237 g., $MgSO_4.7H_2O$, 0·1 g., $CH_3CO_2Na.3H_2O$, 0·5 g., $CaCl_2.6H_2O$, 25 mg., $MnSO_4.4H_2O$, 1 mg., KI, 10 μg., trace elements, 0·1 ml. (Emery, McLeod & Robinson, 1946), calcium-D-pantothenate, 240 μg., thiamine hydrochloride, 200 μg., nicotinic acid, 200 μg., pyridoxine hydrochloride, 250 μg., D-biotin, 0·22 μg., inositol, 1 mg. The medium was adjusted to pH 5·5. This medium, termed 'complete medium', supported maximum yeast growth. Inositol-deficient medium contained no inositol. Yeasts grown in these media and cell walls prepared from them are referred to as 'normal' and 'deficient' respectively. Stock cultures were maintained on complete medium solidified with agar (2 g./100 ml.).

Cultures for cell wall preparation were grown in 2·5 l. bottles (Glaxo pattern) containing 300 ml. of medium inoculated with 1 ml. of a well washed (× 3) suspension containing 10 μg. dry weight of yeast/ml. obtained from a 24 hr complete medium liquid culture grown at 30°. After incubation at 30° for 96 hr for normal yeast or 120 hr for deficient yeast, the cells were harvested by centrifugation at 1000 *g* for 10 min. The cells were then washed twice in saline and once in distilled water. Yields were dry wt equiv. 2 mg. normal yeast and 1 mg. deficient yeast/ml.

Preparation of cell walls

A thick yeast suspension was prepared in distilled water (approx. 40 mg./ml. dry wt). A portion (5 ml.) was delivered into a Mickle cell (capacity 20 ml.) and Ballotini beads (1 mm., Chance Ltd.) were then added to a total volume of 15 ml. Cells were then sealed with polythene caps and vibrated at mains frequency in a Mickle disintegrator. The deficient yeast required 30 min. for complete disintegration as judged by microscopic examination. Normal yeast, however, required shaking for at least 1 hr. A metal cage containing solid carbon dioxide supported directly above the vibrating cells maintained their temperature just above freezing. The glass beads were separated from cell contents by filtration through a fine network of glass-wool. The beads and glass-wool were thoroughly washed with distilled water until the washings became clear. Centrifugation at 1500 *g* for 10 min. at 4° (Northcote & Horne, 1952) resulted in sedimentation of the walls while other debris remained in suspension. The supernatant fluid was discarded, cell walls resuspended in distilled water and the process repeated. At least 50 washings were required before centrifugation produced a perfectly clear supernatant fluid. The cell walls were freeze-dried and stored *in vacuo* over phosphorus pentoxide at room temperature.

Fractionation of cell walls

Cell walls (1 g.) were incubated for 3 days at 37° with anhydrous ethylenediamine (250 ml.) and fractions isolated as described by Korn & Northcote (1960).

Analysis of cell walls and fractions

(*a*) *Carbohydrate analyses*

Total carbohydrate was determined using the anthrone method (Chung & Nickerson, 1954).

Glucose. Samples (10 mg.) were stood at room temperature with concentrated sulphuric acid (0·2 ml.) until dissolved. Water (2 ml.) was then added, the tube sealed and heated at 95° for 6 hr. The acid was neutralized with barium carbonate and the precipitated barium sulphate removed by centrifugation. The residue was washed three times with water and the supernatant and washings combined. The solution was then passed down a 9 × 1 cm. column of Amberlite I.R. 120 resin to remove barium ions completely. The eluate was adjusted to pH 7 and diluted to 100 ml. Glucose was determined by enzymic assay with glucose oxidase (The Boehringer Corporation (London) Ltd.). Maximum glucose liberation occurred after 6 hr hydrolysis.

Mannose. The mannose content was obtained by subtracting the value obtained for glucose by glucose oxidase from that obtained by the anthrone method for total hexose (expressed as glucose). This value, mannan content expressed as glucose, was converted to a true mannose reading by reference to a standard mannose curve determined by the anthrone method.

Amino sugars. Samples (5–10 mg.) were hydrolysed in 4 N-HCl for 12 hr at 100° in sealed tubes. After evaporating to dryness, samples were redissolved in distilled water and the amino sugar content determined as described by Cessi & Piliego (1960).

Chromatography. The identification of sugars was achieved by one-dimensional chromatography on paper (Whatman No. 3) using a single-phase solvent *n*-butanol + acetic acid + water (12 + 3 + 5, by vol.). Sugars were detected by dipping in benzidine + trichloroacetic acid reagent (Smith, 1960) and heating to 100°.

(*b*) *Nitrogen analyses*

Total nitrogen was determined by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943). Protein nitrogen values were obtained by subtraction of the amino sugar nitrogen content, determined separately, from total nitrogen. For amino acid analysis, samples (5 mg.) were hydrolysed at 100° with 6 N-HCl (0·5 ml.) for 18 hr. The hydrolysates were evaporated to dryness and redissolved in 0·1 N-HCl, before analysis. Quantitative amino acid analysis was done by ion-exchange chromatography according to Moore, Spackman & Stein (1958) using a Technicon Amino Acid Autoanalyser.

(*c*) *Lipids*

Cell walls were extracted first with neutral chloroform + methanol (2 + 1, v/v) and then with acidic (1 % HCl) chloroform and methanol. All extractions were made on fresh cell wall material under nitrogen at room temperature. Removal of solvents was effected by distillation under reduced pressure. The crude extracts were purified by means of the procedure of Folch, Lees & Sloane-Stanley (1957). Fatty acid methyl esters were prepared from cell wall lipids as described by Stoffel, Chu & Ahrrens (1959). Standard reference fatty acid methyl esters were prepared by the more rapid esterification procedure with diazomethane. Analysis of the methyl esters was done using a column packed with acid and alkali washed Celite '545' (100–120 mesh, B.D.H. Ltd.) as solid support and 5 % polyethylene glycol adipate as a stationary liquid. The analyses were carried out on a Pye-Argon gas chromatograph.

(d) Phosphorus

Samples (1 mg.) were boiled with 5 N-H_2SO_4 in a micro-Kjeldahl digestion apparatus. Phosphorus estimations were done on the hydrolysates by the micro-method of Dryer, Tammes & Routh (1957).

RESULTS

Cell walls constituted 10·5 and 10·3 % of the dry weight of the deficient and normal yeasts respectively. Treatment with ethylenediamine yielded three fractions, A, B and C, from both types of cell wall (Table 1). The corresponding fractions of normal and deficient walls were indistinguishable on the basis of macroscopic appearance. In agreement with the findings of Korn & Northcote (1960) for bakers' yeast cell walls, phase-contrast microscopy revealed that fraction C of normal and deficient walls retained the shape of the original cell wall.

Table 1. *Fractionation of cell walls with ethylenediamine*

Fractions A, B and C were isolated after incubation of cell walls with ethylenediamine for three days at 37°. Yields in g./100 g. cell walls.

	Solubility		Yield	
Fraction	In water	In ethylene-diamine	Normal cell walls	Deficient cell walls
A	+	+	23·7	23·4
B	−	+	10·4	13·4
C	−	−	59·9	52·5

Table 2. *Carbohydrate content of cell walls and fractions*

Total hexose was estimated by the anthrone method, glucose by glucose oxidase. The values for mannose were obtained by difference as described under Methods. Hexosamine was estimated by the method of Cessi & Piliego (1960). Values are expressed in g./100 g. sample.

	Fraction A		Fraction B		Fraction C		Unfractionated cell walls	
	Normal	Deficient	Normal	Deficient	Normal	Deficient	Normal	Deficient
Total hexose	59·0	56·7	69·9	84·0	75·7	70·3	69·1	63·3
Glucose	0·0	0·0	14·7	76·4	57·4	68·5	37·5	47·9
Mannose	59·0	56·7	55·2	7·6	18·3	1·8	31·6	15·4
Hexosamine	0·4	0·7	< 0·1	1·4	1·0	7·2	0·7	4·2

Carbohydrate composition of cell walls and fractions

Chromatographic analysis of the hydrolysates of normal and of deficient cell wall fractions showed that mannose was the only hexose present in fractions A whereas both mannose and glucose were detected in fractions B and C.

The results of quantitative analysis of carbohydrates are shown in Table 2. The mannose content of normal and deficient fractions A was very similar. Deficient fractions B and C, however, contained less mannose, but more glucose than the corresponding normal fractions. Overall, the total hexose content of deficient cell walls was only slightly less than that of normal walls. The values obtained in the

present study for the mannose and glucose content of normal cell walls agree well with those obtained by Northcote & Horne (1952) for bakers' yeast cell walls. Unfractionated deficient cell walls contained very much more amino sugar than normal cell walls. This fact is reflected in the amino sugar content of deficient cell wall fractions all of which contained more hexosamine than the corresponding normal fractions.

Table 3. *The protein content of cell walls and fractions*

The values given represent (total nitrogen-hexosamine nitrogen) × 6·25. Values are expressed in g. protein/100 g. sample. Total nitrogen was estimated by the micro-Kjeldahl procedure.

	Normal	Deficient
Fraction A	30·4	29·6
Fraction B	31·2	6·8
Fraction C	8·9	5·9
Unfractionated cell walls	15·9	11·1

Table 4. *The amino acid composition of cell wall proteins*

Values in μmole/100 mg. cell wall protein.

	Normal cell walls	Deficient cell walls
Aspartic acid	52·5	80·8
Threonine	105·8	166·3
Serine	110·4	151·8
Glutamic acid	56·4	93·8
Proline	37·1	56·0
Glycine	44·8	62·0
Alanine	84·6	115·3
Valine	55·6	59·6
Cystine	2·5	1·7
Methionine	2·6	Trace
iso-Leucine	40·0	39·8
Leucine	47·9	37·8
Tyrosine	14·5	14·1
Phenylalanine	20·7	22·2
Lysine	44·3	39·8
Histidine	18·4	8·6
Arginine	15·8	9·6
Ammonia	63·9	79·3

The protein content of cell walls and fractions

Deficient cell walls and fractions all contained less protein than the corresponding normal materials (Table 3). This overall decrease was most marked in the case of deficient fraction B which contained 24·4% less protein than normal fraction B.

Quantitative amino acid analysis (Table 4) revealed striking differences in the protein composition of normal and deficient cell walls. Thus, the deficient cell wall protein was much richer in threonine, serine, glutamic and aspartic acids, alanine, glycine and proline, but contained less lysine, histidine, and arginine than normal cell wall protein.

Lipid analyses

Normal and deficient cell walls contained the same percentage of lipid. The fatty acids present in normal and in deficient cell wall lipids were predominantly unsaturated

(Table 5). The major components palmitoleic and oleic acids comprised approximately the same percentage of the fatty acids of normal and deficient cell walls.

Phosphorus content of cell walls and fractions

The phosphorus content of the deficient cell wall and of each of the deficient cell wall fractions was less than that of the normal materials (Table 6).

Table 5. *Identity and percentage composition of fatty acids from cell wall lipids*

Analyses were carried out at 175° on a 5% polyethylene glycol adipate column. Gas flow 45 ml./min.. Pressure 15 lb./in^2.

Identity of fatty acid		Total fatty acid (%)	
Shorthand designation	Familiar name	Normal lipid	Deficient lipid
12:0	Lauric	0·4	0·6
13:0	—	0·5	0·3
Unidentified		0·3	0·3
14:0	Myristic	0·7	1·2
Unidentified		0·7	2·1
15:0	—	Trace	Trace
Unidentified		0·2	0·3
16:0	Palmitic	16·7	14·4
16:1	Palmitoleic	42·4	42·1
18:0	Stearic	4·3	4·9
18:1	Oleic	33·7	33·8

Table 6. *The phosphorus content of cell walls and fractions*

Values in μg./mg. sample.

	Normal	Deficient
Fraction A	7·4	6·1
Fraction B	3·2	2·0
Fraction C	1·4	1·1
Unfractionated cell walls	3·8	2·7

DISCUSSION

Growth of *Saccharomyces cerevisiae* W.S. in media without inositol resulted in the formation of an aberrant cell wall which contained less mannose but more glucose than normal cell walls. This finding is consistent with the suggestion (Chung & Nickerson, 1954) that inositol is required for the interconversion of mannose and glucose phosphates which are important intermediates in the biosynthesis of mannan and glucan from glucose. Gosh, Charalampous, Sison & Borer (1960) reported that a strain of *S. carlsbergensis* when grown in inositol-deficient media formed an abnormal cell wall containing greatly increased amounts of glucan. However, no significant change in mannan content occurred with this yeast.

Inositol-deficiency results in a large increase in the whole cell lipid content of *Saccharomyces cerevisiae* W.S. (Challinor & Daniels, 1955). It is surprising, therefore, that the content and fatty acid composition of the normal and deficient cell wall lipids were found to be so similar.

The cell wall of inositol-deficient yeast contained less protein than the normal cell wall. Furthermore, determination of the amino acids present showed that deficient cell wall protein was quite different in composition. Kessler & Nickerson (1959) proposed that combination between the two moieties of polysaccharide-protein complexes is effected by esterification involving the carbonyl groups of the protein and the hydroxyl groups of the polysaccharide. Thus, the large increase in concentration of aspartic and glutamic acids in deficient yeast cell wall protein possibly reflects an essential requirement for these amino acids to maintain the integrity of the cell wall. Glucosamine may play a part in binding polysaccharide-protein complexes in the yeast cell wall (Eddy, 1958; Korn & Northcote, 1960). In the present study, a large increase in hexosamine was found in the deficient cell wall, and it is possible that this increase compensates in part for the relatively low content of the basic amino acids lysine, histidine and arginine. Eddy & Williamson (1959) showed that yeast protoplasts could give rise to a superficial membrane which somewhat resembled the intact cell wall but which contained N-acetyl glucosamine as the principal nitrogenous material rather than amino acid residues.

The electron micrographs of several workers including Northcote & Horne (1952), Agar & Douglas (1955) and Bartholomew & Levin (1955), suggest that the yeast cell wall is a layered structure. Although the chemical composition of the individual layers is unknown, mannan occupies a large proportion of the surface of the wall (Eddy, 1958). The work of Millbank & Macrae (1964) supports the view that the mannan-protein complex overlays the glucan. Thus, it is reasonable to assume that fractions A isolated here with ethylenediamine represent part of the surface of the intact cell wall. The marked similarity in composition between normal and deficient fractions A probably reflects therefore an attempt on the part of the deficient cell to maintain the surface regions of the wall intact under adverse growth conditions. It is generally agreed that the mannan and protein components of the cell fabric are intimately associated. The ratio of mannose to protein found here is constant (approx. 2) for the normal cell wall and each of the fractions A, B and C isolated from it. However, only A of the deficient wall fractions showed a mannose to protein ratio of 2. This observation underlines the similarity between normal and deficient fractions A.

On the other hand, deficient fractions B and C are very different in composition from corresponding normal cell wall fractions. Large decreases in the mannose and protein content of these fractions are accompanied by equally marked increases in glucose and hexosamine. There is no doubt that such changes result in a much weakened cell wall as indicated by the relative ease of rupture of the deficient cell. However, a complete interpretation of these findings must await a more precise knowledge of the structure of the normal cell wall.

REFERENCES

AGAR, H. D. & DOUGLAS, H. C. (1955). Studies of budding and cell wall structure of yeast-electron microscopy of thin sections. *J. Bact.* **70**, 427.

BARTHOLOMEW, J. W. & LEVIN, R. (1955). The structure of *Saccharomyces carlsbergensis* and *S. cerevisiae* as determined by ultra-thin sectioning methods and electron microscopy. *J. gen. Microbiol.* **12**, 473.

CESSI, C. & PILIEGO, F. (1960). Determination of aminosugars in presence of amino acids and glucose *Biochem. J.* **77**, 508.

CHALLINOR, S. W. & DANIELS, N. W. R. (1955). Fat production by inositol-deficient yeast. *Nature, Lond.* **176**, 1267.

CHALLINOR, S. W., DANIELS, N. W. R. & HALL, J. C. (1958). Recent studies in yeast and their significance in industry. *Soc. Chem. Indust. Mon.* No. 3.

CHALLINOR, S. W., POWER, D. M. & TONGE, R. J. (1964). Effects of inositol-deficiency on yeast, with particular reference to chemical composition of the cell and of the cell wall. *Nature, Lond.* **203**, 250.

CHIBNALL, A. C., REES, M. W. & WILLIAMS, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354.

CHUNG, W. & NICKERSON, W. J. (1954). Polysaccharide synthesis in growing yeasts. *J. biol. Chem.* **208**, 395.

DRYER, R. L., TAMMES, A. R. & ROUTH, J. I. (1957). The determination of phosphorus and phosphatase with N-phenyl-p-phenylene diamine. *J. biol. Chem.* **225**, 177.

EDDY, A. A. (1958). The structure of the yeast cell wall. II. Degradative studies with enzymes. *Proc. Roy. Soc.* B **149**, 435.

EDDY, A. A. & WILLIAMSON, D. H. (1959). Formation of aberrant cell walls and of spores by the growing yeast protoplast. *Nature, Lond.* **183**, 1101.

EMERY, W. B., MCLEOD, N. & ROBINSON, F. A. (1946). Comparative microbiological assays of members of the vitamin B complex in yeast and liver extracts. *Biochem. J.* **40**, 426.

FALCONE, G. & NICKERSON, W. J. (1956). Cell wall mannan-protein of baker's yeast. *Science N.Y.* **124**, 272.

FOLCH, J., LEES, M. & SLOANE-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.* **226**, 497.

GOSH, A., CHARALAMPOUS, F. C., SISON, Y. & BORER, R. (1960). Metabolic functions of myo-inositol. I. Cytological and chemical alterations in yeast resulting from inositol deficiency. *J. biol. Chem.* **235**, 2522.

HOUWINK, A. L. & KREGER, D. R. (1953). Cell wall of yeasts—electron microscope and X-ray diffraction. *Antonie van Leeuwenhoek* **19**, 1.

KESSLER, G. & NICKERSON, W. J. (1959). Glucomannan-protein complexes from cell walls of yeasts. *J. biol. Chem.* **234**, 2281.

KORN, E. C. & NORTHCOTE, D. H. (1960). Physical and chemical properties of polysaccharides and glycoproteins of the yeast cell wall. *Biochem. J.* **75**, 12.

MILLBANK, J. W. & MACRAE, R. M. (1964). Degradation of yeast cell wall by fractionated snail-gut enzyme. *Nature, Lond.* **201**, 1347.

MOORE, S., SPACKMAN, D. H. & STEIN, W. H. (1958). Chromatography of amino acids on sulphonated polystyrene resins. *Analyt. Chem.* **30**, 1185.

NORTHCOTE, D. H. & HORNE, R. W. (1952). The chemical composition and structure of the yeast cell wall. *Biochem. J.* **51**, 232.

ROELOFSEN, P. A. (1953). Yeast mannan, a cell wall constituent of bakers' yeast. *Biochim. biophys. Acta* **10**, 477.

ROELOFSEN, P. A. & HOETTE, I. (1951). Chitin in the cell wall of yeasts. *Antonie van Leeuwenhoek* **17**, 297.

SMITH, I. (1960). *Chromatographic and Electrophoretic Techniques*, 2nd Ed., by I. Smith. London: William Heinemann.

STOFFEL, W., CHU, F. & AHRRENS, E. H. (1959). Analysis of long-chain fatty acids by gas–liquid chromatography-micromethod for preparation of methyl esters. *Analyt. Chem.* **31**, 307.

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Multivesicular Bodies in *Sclerotinia fructigena* and their Possible Relation to Extracellular Enzyme Secretion

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SUMMARY

The occurrence of multivesicular bodies (m.v. bodies) in *Sclerotinia fructigena*, both *in vivo* and *in vitro*, was studied by electron microscopy. This investigation was an attempt to correlate the existence of m.v. bodies with extracellular enzyme production. It was observed that when the fungus grew in its natural hosts or in media containing pectin or sodium pectate, where extracellular pectolytic enzyme secretion was high, m.v. bodies were present in the hyphae. However, when the fungus grew in synthetic media without pectin or sodium pectate, these m.v. bodies were absent, and the extracellular enzyme activity was very low. Multivesicular bodies in *S. fructigena* seemed to originate from the endoplasmic reticulum, moving later to the periphery of the cell.

A new histochemical technique was used at the electron microscope level; this provided supporting evidence that m.v. bodies are the cytoplasmic constituents involved in extracellular enzyme secretion by this fungus.

INTRODUCTION

Since the first observation by Girbardt (1958) on vesicular structures associated with the plasmalemma in *Polyporus versicolor*, several reports have been published on such organelles in different organisms. In 1959, Glauert & Hopwood described membranous structures in the vicinity of growing cross-walls of bacteria, interpreting them as organelles responsible for cell-wall synthesis. Moore & McAlear (1961) reported similar vesicles in other fungi, which they termed 'lomasomes'. Since then, lomasome-like bodies have been described in *Botrytis cinerea* (Hawker & Hendy, 1963; Pitt, 1968); in *Peronospora manshurica* (Peyton & Bowen, 1963); in *Albugo candida* (Berlin & Bowen, 1964); in *Puccinia graminis tritici* (Shaw & Manocha, 1965) and in *Phytophthora infestans* and *P. parasitica* (Ehrlich & Ehrlich, 1966). Lomasome-like bodies have also been reported in algae (Barton, 1965; Crawley, 1965). Their occurrence in higher plants has been described by Manocha & Shaw (1964), and reviewed by Esau, Cheadle & Gill (1966). The latter termed these structures 'boundary bodies', which were always found between plasmalemma and cell wall, but never in the protoplast.

The name 'multivesicular bodies' has been given to certain small vesicles limited by a membrane, itself 50 to 60 Å thick, present in the rat ovum (Sotelo & Porter, 1959).

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Marchant, Peat & Banbury (1967) postulated multivesicular (m.v.) bodies as structures present in the protoplast, from which lomasomes are formed, and having their origin in the endoplasmic reticulum. However, it is difficult to verify these conclusions from the published micrographs. Structures resembling multivesicular bodies were found in the alga *Chlamydomonas reinhardi* (Sager & Palade, 1957) and similar bodies have also been described in higher plants by Walker & Bisalputra (1967).

In the course of work on the effects of *Sclerotinia fructigena* on host tissues, it was noticed that multivesicular bodies were present in great numbers in the invading hyphae; this observation encouraged further investigations *in vitro* on the possible origin and physiological significance of these structures, including an attempt to detect localized enzyme secretion from them.

MATERIALS AND METHODS

The fungus *Sclerotinia fructigena* Aderh. & Ruhl. used for these experiments was isolated by H. J. Willetts from mummified apple fruits collected at Long Ashton Research Station in February 1967.

A monosporic culture was made on potato glucose agar (peeled potato tissue, 200 g.; glucose, 30 g.; agar, 20 g.; to 1 l. with distilled water), and maintained either on this medium or on V-8 medium. This comprised V-8 vegetable juice (Campbell's Soups Ltd.), 200 ml.; agar, 30 g.; to 1 l. with distilled water, in large boiling tubes, incubated at 25°.

The *in vivo* experiments were done by means of inoculations on healthy ripe apple and pear fruits, previously kept in a 25° incubation room for a few days.

The media used for the *in vitro* experiments were: (1) Glucose, 0·2g.; asparagine, 0·2 g.; $MgSO_4 . 7H_2O$, 0·075 g.; K_2HPO_4, 0·125 g.; malic acid, 0·5 g.; distilled water to 100 ml. and the pH adjusted to 4·5 by the addition of N-NaOH. For use as a solid medium, 3% (w/v) agar was added. (2) The V-8 juice-agar as described above. (3) Apple extract media. Apple fruitlets picked in July (20 g.) were stewed to a pulp with 30 ml. distilled water, strained through muslin and the extract made up to 50 ml. For use as a solid medium, an equal quantity of 4% sterilized agar was added after sterilization of the extract. (4) A sodium pectate medium used for pectolytic enzyme production (Byrde & Fielding, 1968). (5) Pectin (Brown Ribbon brand, Union Crystalex Gelatine Ltd., London) (2%, w/v) in distilled water, without mineral salts. (6) Sodium polypectate (Exchange Lemon Products Co., California) (2%, w/v) in distilled water, without mineral salts.

The liquid media were dispensed in 4 oz. medicine bottles, and the solid media into boiling tubes in 16 ml. lots, the tubes being sloped after sterilization.

The inoculum consisted of small mycelial samples from the stock culture.

In all the experiments, both *in vivo* and *in vitro*, incubation was at 25°.

Electron microscopy

Material for ultrastructural studies was collected from infected fruit, 4 days after inoculation; in the *in vitro* experiments from 2-day-old cultures grown in media 1 and 3, from a 7-day-old culture in medium 2, from an 11-day-old culture in medium 4, and from an 8-day-old culture in media 5 and 6.

In all these cultures the fungus was fully grown when harvested. Samples of mycelia from these sources were fixed by one of the following methods:

(1) 6% Glutaraldehyde (Sabatini, Bensch & Barrnett, 1963) in 0·1 M-phosphate buffer (pH 7·0) for 24 hr at 4° followed by thorough washing in the same buffer solution, and post-fixed in 2% OsO_4 (in the same buffer) for 4 hr at 4°.

(2) 2% $KMnO_4$ unbuffered (Luft, 1956), for 30 min. at room temperature, followed by washing in distilled water and staining in 0·5% aqueous uranyl acetate for 3 days at room temperature (Hess, 1966).

The material in both cases was dehydrated in a graded ethanol series and embedded either in Epon 812 or in Araldite mixtures (Luft, 1961). Sections were cut on an LKB Ultratome and stained with lead citrate (Reynolds, 1963); observations were then made with an AEI EM6B electron microscope.

The fixation of the material by the first method was generally better than with 2% $KMnO_4$. Nevertheless, this second method of fixation gave useful confirmatory information on the distribution of membranes and lipid structures within the cell.

Enzyme estimations

Polygalacturonase (PG) was estimated at pH 4·7 by the cup-plate assay of Dingle, Reid & Solomons (1953) with sodium polypectate as substrate. The diameter of the white ring obtained on the agar plate was proportional to the logarithm of enzyme concentration over a range of dilutions. Activities were expressed relative to an aqueous solution (1 mg./ml.) of the commercial enzyme Pectinol 10 M (Rohm and Haas Ltd.), defined arbitrarily as having 100 units (u.) activity/ml.

Arabinofuranosidase (AF) and pectin methyl-*trans*-eliminase (PTE) were estimated at pH 4·7 and 7·0, respectively, by the methods described by Byrde & Fielding (1968); these pH values are approximately optimal.

Histochemical techniques

An attempt was made to detect localized enzyme secretion by a histochemical method involving the enzyme α-L-arabinofuranosidase. This enzyme, known to be secreted by *Sclerotinia fructigena* in media containing sodium pectate, is suitable because hydrolysis of the phenylglycoside liberates phenol which reacts with a ferric chloride–potassium ferricyanide mixture to give a dark blue electron-dense material.

Fresh samples of mycelium were incubated in an aqueous solution of the substrate, phenyl α-L-arabinofuranoside (Börjeson, Jerkeman & Lindberg, 1963; Byrde & Fielding, 1965), 5 mg./ml., for various times at room temperature. The material was then thoroughly washed in distilled water, and briefly immersed in a solution containing equal volumes of 1% potassium ferricyanide and 1% ferric chloride; a final washing in water was made before fixation for electron microscopy as described above. Control samples were subjected to the same procedure, omitting the initial incubation with phenyl α-L-arabinofuranoside.

RESULTS

Experiments in vivo. When grown on pear fruits, the ultrastructure of hyphae of *Sclerotinia fructigena* showed numerous multivesicular bodies (m.v. bodies). These bodies were usually located in the vicinity of the cell wall and septum in close associa-

tion with the plasmalemma (Pl. 1, fig 1, 2) and less frequently inside the protoplast (Pl. 1, fig. 3). An observation on such bodies at higher magnification indicated that their internal vesicles are connected to each other and with the plasmalemma by means of thin threads (Pl. 2, fig. 4). These vesicles had an average diameter of 500 to 2000 Å and presented a more or less spherical shape with granular material inside. They were surrounded by a unit membrane about 60 Å thick.

The m.v. bodies were not so numerous when the fungus was grown on apple (Pl. 2, fig. 5), but the general appearance of the ultrastructure of the hyphae was similar.

Experiments in vitro. No typical m.v. bodies, as described above, were observed in *Sclerotinia fructigena* when the fungus was grown on either solid or liquid defined medium, with glucose and asparagine as the sole carbon and nitrogen sources (Pl. 2, fig. 6). The hyphal ultrastructure was seen to consist of ribosomes and glycogen with some vacuoles.

No m.v. bodies were observed in *Sclerotinia fructigena* hyphae grown on V-8 juice agar medium. Plate 3, fig. 7, shows a similar feature to that already described in Pl. 1, fig. 2, but while m.v. bodies could clearly be seen close to the septum in the latter, none appeared in the former. Plate 3, fig. 7, also shows a nucleus at the moment of crossing the septal pore. The ultrastructure of this material at higher magnification showed some kind of vacuoles with amorphous material, perhaps from abortive m.v. bodies (Pl. 3, fig. 8), and also unusual tubular structures (tubules of about 200 Å diameter) composed of a membrane similar in thickness to the plasmalemma, in which the 'hole' seemed to be of about 70 Å diameter (arrow).

The same material as in Pl. 3, fig. 8, but fixed with permanganate, showed no traces of m.v. bodies (Pl. 3, fig. 9), nor were m.v. bodies observed when *Sclerotinia fructigena* was grown on potato glucose agar with added apple extract (Pl. 3, fig. 10). The ultrastructure of the vegetative hyphae in this case was similar to that presented in Pl. 2, fig. 6, when the fungus grew on glucose asparagine medium.

When the fungus was cultured on a medium with sodium pectate, m.v. bodies could be observed (Pl. 4, fig. 11 to 14); however, these bodies presented differences in structure and were less numerous than in the hyphae from the *in vivo* experiments. Plate 4, fig. 11 and 12, also suggest the possible origin of m.v. bodies from the endoplasmic reticulum.

Finally, the ultrastructure of the hyphae maintained in 2% pectin or sodium pectate alone showed m.v. bodies of similar appearance to those described in the previous case (Pl. 5, fig. 15, 16).

In attempts to elucidate the function of m.v. bodies as possible sources of enzyme secretion, hyphae from medium 4 were examined after treatment by the methods described above. Electron-dense areas appeared to be located in the vicinity of the cell wall, sometimes showing a structure resembling the original m.v. bodies (Pl. 6, fig. 17), although the general preservation of the cytoplasmic components was not well maintained by this method. Similar deposition of electron-dense material occurred outside the cell wall (Pl. 6, fig. 18).

The appearance of control hyphae was markedly different (Pl. 6, fig. 19) showing a few spots only in the cytoplasm but nothing in the proximity of the cell wall.

Table 1 records the enzyme activities in various media.

Table 1. *Enzyme activity of culture filtrates and formation of multivesicular bodies by S. fructigena grown on various media*

Source of extract	PTE (ΔE_{240}/ml./hr)	PG (u.)	AF (ΔA/ml./hr)	pH (after growth)	M.v. bodies*
Rotted apple tissue (dialysed 5% NaCl extract) (1 ml./g. fresh wt)	0	36	0·06	3·8	+
Medium 1 liquid	0	4	0·04	4·3	−
1 solid†	0	32	0	4·6	−
2 solid†	0	8	0·05	4·8	−
3 liquid	0	5	0·38	3·7	−
3 solid†	0	0	0·12	4·1	−
4 liquid	4·05	316	2·93	7·0	+
5 liquid‡	0	8	0·01	3·4	+
6 liquid	0	128	0·19	5·0	+

* Multivesicular bodies; +, present; −, absent.
† After aqueous extraction (1:1). Values corrected for dilution.
‡ In this medium the total growth of mycelium was very small, relative to the volume of medium.

DISCUSSION

Literature on vesicular structures in plant organisms at present gives a variety of interpretations concerning their possible origin. Wardrop & Foster (1964) suggested that vesicles, present in the protoplast of epidermal cells of oat coleoptile, seemed to be secreted by the Golgi complex because of their lipid nature. Membranous organelles such as vacuoles and m.v. bodies could be formed by the utilization of stored phospholipids in germinating conidia (Buckley, Sjholm & Sommer, 1966; Marchant, 1966). The results obtained during this investigation with *Sclerotinia fructigena* support the evidence that m.v. bodies originate from the endoplasmic reticulum (Pl. 4, fig. 11, 12) as has recently been reported in other fungi (Marchant *et al.* 1967). Thus it is possible that the m.v. bodies so originated may move towards the cellular periphery by a type of reverse pinocytosis and there empty its content outside the membrane, after fusion with the plasmalemma. This is one of the hypotheses suggested by Lampen (1968) to explain the possible mechanisms of external enzyme formation in yeast.

The views on the physiology of these vesicle-like structures are that they are involved in the synthesis of cell-wall material in the different organisms studied (Glauert & Hopwood, 1959; Crawley, 1965; Esau *et al.* 1966; Walker & Bisalputra, 1967; Marchant *et al.* 1967). Multivesicular bodies appear to be located, in general, near the plasmalemma and cell wall (Fig. 1–5) when they are present in the vegetative hyphae. No traces of m.v. bodies were observed in macro or microconidia of *S. fructigena* (Willetts & Calonge, 1969).

Apart from their suggested role in cell-wall synthesis, membranous structures in *Streptomyces coelicolor* (Glauert & Hopwood, 1959) and tubular organelles in Chara (Barton, 1965) are thought to be associated with some specific enzyme activity and they may be sites of extracellular enzyme synthesis in several fungi (Marchant *et al.* 1967). In *Botrytis cinerea*, Pitt (1968) demonstrated the location of several acid and neutral hydrolases in cytoplasmic particles by means of histochemical methods. Very recently,

m.v. bodies have been presumed to be concerned in protein turnover in an insect (Locke & Collins, 1968) and it has also been shown that the red alga Laurencia can produce a type of vesicle which is able to cross the cell wall (Bisalputra, Rusanowski & Walker, 1967).

Evidence presented above indicates a similar role for m.v. bodies in *Sclerotinia fructigena* for the following reasons. If the fungus is grown on certain synthetic media where extracellular PG secretion is very low, the bodies are absent in vegetative hyphae (Pl. 2, fig. 6; Pl. 3, fig. 7, 9, 10) and sometimes they appear aborted, or degenerated (Pl. 3, fig. 8). (Pl. 3, fig. 8 also shows the existence of certain tubular structures which could replace the function of the degenerated m.v. bodies). However, when the fungus is grown on host tissue or various pectic media in each of which extracellular PG enzyme secretion is known to occur (Table 1) m.v. bodies are present. Apart from anomalous results with autoclaved apple extract a similar pattern emerges for the relationship between AF activity and the presence of m.v. bodies. Moreover, preliminary histochemical examination of such fungal hyphae with the electron microscope gives a positive reaction in bodies which in some cases appear to resemble m.v. bodies. Further histochemical studies seem desirable, using a more specific reagent for the liberated phenol and which at the same time yields an electron-dense product.

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REFERENCES

BARTON, R. (1965). An unusual organelle in the peripheral cytoplasm of Chara cells. *Nature, Lond.* **205**, 201.

BERLIN, J. D. & BOWEN, C. C. (1964). The host-parasite interface of *Albugo candida* on *Raphanus sativus*. *Am. J. Bot.* **51**, 445.

BISALPUTRA, T., RUSANOWSKI, P. C. & WALKER, W. S. (1967). Surface activity, cell wall, and fine structure of pit connection in the red alga *Laurencia spectabilis*. *J. Ultrastruct. Res.* **20**, 277.

BÖRJESON, H., JERKEMAN, P. & LINDBERG, B. (1963). Phenyl glycofuranosides. 1. Synthesis of phenyl β-D-xylofuranoside and phenyl α-L-arabinofuranoside. *Acta chem. scand.* **17**, 1705.

BUCKLEY, P. M., SJHOLM, E. & SOMMER, N. F. (1966). Electron microscopy of *Botrytis cinerea* conidia. *J. Bact.* **91**, 2037.

BYRDE, R. J. W. & FIELDING, A. H. (1965). An extracellular α-L-arabinofuranosidase secreted by *Sclerotinia fructigena*. *Nature, Lond.* **205**, 39c.

BYRDE, R. J. W. & FIELDING, A. H. (1968). Petin methyl-*trans*-eliminase as the maceration factor of *Sclerotinia fructigena* and its significance in brown rot of apple. *J. gen. Microbiol.* **52**, 287.

CRAWLEY, J. C. W. (1965). A cytoplasmic organelle associated with the cell walls of Chara and Nitella. *Nature, Lond.* **205**, 200.

DINGLE, J., REID, W. W. & SOLOMONS, G. L. (1953). The enzymic degradation of pectin and other polysaccharides. II. Application of the 'cup-plate' assay to the estimation of enzymes. *J. Sci. Fd Agric.* **4**, 149.

EHRLICH, M. A. & EHRLICH, H. G. (1966). Ultrastructure of the hyphae and haustoria of *Phytophthora infestans* and hyphae of *P. parasitica*. *Can. J. Bot.* **44**, 1495.

ESAU, K., CHEADLE, V. I., & GILL, R. H. (1966). Cytology of differentiating tracheary elements. II. Structures associated with cell surfaces. *Am. J. Bot.* **53**, 765.

GIRBARDT, M. (1958). Über die Substruktur von *Polystictus versicolor*. *Arch. Mikrobiol.* **28**, 255.

GLAUERT, A. M. & HOPWOOD, D. A. (1959). A membraneous component of the cytoplasm in *Streptomyces coelicolor*. *J. biophys. biochem. Cytol.* **6**, 515.

HAWKER, L. E. & HENDY, R. J. (1963). An electron-microscope study of germination of conidia of *Botrytis cinerea*. *J. gen. Microbiol.* **33**, 43.

HESS, W. M. (1966). Fixation and staining of fungus hyphae and host plant root tissues for electron microscopy. *Stain Technol.* **41**, 27.

LAMPEN, J. O. (1968). External enzymes of yeast; their nature and formation. *Antonie van Leeuwenhoek* **34**, 1.

LOCKE, M. & COLLINS, J. V. (1968). Protein uptake into multivesicular bodies and storage granules in the fat body of an insect. *J. Cell Biol.* **36**, 453.

LUFT, J. H. (1956). Permanganate: a new fixative for electron microscopy. *J. biophys. biochem. Cytol.* **2**, 799.

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.* **9**, 409.

MANOCHA, M. S. & SHAW, M. (1964). Occurrence of lomasomes in mesophyll cells of 'Khapli' wheat. *Nature, Lond.* **203**, 1402.

MARCHANT, R. (1966). Fine structure and spore germination in *Fusarium culmorum*. *Ann. Bot.* **30**, 441.

MARCHANT, R., PEAT, A. & BANBURY, G. H. (1967). The ultrastructural basis of hyphal growth. *New Phytol.* **66**, 623.

MOORE, R. T. & MCALEAR, J. H. (1961). Fine structure of mycota. V. Lomasomes—previously uncharacterised hyphal structures. *Mycologia* **53**, 194.

PEYTON, G. A. & BOWEN, C. C. (1963). The host-parasite interface of *Peronospora manshurica* on *Glycine max*. *Am. J. Bot.* **50**, 787.

PITT, D. (1968). Histochemical demonstration of certain hydrolytic enzymes within cytoplasmic particles of *Botrytis cinerea* Fr. *J. gen. Microbiol.* **52**, 67.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. biophys. biochem. Cytol.* **17**, 208.

SABATINI, D. D., BENSCH, K. & BARRNETT, R. J. (1963). Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**, 19.

SAGER, R., & PALADE, G. E. (1957). Structure and development of the chloroplast in Chlamydomonas. *J. biophys. biochem. Cytol.* **3**, 463.

SHAW, M. & MANOCHA, M. S. (1965). The physiology of host-parasite relations. XV. Fine structure in rust-infected wheat leaves. *Can. J. Bot.* **43**, 1285.

SOTELO, J. R. & PORTER, K. R. (1959). An electron-microscope study of the rat ovum. *J. biophys. biochem. Cytol.* **5**, 327.

WALKER, W. S. & BISALPUTRA, T. (1967). The fine structure of vesicles associated with the cell surface in Helianthus shoot tissue. *Can. J. Bot.* **45**, 2103.

WARDROP, A. B. & FOSTER, R. C. (1964). A cytological study of the oat coleoptile. *Aust. J. Bot.* **12**, 135.

WILLETTS, H. J. & CALONGE, F. D. (1969). Spore development in the brown rot fungi (*Sclerotinia* spp.). *New Phytol.* **67** (in the Press).

EXPLANATION OF PLATES

Key to symbols: E.R. = endoplasmic reticulum, H = hypha, H.C. = host cell, M = mitochondrion, M.V.B. = multivesicular body (or bodies), N = nucleus, NU = nucleolus, P = plasmalemma, S = septum, V = vacuole, VE = vesicle, W = wall.

PLATE 1

Fig. 1. Longitudinal section of *Sclerotinia fructigena* hypha growing in pear. M.V.B. are present (arrows). × 9,000.

Fig. 2. Section showing several M.V.B. in the vicinity of the septum. × 20,000.

Fig. 3. M.V.B. with abundant vesicles filled with a granular material. × 120,000.

PLATE 2

Fig. 4. Multivesicular bodies at higher magnification. The vesicles inside are attached to the plasmalemma (arrows). × 100,000.

Fig. 5. Part of a hypha of *Sclerotinia fructigena* growing in apple. Two M.V.B. can be seen among cellular organelles. × 20,000.

Fig. 6. Ultrastructural feature of two hyphae of *Sclerotinia fructigena* grown in glucose asparagine medium. No M.V.B. can be observed. × 34,500.

PLATE 3

Fig. 7. Longitudinal section of a hypha grown in 'V-8' agar medium. No M.V.B. can be seen (cf. with fig. 2). A nucleus is crossing the septal pore. × 15,000.

Fig. 8. Part of a hypha grown on 'V-8' medium. Some tubular structures are present (arrows). There are several vacuoles with an amorphous content with slight resemblance to aborted M.V.B. × 90,000.

Fig. 9. The same material as in fig. 8. but fixed with $KMnO_4$. × 20,000.

Fig. 10. Transverse section of a hypha grown in apple extract liquid medium. M.V.B. are absent. × 20,000.

PLATE 4

Fig. 11. *Sclerotinia fructigena* hypha grown in a liquid medium with sodium pectate. This cross-section shows some areas of enlarged endoplasmic reticulum with vesicles inside (arrows), resembling those of M.V.B. $KMnO_4$ fixation. × 40,000.

Fig. 12. Section from similar material as in fig. 11 suggesting the formation of M.V.B. from the endoplasmic reticulum. $KMnO_4$ fixation. × 69,000.

Fig. 13, 14. Different areas from the same material as in fig. 11, and 12, showing several M.V.B. dispersed in the cytoplasm. $KMnO_4$ fixation. Both × 60,000.

PLATE 5

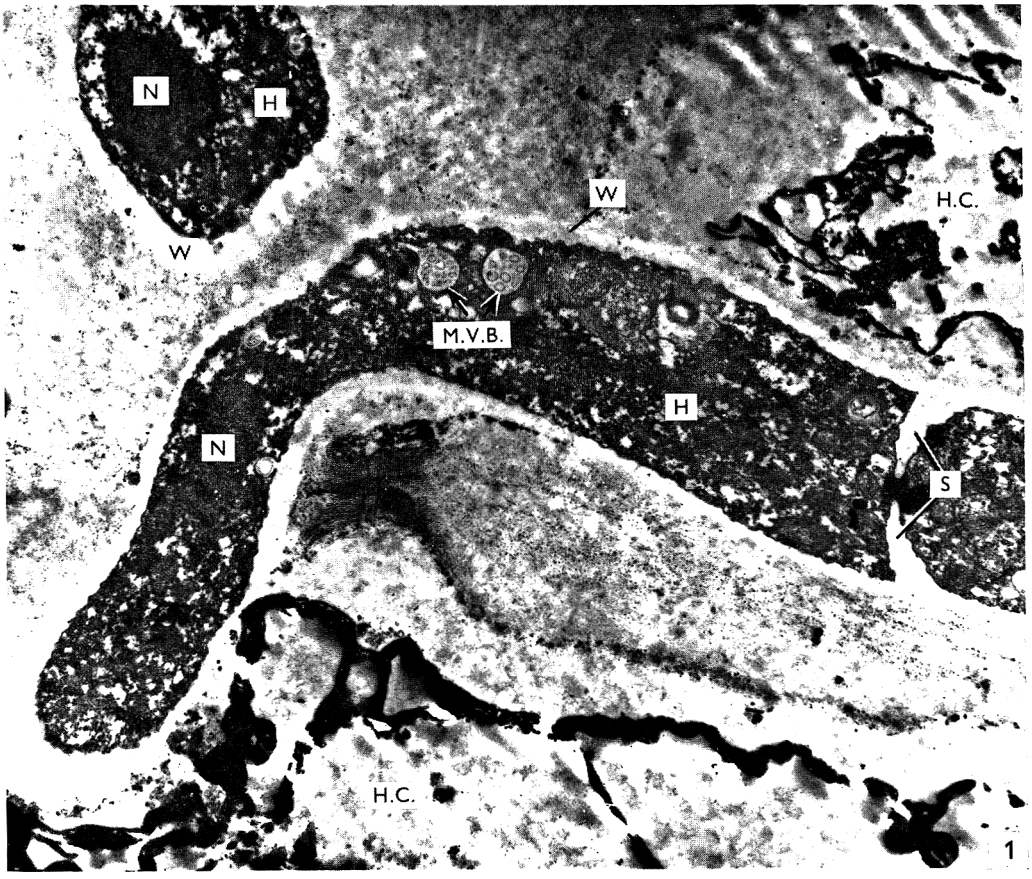
Fig. 15. Section of part of a hypha grown in 2% pectin in distilled water. An M.V.B. can be seen near the plasmalemma with numerous vesicles inside. × 62,500.

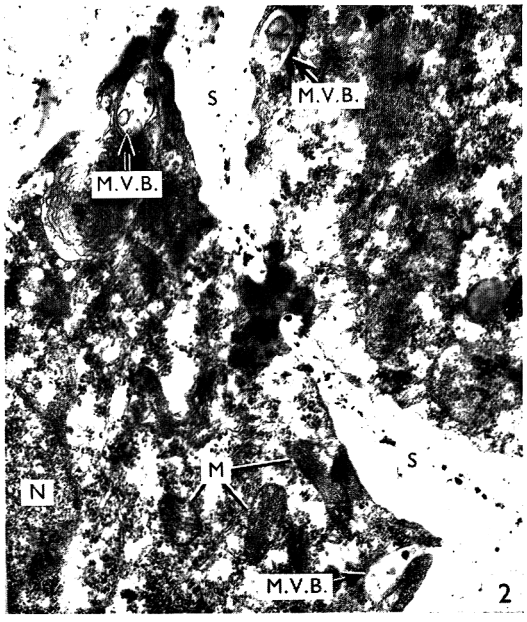
Fig. 16. Longitudinal section of part of a cell at the septum level. An M.V.B. is present near the plasmalemma. × 69,000.

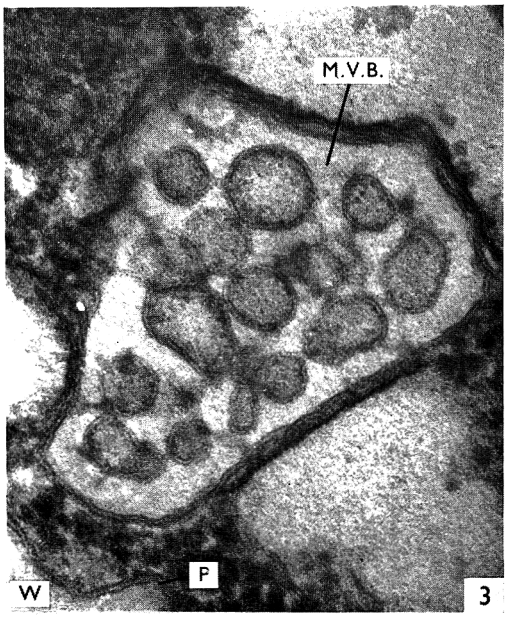
PLATE 6

Fig. 17, 18. Transverse sections of hyphae grown in the sodium pectate medium (no. 4) stained for enzyme detection. The black electron-dense areas, corresponding to the Fe deposition, are located both at the periphery of the cell and outside the wall (arrows). × 60,000 and 45,000 respectively.

Fig. 19. Longitudinal section of a hypha from the same source as in fig. 17 and 18. (Control). × 92,000.







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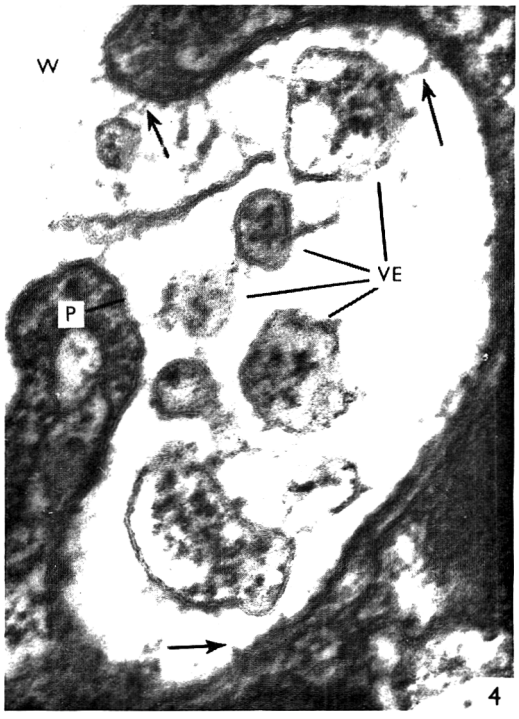

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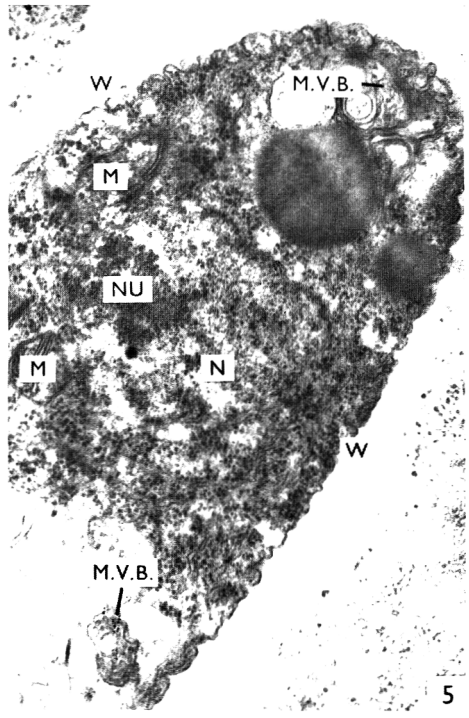

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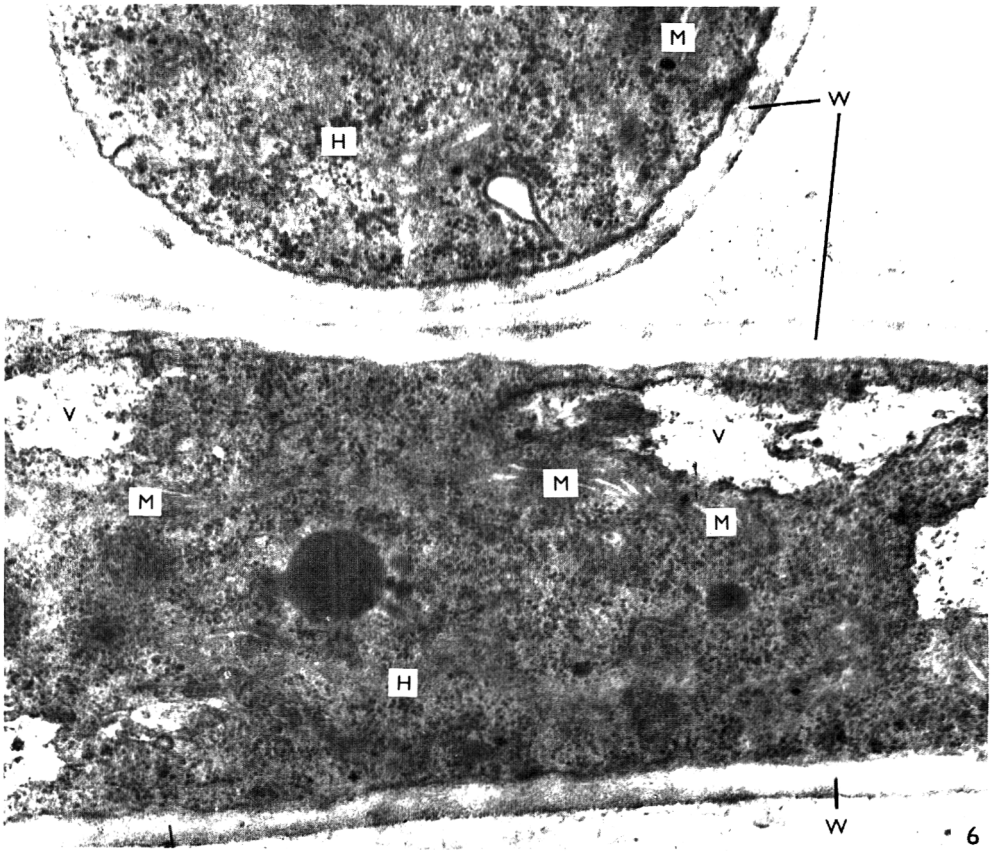

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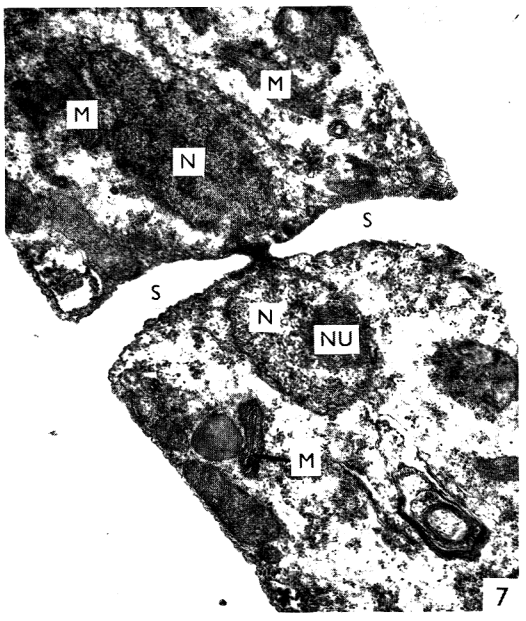
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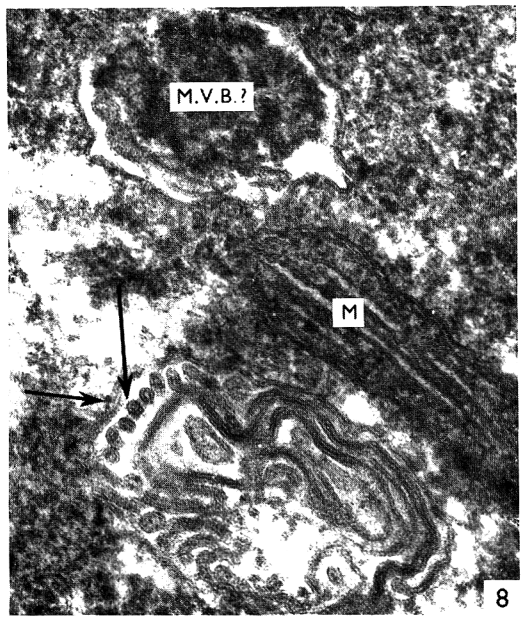
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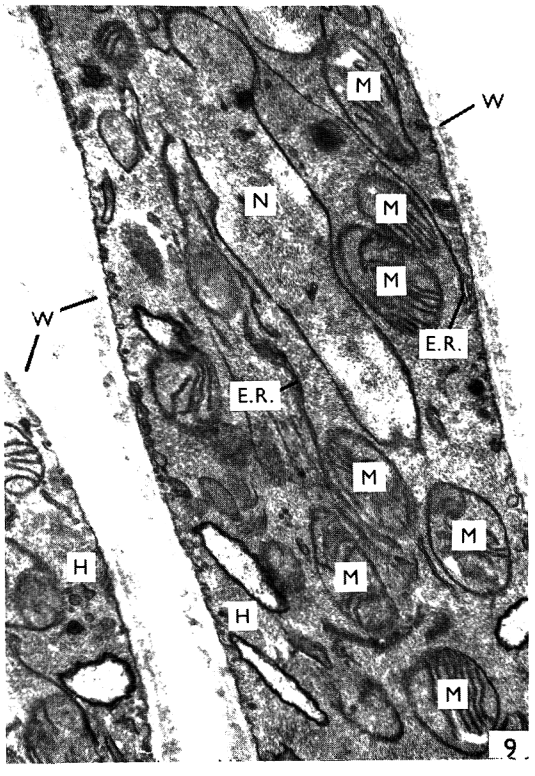
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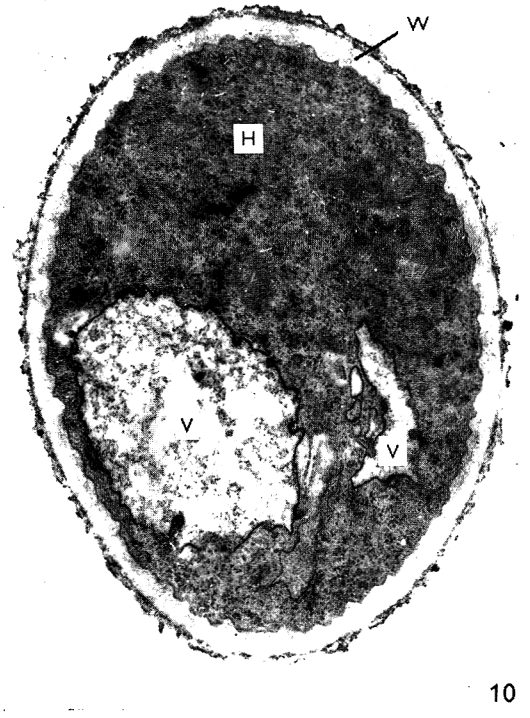
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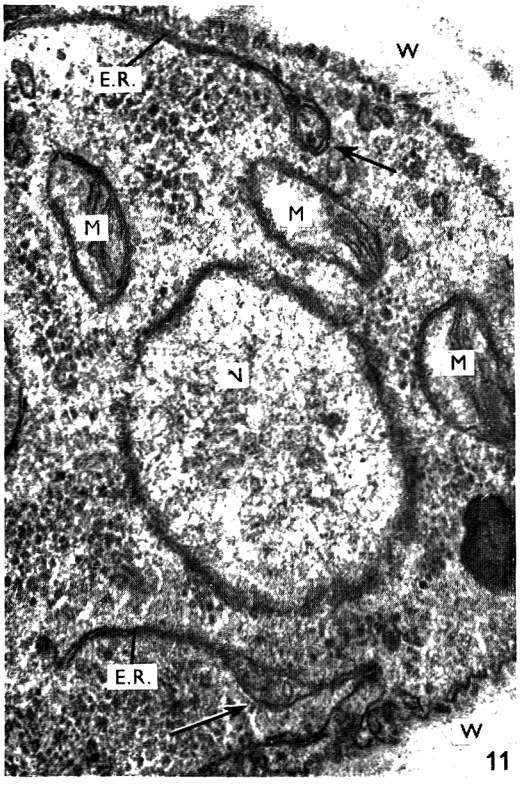

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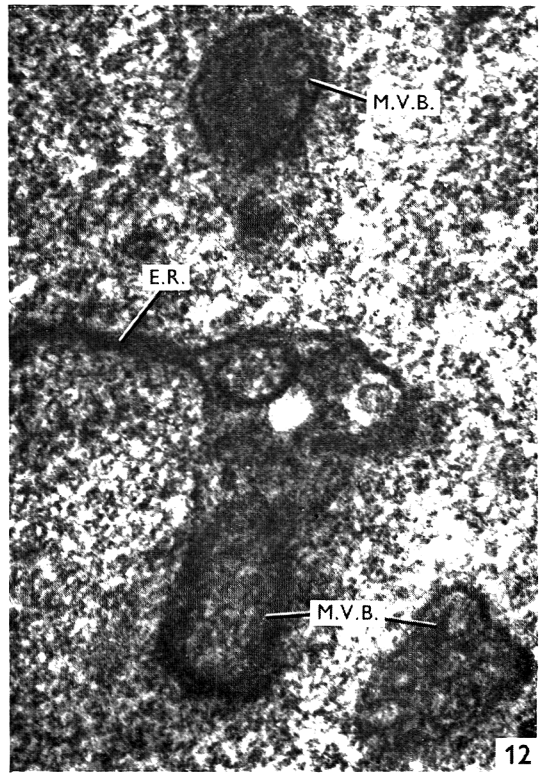

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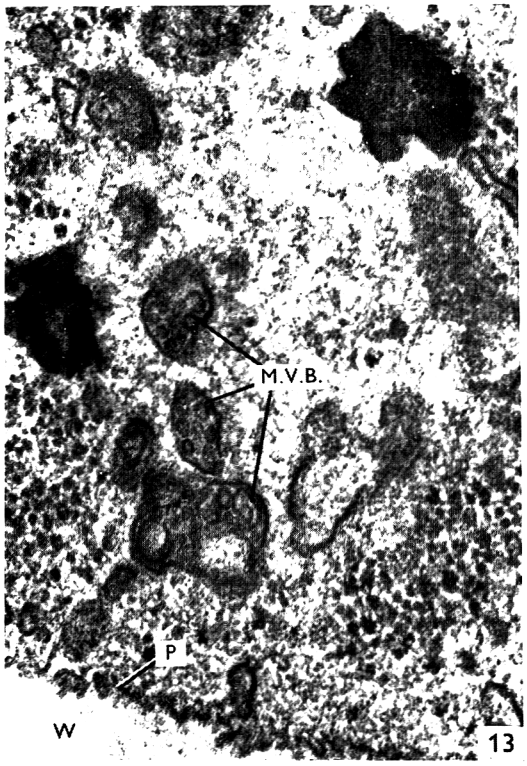

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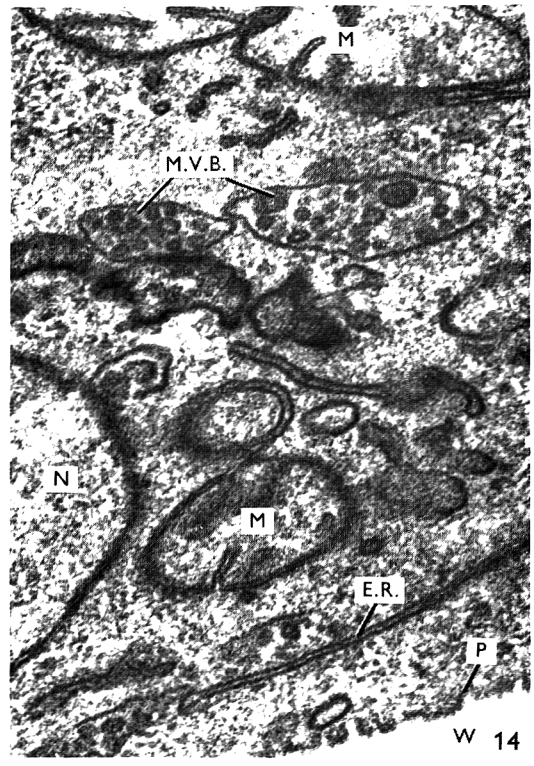

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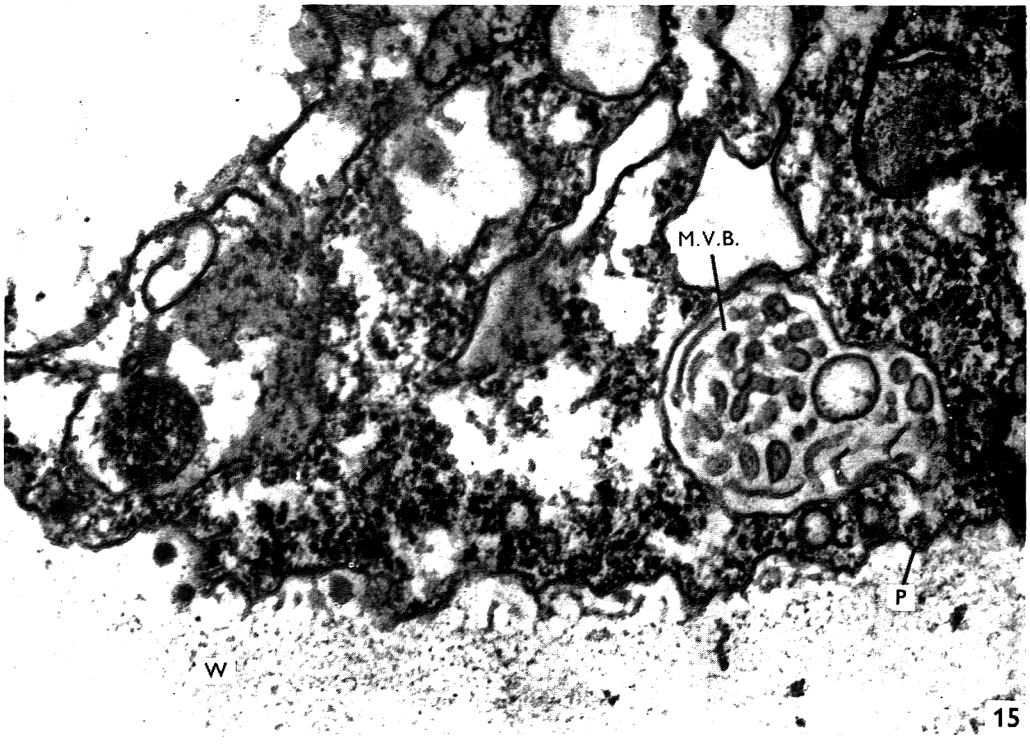

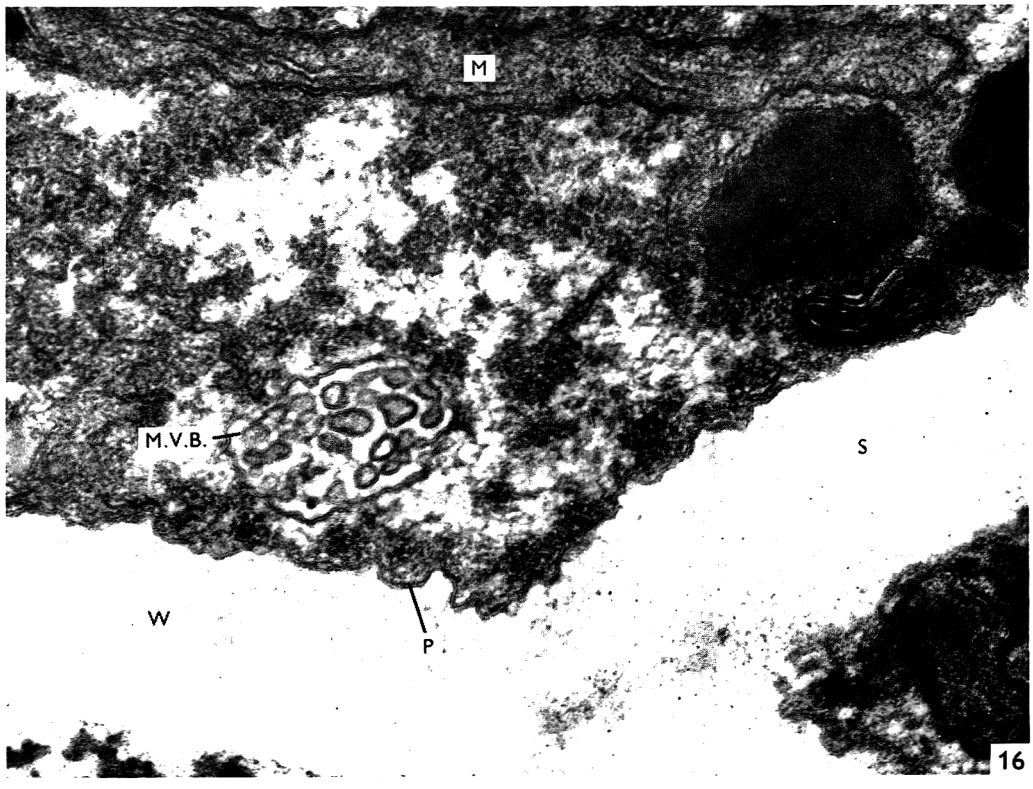

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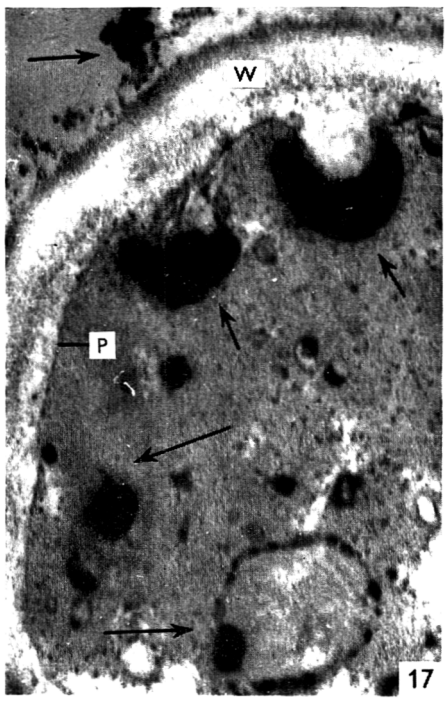

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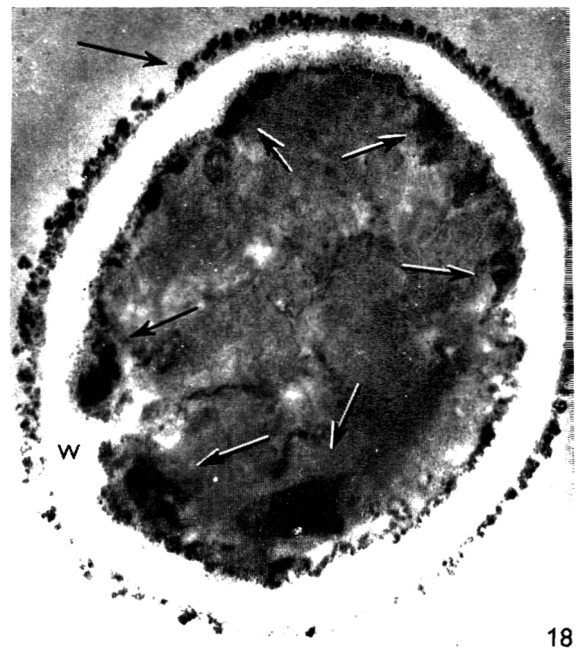

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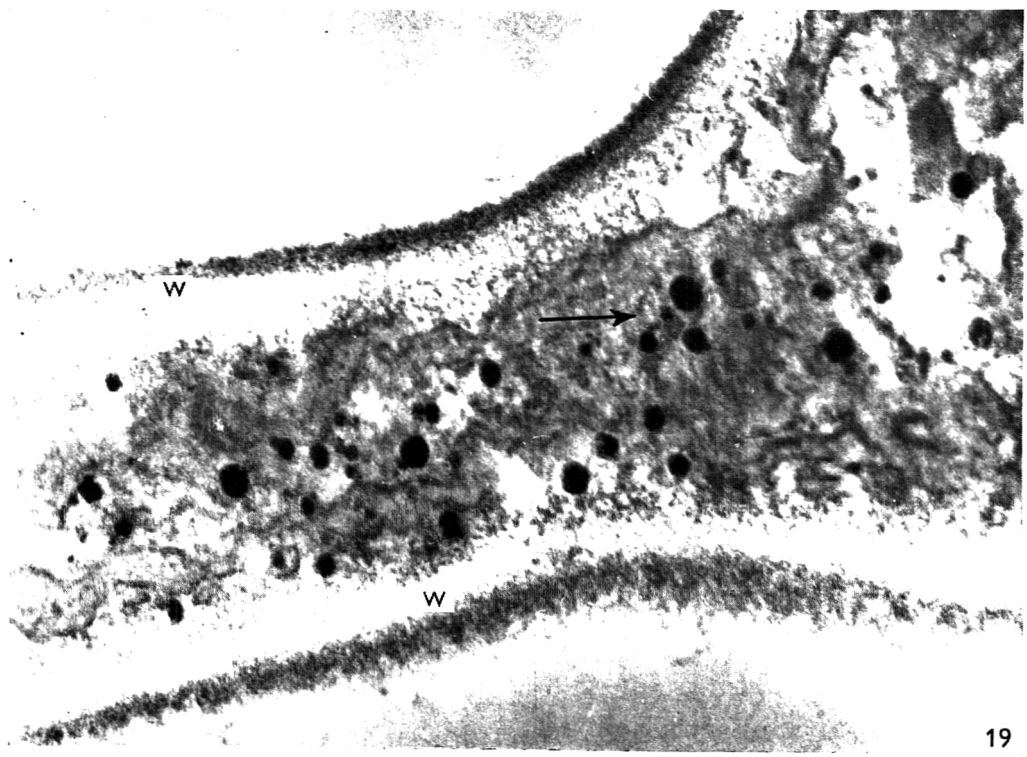

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Observations on the Action of Benzylpenicillin on a Strain of *Streptococcus lactis*

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(*Accepted for publication* 19 *September* 1968)

SUMMARY

Streptococcus lactis was grown in media containing various concentrations of benzylpenicillin; 0·05 μg./ml. was without effect and 0·40 μg./ml. inhibited growth. Intermediate concentrations in increasing steps of 0·05 μg./ml. were investigated. The Gram-positive cocci tended to become Gram-negative rods, the most effective concentration of penicillin for this effect was 0·15 ± 0·05 μg./ml. The rod forms frequently had surface vesicles which appeared to be membrane-bounded but were osmotically stable; the dry wt yield/ml. medium was diminished. Such forms contained up to 10 times more nisin/unit dry wt than the control. At penicillin 0·20 to 0·25 μg./ml. growth was followed by lysis which was followed by new growth after a long delay; morphologically these forms resembled the controls and the dry wt yield increased. Higher concentrations of penicillin (0·35 μg./ml.) decreased the dry wt yield/ml. medium and growth appeared only after a delay of 16 hr. Morphologically these forms tended to elongate again and the cell nisin/unit dry wt was 8 times higher than the control. Single-colony isolates obtained from the higher penicillin concentrations were grown in penicillin-free medium and when again challenged with penicillin no longer formed rods at 0·15 μg./ml. This more penicillin-resistant population occurred at a frequency of 1:500 of the parent population and was distinguished from it by a number of physiological properties. These results suggest that penicillin acted by selecting resistant individuals already present in the parent population.

INTRODUCTION

Previous studies on the synthesis and function of nisin, a basic protein with antibiotic activity, suggested that it plays a regulatory role concerned with the initiation and halting of growth of the organism which produces it. Hurst & Dring (1968) showed that the length of lag phase of growth in *Streptococcus lactis* was directly related to the nisin content of the organism. White & Hurst (1968) examined the location of nisin within cells of the producer organism and demonstrated that its principal association was with the wall and membrane. They fractionated cells by chemical and mechanical methods but were unable to obtain membranes free from contaminating wall material. Protoplasts would have provided ideal material for studies of the membrane-associated nisin but we were unable to produce them in preliminary studies. We tried unsuccessfully treatments with sodium lauryl sulphate, deoxycholic acid, glycine, lysozyme, snail and spore enzyme and mixtures of these reagents. We found that crude enzyme preparations from *Streptomyces albus* (Maxted, 1948) led to the total uncontrollable digestion of the organism and its structures. Brown, Sandine & Elliker (1962) were able to lyse *Streptococcus lactis* and other lactic acid bacteria by

digestion with lysozyme in the presence of EDTA at pH 8·0, but application of this method was undesirable since the antibiotic activity of nisin is lost in alkaline solutions.

Penicillin is known to affect the cell walls of sensitive bacteria and has been used for making protoplasts or spheroplasts (McQuillen, 1960). We therefore tried the effect of penicillin on our strain of *Streptococcus lactis* which is highly penicillin-sensitive. Although we have not succeeded in obtaining protoplasts we have obtained unexpected results. With increasing concentrations of penicillin in the culture medium the terminal dry-weight yield of organism did not decrease proportionately with the penicillin concentration over the range examined. The data suggested that the parent strain contained some individuals of higher penicillin-resistance (type II culture). Some of the physiological properties of the parent strain and of the type II culture derived from it were studied; first, by testing the effect of increasing concentrations of penicillin on the dry-weight yield, morphology and nisin content; secondly by testing the effect of increasing concentrations of penicillin on growth kinetics. We then isolated a pure clone of the type II culture and compared the heat and acid tolerance of the parent with the type II cultures. A preliminary account of this work has already been published (Dring & Hurst, 1967).

METHODS

Organism. Streptococcus lactis 354/07 (NCDO 497) was used throughout; it was maintained on slopes at 2° prepared from a culture derived from a single-colony isolate.

Morphology. Cell morphology was examined and photographed by using a Zeiss photomicroscope fitted with phase-contrast. For electron microscopy, organisms were fixed and stained according to the method of Hamilton & Stubbs (1967). Sections were made on a L.K.B. Ultratome III and examined in a J.E.M. 7A electron microscope.

Medium and estimation of minimum inhibitory concentration. All cultivation was done in the medium of Hurst (1966) used either as a liquid or solidified with agar; it had the following composition (%, w/v): meat extract (Lemco), 1; yeast extract (Difco), 1; tryptone (Difco), 1; glucose, 1; NaCl, 0·5; Na_2HPO_4, 0·2; pH 7; solidified when required with Agar (Difco), 1·5.

For the determination of the minimum inhibitory concentration (m.i.c.) of benzylpenicillin (Penicillin, sodium salt of crystalline benzylpenicillin; Crystapen, Glaxo Laboratories, England) against the organism, cultures were grown for 18 hr at 30° and were then inoculated at 0·1% (v/v) into liquid medium containing penicillin graded in increments of 0·05 μg./ml. over the range 0·05 to 0·40 μg./ml. Only fresh penicillin solutions were used; they were prepared at a concentration of 50 μg./ml. in distilled water, sterilized by Seitz filtration and diluted in the growth medium as required.

Estimations. The growth response of the organism at the various concentrations of penicillin was determined by measuring the extinction of growing cultures at 600 mμ in a Unicam SP 600 spectrophotometer (Unicam Instruments, Cambridge, England) against distilled water in 3·0 ml. cuvettes with 1 cm. light path, and by gravimetric determination of terminal organism dry weights. pH values were determined electrometrically and nisin by bioassay (Hurst, 1966).

Most probable numbers. The number of type II organisms in the parent population was estimated by diluting an overnight culture in decimal dilution series to 10^{-9} organisms/ml. and pipetting 5 × 1·0 ml. samples of each dilution to 9·0 ml. volumes of

liquid medium with and without 0·25 μg. penicillin/ml. The tubes were incubated at 30° for 48 hr and most probable number (m.p.n.) were estimated from probability tables. Those organisms developing in medium containing penicillin were scored as type II organisms.

Acid and heat tolerance test. An overnight culture was harvested, washed and the organisms were resuspended at 10^7 viable organisms/ml. For the heat test the organism was resuspended in fresh medium held at 60°. For the acid test the organism was resuspended in distilled water or 0·04 N-lactic acid held at 37°. Exposure of the organism to heat or acid was continued until the viable count decreased from 10^7 to about 10^5 organisms/ml., numbers being estimated by surface plating (Miles & Misra, 1938). When this decrease in number was achieved the colonies which developed were screened for penicillin resistance. Discrete colonies were picked with sterile hypodermic needles and replicated by touching the surface of agar medium containing penicillin (0·00 to 0·5 μg./ml.). The plates were incubated at 30° for 48 hr and the number of developing colonies at each concentration of penicillin counted.

RESULTS

Effects of penicillin, added at time of inoculation, observed in cultures which had reached stationary phase

Minimum inhibitory concentration

Penicillin at 0·40 μg./ml. inhibited growth during incubation for 48 hr at 30°. The precise end-point varied in different experiments, but the cause of the variation was not studied further. During a typical m.i.c. determination, growth was completed within 8 hr at 30° in the medium containing penicillin at 0·00–0·15 μg./ml. with slight indication of growth at 0·20 μg./ml. After a further 20 hr of incubation, growth occurred at concentrations up to the m.i.c.

Dry-weight yield and terminal pH value

A progressive decrease of dry-weight yield at 48 hr occurred at penicillin concentrations up to 0·20 μg./ml. There was a slight enhancement of growth at 0·25 μg./ml. which resulted in some increase in dry-weight yield. Above 0·25 μg./ml., and as the m.i.c. was approached, the dry weight again decreased (Fig. 1). The terminal pH values of the cultures were inversely related to the dry weight.

Nisin content

Total and cell-bound nisin values in cultures incubated for 48 hr at 30° are shown in Fig. 1. The total nisin synthesized (per unit dry weight organism) was similar at all concentrations of penicillin. However, the cell-bound nisin (also per unit dry weight) increased as the concentration of penicillin increased from 0·00 to 0·15 μg./ml.; the latter cultures contained about 10 times more cellular nisin than control organisms grown without penicillin. The cell nisin content was lower at 0·25 μg./ml., but again showed an increase to values 8 times greater than the control organisms at concentrations of penicillin immediately below the m.i.c. Following an m.i.c. estimation the organisms were harvested, resuspended in the same volume of fresh penicillin-free medium, adjusted with lactic acid to pH 4·2, and left standing at 30° for 24 hr. The cell nisin was then again estimated and found to be unchanged.

Morphology

The phase-contrast appearance of the organism obtained after growth for 48 hr at the various concentrations of penicillin is shown in Pl. 1, fig. 1 to 8. The photographs show that in the presence of up to 0·20 μg. penicillin/ml. the organisms became progressively more rod shaped. Concomitant with this development they became Gram-negative. Some of the rod-shaped forms were phase dark and appeared intact, whereas

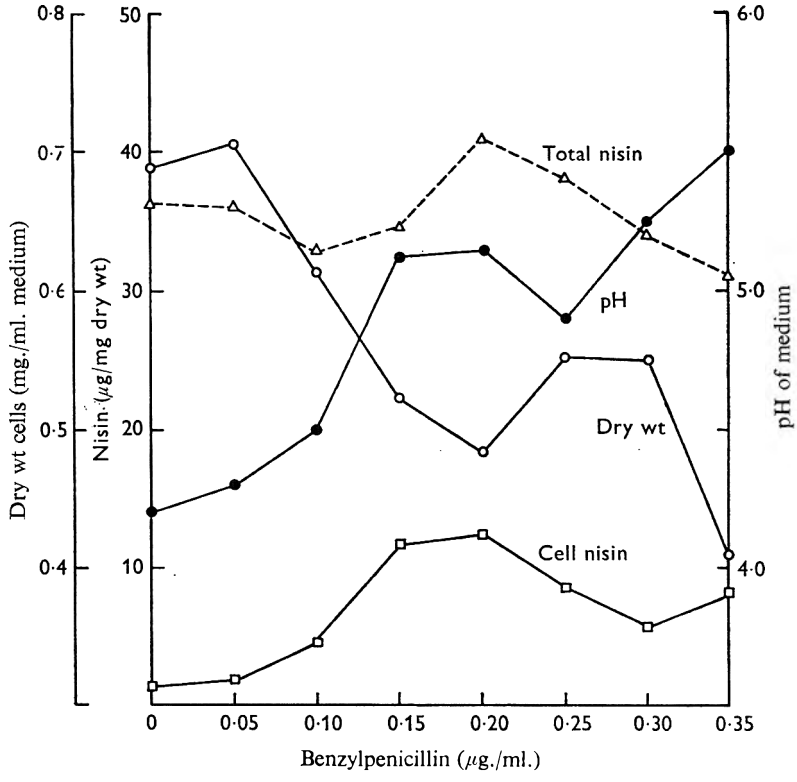


Fig 1. Effect of benzylpenicillin on the growth and biosynthetic activities of *Streptococcus lactis*. Estimations were made after 48 hr growth at 30°.

others were ghost-like and had granules within them. In some experiments, although not all, together with these rod-shaped forms there was also a small proportion of coccobacilliary forms. In the presence of 0·25 μg. penicillin/ml. the organisms showed a marked resemblance to those grown without penicillin. At this concentration only coccobacilli developed which were mainly Gram-positive. However, at concentrations approaching the m.i.c. cell elongation took place and the organisms were Gram-negative. Together with the slight variation in actual m.i.c. encountered in different experiments the precise concentration of penicillin at which the reversion from the rod-shaped form to the coccobacillary form occurred varied slightly (±0·05 μg./ml.). Subcultures from tubes containing rod-shaped predominately Gram-negative organisms into fresh medium without penicillin grew as normal Gram-positive coccobacilli.

Osmotic fragility

The rod-shaped forms obtained after growth of the test organism in medium containing 0·10–0·15 μg. penicillin/ml. were not osmotically fragile because on harvesting them from the growth medium they did not burst when suspended in distilled water. They were also interesting in that many showed small bubbles or vesicles protruding from the surface of the cell (see Pl. 1, fig. 4); some forms had as many as six vesicles. Vesicle formation was also observed on some forms which grew at 0·35 μg. penicillin/ml. Electron micrographs of fixed and sectioned organisms showed that the vesicles were formed by the extrusion of membrane through a damaged point of the cell wall (see Pl. 2, fig. 9).

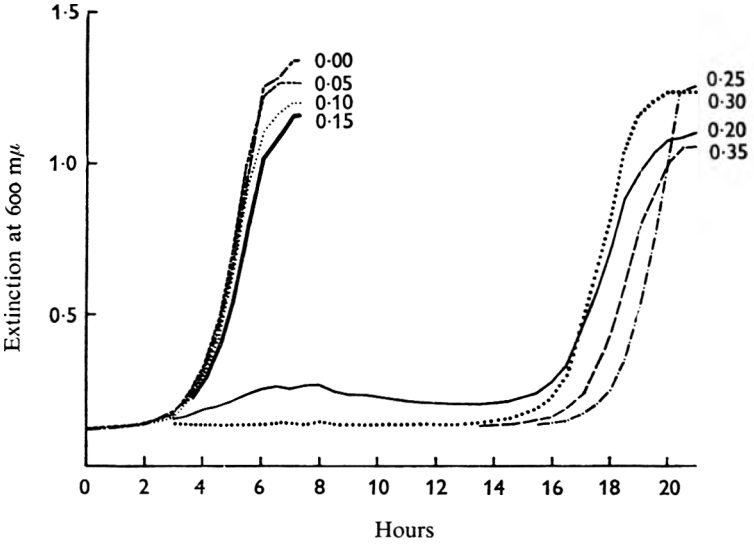


Fig. 2. Growth curves of *Streptococcus lactis* in various concentrations of benzylpenicillin showing development of two populations from the parent culture. Figures 0·00 to 0·35 show concentrations of penicillin (μg/ml.) used.

Effect of penicillin on growth kinetics

The growth cycles of the organism cultured at the various concentrations of penicillin (0·05 to 0·35 μg./ml.) were then studied (Fig. 2). Initiation of growth in cultures in medium containing penicillin 0·00 to 0·15 μg./ml. occurred after a lag period lasting about 2 hr and these low concentrations of penicillin had little effect on the rate of growth. After 4 hr of incubation growth started in medium containing 0·20 to 0·25 μg. penicillin/ml. The rate of growth was much slower than at the lower concentrations of penicillin and continued up to 6 to 7 hr, after which the extinction value decreased. Examination of hanging-drop preparations under phase contrast showed that lysis was occurring at this time. After a prolonged delay of 14 to 16 hr, growth began again. At penicillin concentrations of 0·30 to 0·35 μg./ml. there was no intermediate growth and lysis; growth started after a delay of 16 hr. Once started the growth rate was the same at all penicillin concentrations from 0·20 to 0·35 μg./ml. Nisin synthesis in a control culture and one growing in medium containing 0·15 μg. penicillin/ml. began when some 50% of the total organism dry weight had been formed. Elongation to long

forms in medium containing penicillin was observed as soon as growth began. There was thus no correlation between elongation and the increase in cell-nisin content.

Isolation of type II cultures

The effect of increasing concentrations of penicillin on stationary phase organisms was discontinuous in that intermediate concentrations of penicillin caused unexpected changes in morphology, dry weight, terminal pH value and cellular nisin content. For example, penicillin at 0·20 μg./ml. initially permitted growth which after lysis was followed by further growth. These results clould be most easily explained by assuming that the parent population was heterogeneous as regards the penicillin resistance of individuals. Accordingly, organisms from medium containing 0·30 and 0·35 μg. penicillin/ml. were subcultured to fresh medium without penicillin and incubated overnight. The cultures were streaked on agar and single colonies were isolated and grown in penicillin-free liquid medium. Their response to graded amounts of penicillin (0·05 to 0·40 μg./ml.) was then tested. The discontinuous effect observed with the parent culture could not be reproduced; the m.i.c. was 0·40 μg. penicillin/ml. after only overnight incubation. These were called type II cultures; their response when challenged by graded amounts of penicillin was not altered by up to five daily serial subcultures in penicillin-free medium.

The type II cultures differed from the parent population in that the organisms, although of coccobacillary morphology, were slightly larger than normal. There was a small, but consistent difference between the terminal pH values of the type II cultures (pH 4·5) and the parent cultures (pH 4·2). When grown in the absence of penicillin, the total and cell nisin synthesized by the type II culture were the same as those of the parent population; the growth rates were also the same.

The parent population thus consisted of a majority of individuals which could grow in liquid medium broth up to 0·15 μg. penicillin/ml.; at penicillin concentrations of 0·20 to 0·35 μg./ml. only type II individuals grew. The frequency of occurrence of type II individuals in the parent population was tested by most probable number counts (m.p.n.). Total numbers were estimated by growing in liquid medium alone and type II numbers by growing in liquid medium containing 0·25 μg. penicillin/ml. Type II organisms were 1/500 of the parent population. Counting on nutrient agar media gave similar results.

That a hitherto 'pure' culture of *Streptococcus lactis* should harbour two strains, the progeny of which could be readily screened on the basis of their relative resistance to penicillin, prompted studies to determine whether the type II organisms possessed other characteristics distinguishable from the parent population. The heat resistance of type II organisms and the parent population were tested and found to be the same.

The effect of suspending organisms in distilled water and 0·04 N-lactic acid was also tested and the results subjected to analysis of variance. One hr in distilled water at 37° did not alter the number of organisms as revealed by surface plate counts. Replication of these colonies on agar containing increasing concentrations of penicillin showed that 0·3 to 0·5 μg./ml. did not affect the number of colonies able to develop (Table 1). However, 0·04 N-lactic acid caused a 99% kill, as revealed by surface plate counts. Replication of these colonies on agar containing increasing concentrations of penicillin showed, as before, that the number of colonies which developed was independent of the penicillin concentration (0·3 to 0·5 μg./ml.). The initial F value for this distribution

at the 5% level was 4·41 and the value found of 2·51 was therefore not significant. On the other hand, comparison of the number of colonies developing at the different penicillin concentrations revealed a highly significant difference between the two treatments (distilled water and lactic acid). The initial value of the F distribution at the 1% level was 8·29; the value found was 12·34 and was therefore highly significant. These results show that the lactic acid treatment selected a homogeneous population which was significantly different from the parent population by its penicillin resistance.

Table 1. *The selection of penicillin resistant colonies by lactic acid*

Overnight cultures of *Streptococcus lactis* 354/07 (NCDO 497) were centrifuged, the deposit washed once with distilled water and suspended at 10^7 organisms/ml. in distilled water or in 0·04 N-lactic acid at 37° for 1 hr. Survivors were then counted by surface plating and resulting colonies screened for penicillin resistance.

		Penicillin in medium (μg./ml.)						
		0·00	0·25	0·30	0·35	0·40	0·45	0·50
		No. of colonies developing out of 50 subcultured at different concentrations of penicillin						
Treatment	Replicate nos.							
Distilled water	1	48	38	22	17	18	16	16
	2	50	38	26	28	12	7	11
	3	50	39	32	9	27	5	11
Lactic acid	1	50	40	29	31	28	21	17
	2	50	38	27	26	47	20	34
	3	50	33	31	16	22	21	33

DISCUSSION

On the basis of penicillin resistance two strains could be distinguished in a culture of *Streptococcus lactis* which was known to be otherwise pure, and which explained the discontinuous growth response of the culture to penicillin. Cultures which were inhibited by concentrations of penicillin of 0·15 to 0·20 μg./ml. were designated type I, and those inhibited by 0·35 to 0·40 μg./ml. type II. Penicillin probably acted by selecting the resistant individuals already in the parent population. Once isolated from single colonies, type II cultures retained their higher penicillin resistance when grown in the absence of penicillin. Based on penicillin resistance type II organisms formed 1/500 of the population. The results of the tests with lactic acid confirmed that type II organisms were present in the parent population before contact with penicillin. However, this test did not tell us how many of the lactic acid resistant organisms were also penicillin resistant.

Comparison of type II cultures with the parent population showed that the former differed in that they had a higher terminal pH value in cultures and tended to be larger. The latter property could be due to an over-all increase in size or to a change in cell-wall thickness. The experiment in which organisms surviving lactate were also more penicillin resistant suggests that type II organisms may have an altered surface structure, causing changes in the permeability characteristics. Such effects were particularly evident when *Streptococcus lactis* was grown in sublethal amounts of penicillin. For example, Hurst & Dring (1968) showed that the percentage of nisin retained by the producer organism was pH dependent; at pH > 6·0 more than 80% of the nisin was cellular, whereas at PH < 6·0 more than 80% of the nisin was in the medium. The data

of Fig. 1 have been replotted in Fig. 3 and show that in the presence of penicillin the distribution of nisin was no longer dependent on pH value but on the concentration of penicillin in which the organism had been grown. Rogers (1967) showed that when *Staphylococcus aureus* was grown in penicillin, membrane permeability was affected.

Various studies have been made of intracellular membranous structures or mesosomes in bacteria (Gel'man, Lukoyanova & Ostrovskii, 1967). Fitz-James (1964), Ryter & Landman (1964) and Kakefuda, Holden & Utech (1967) showed the association of mesosomes with the cell membrane in various bacteria. Fitz-James (1964) and

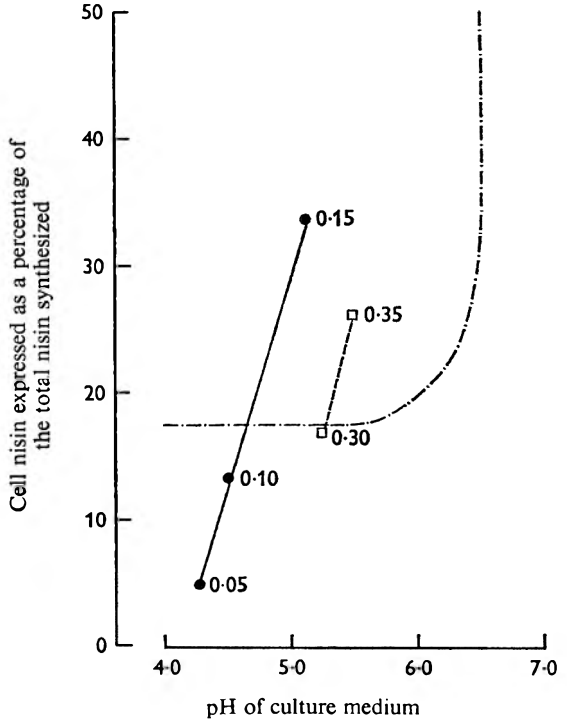


Fig. 3. The effect of benzylpenicillin at various concentrations on the synthesis and binding of nisin by *Streptococcus lactis*. Organism grown without penicillin showing the binding of cell nisin as a function of the pH of the medium. Data from Hurst & Dring (1968) –·–·–·–; cell-bound nisin of type I cells growing in 0·05 to 0·15 μg. penicillin/ml., ●——●; cell-bound nisin of type II cells growing in 0·30 and 0·35 μg. penicillin/ml, □ - - - - □.

Ryter & Landman (1964) showed that such mesosomes were extruded into the wall-membrane interspace (periplasm) during protoplast formation and that they were released into the medium when the cell wall was ruptured. Plate 1, fig. 4, and Pl. 2, fig. 9, probably depict the same phenomenon of mesosome extrusion when the cell wall of *Streptococcus lactis* was damaged by penicillin. Although the vesicles (Pl. 2, fig. 9) appeared to be bounded only by membrane they were not osmotically fragile. If the retaining structure of the vesicle were solely a lipoprotein membrane, then lysis of the vesicle would have been expected. As this did not occur it is possible that a strengthening, although non-rigid, layer was also present. Fitz-James (1964), from the appearance of *Bacillus megaterium* mesosomes as shown by electron microscopy, suggested that they consisted of vesicles with associated wall-like material. White & Hurst (1968) were

unable, by using mechanical means, to prepare a membrane fraction from *S. lactis* free from amino sugar and suggested that the cell wall and membrane of this organism formed a closely integrated structure. The presence of the stable extruded vesicles is in accordance with these suggestions. Briggs, Crawford, Abraham & Gladstone (1957) obtained results with *Staphylococcus aureus* which were similar to ours. However, later work cast doubt on the original findings, suggesting that the results were due to contamination (Hilson & Elek, 1959). If in our study twofold increments in penicillin concentrations had been used, which are customary in m.i.c. determinations (Briggs *et al.* 1957) the stable type II population might have been missed. It is possible that this accounts for the failure of some workers to reproduce the conditions necessary for the isolation of two forms of *Staphylococcus aureus*.

The authors wish to thank Mr J. M. Stubbs, who did the electron microscopy, Mr I. W. Tully for statistical analysis and Mr M. E. Pugh for technical assistance.

REFERENCES

Briggs, S., Crawford, K., Abraham, E. P. & Gladstone, G. P. (1957). Some properties of Gram-negative bacilli obtained from a strain of *Staphylococcus aureus* in the presence of benzylpenicillin. *J. gen. Microbiol.* **16**, 614.

Brown, W. C., Sandine, W. E. & Elliker, P. R. (1962). Lysis of lactic acid bacteria by lysozyme and ethylenediaminetetraacetic acid. *J. Bact.* **83**, 697.

Dring, G. J. & Hurst, A. (1967). The effect of penicillin on a nisin-producing strain of *Streptococcus lactis*. *Biochem. J.* **106**, 45P.

Fitz-James, P. C. (1964). Fate of the mesosomes of *Bacillus megaterium* during protoplasting. *J. Bact.* **87**, 1483.

Gel'man, N. S., Lukoyanova, M. A. & Ostrovskii, D. N. (1967). In *Respiration and Phosphorylation of Bacteria*. Ed. by G. B. Pinchot. New York: Plenum Press.

Hamilton, W. A. & Stubbs, J. M. (1967). Comparison of the germination and outgrowth of spores of *Bacillus cereus* and *Bacillus polymyxa*. *J. gen. Microbiol.* **47**, 121.

Hilson, G. R. F. & Elek, S. D. (1959). An investigation into the development of Gram-negative rods in penicillin-treated cultures of *Staphylococcus aureus*. *J. gen. Microbiol.* **21**, 208.

Hurst, A. (1966). Biosynthesis of the antibiotic nisin by whole *Streptococcus lactis* organisms. *J. gen. Microbiol.* **44**, 209.

Hurst, A. & Dring, G. J. (1968). The relation of the length of the lag phase of growth to the synthesis of nisin and other basic proteins by *Streptococcus lactis* grown under different cultural conditions. *J. gen. Microbiol.* **50**, 383.

Kakefuda, T., Holden, J. T. & Utech, N. M. (1967). Ultrastructure of the membrane system in *Lactobacillus plantarum*. *J. Bact.* **93**, 472.

Maxted, W. R. (1948). Preparation of streptococcal extracts for Lancefield grouping. *Lancet*, ii, 255.

McQuillen, K. (1960). Bacterial Protoplasts. In *The Bacteria*. Ed. by I. C. Gunsalus and R. Y. Stanier. Vol. I. New York: Academic Press.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

Rogers, H. J. (1967). Killing of staphylococci by penicillins. *Nature, Lond.* **213**, 31.

Ryter, A. & Landman, O. E. (1964). Electron microscope study of the relationship between mesosome loss and the stable L state (or protoplast state) in *Bacillus subtilis*. *J. Bact.* **88**, 457.

White, R. J., & Hurst, A. (1968) The location of nisin in the producer organism, *Streptococcus lactis*. *J. gen. Microbiol.* (in the Press).

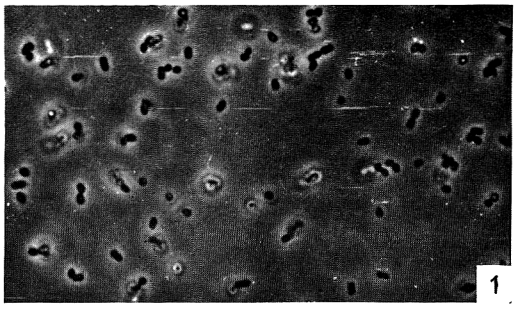
EXPLANATION OF PLATES

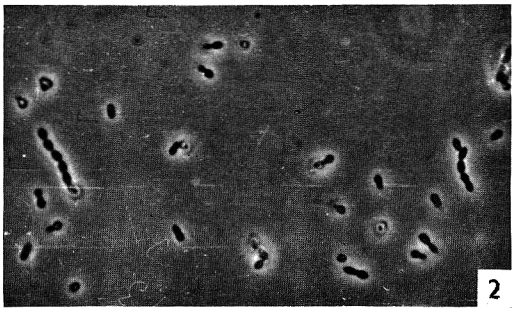
PLATE 1

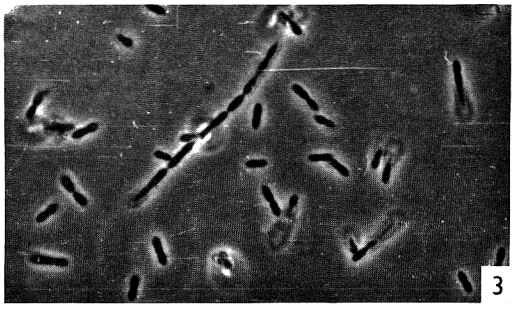
Fig. 1–8. Morphology of stationary phase whole *Streptococcus lactis* grown in medium containing 0·00, 0·05, 0·10, 0·15, 0·20, 0·25, 0·30 and 0·35 μg. penicillin/ml. Phase contrast: × 2000 (approx.). Note vesicle formation (arrowed) in 0·15 μg. penicillin/ml.

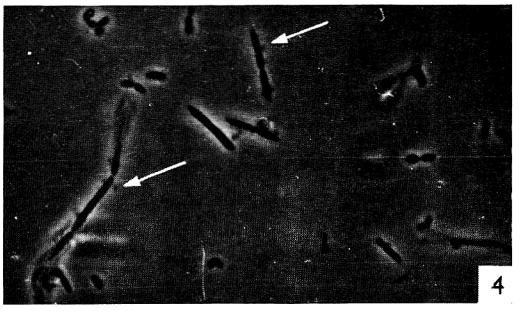
PLATE 2

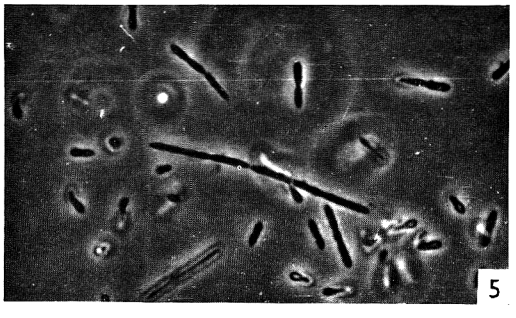
Fig. 9. Section of vesicle on a *Streptococcus lactis* organism grown in 0·15 μg. penicillin/ml. × 60 000.

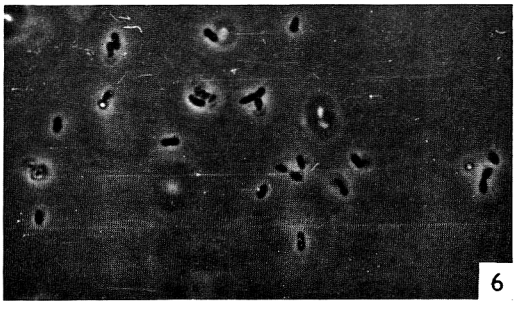


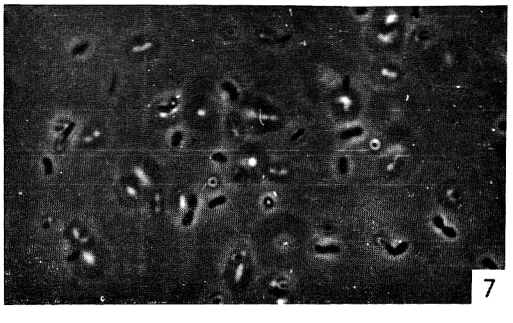


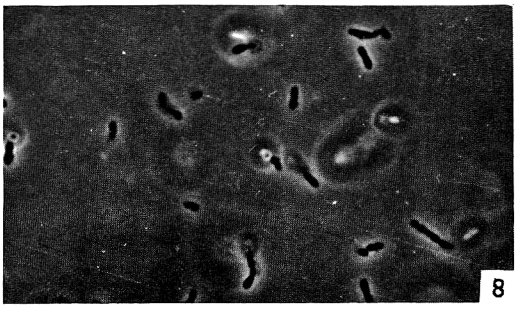












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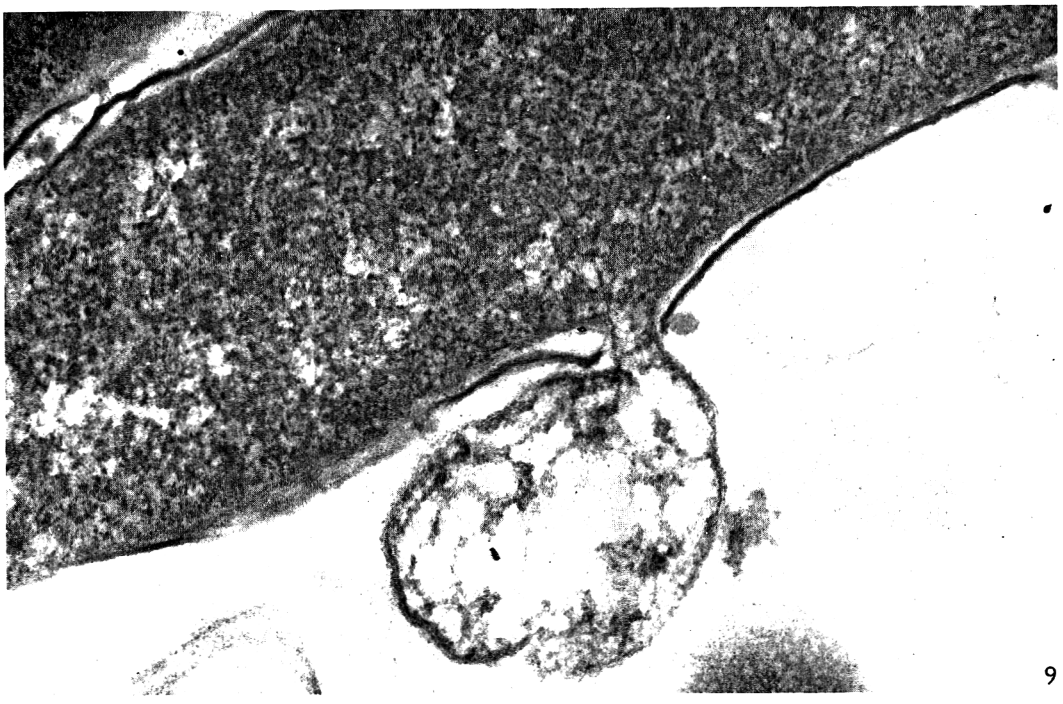

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Transformation of Nutritionally Deficient Mutants of *Aspergillus niger*

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SUMMARY

Reversions to wild type occurred when conidia of a number of biochemically deficient mutants of *Aspergillus niger* were treated with DNA from the wild-type strain. No reversions or wild-type colonies were obtained when an equivalent number of conidia from deficient strains were either treated with the same DNA as the recipient, or were plated without any DNA treatment. Increase in the percentage of transformation was observed up to 6 μg./ml. of donor DNA. The transforming activity of the donor DNA was found to be inhibited by the action of u.v. radiation, heat and DNase. The frequency of transformation was low which could be attributed to the method of extraction of DNA which involved crushing of cells in the presence of alumina, possibly breaking the DNA into small fragments thus making it biologically less active.

INTRODUCTION

Very little information is available on transformation in moulds. Shamoian, Canzanelli & Melrose (1961) described the transforming activity of a nucleic acid complex in *Neurospora crassa*. Shockley & Tatum (1962) attempted the reversion of biochemically deficient mutants of *N. crassa* through transformation. The results of these authors were not very conclusive in nature. The present authors have tried to demonstrate the presence of the process of transformation in the mould *Aspergillus niger* and have tried to show that reversions occurring in the nutritionally deficient mutants were due to transformation of the mutants to wild type and that this reversion rate was affected if the donor DNA was treated with u.v., heat or DNase.

METHODS

Six biochemical mutants derived through u.v. radiation (Mishra & Nandi, 1959) of a laboratory-isolated strain of *Aspergillus niger*, designated v35 were used as the recipient strains. The strains were 2 *nic*$^-$, 3 *lys*$^-$, 4 *leu*$^-$, 14 *met*$^-$, 21 *cho*$^-$ and 45 *arg*$^-$ requiring nicotinic acid, lysine, leucine, methionine, choline and arginine respectively. Donor DNA was prepared in the following manner: a number of Roux bottles containing 100 ml. of Czapek Dox liquid medium (Clutterbuck, Lovell & Raistrick, 1932) were inoculated with a heavy conidial suspension of v35 and incubated at 28°. A very thin transparent mycelial layer appeared at the surface of the medium in about 24 to 36 hr. This layer was collected by filtration and ground in a mortar in the presence of alumina (mycelium + alumina; 1 + 4) at 5° for about 1 hr. During grinding 10% Na-deoxycholate (0·5 ml./g. mycelium) and citrate saline buffer (1 ml./g. mycelium) were added.

After thorough grinding the mass was finally extracted with citrate saline buffer, from which the protein and RNA were removed by treatment with chloroform and amyl alcohol mixture and RNase respectively. The final DNA preparation which was obtained by precipitation with alcohol was resuspended in 5 ml. of sterile citrate saline buffer and kept at 5°. Purified DNA sample contained about 0·1 mg of DNA per ml. of the solution. The transforming DNA preparations were sterile and did not give rise to any colonies of the donor type when plated alone on Czapek Dox agar medium.

Transformation reaction was carried out in 25 ml. Erlenmeyer flasks by adding 1 ml. of conidial suspension to 8 ml. of Czapek Dox broth, to which DNA solution to make 25 μg/ml had been added. Two controls were usually set up for each lot of experiments. In one control DNA was omitted and, in the other, DNA isolated from the recipient strain was added. The flasks were incubated for 18 to 20 hr at 28° after which the reaction was terminated by adding 0·004 μg. Mg-activated DNase/ml. The transformation mixture was centrifuged and the conidia washed several times before plating on Czapek Dox agar medium. The plates were incubated at 28° for 4 to 5 days when revertants to wild type were observed to appear. Heat inactivation of DNA was done by suspending a number of tubes containing 3 ml. of DNA samples for 30 min at 50°, 60°, 70°, 80°, 90° and 100° in a water bath and u.v. inactivation of DNA was studied by subjecting 5 ml. of DNA solutions contained in a number of 9 cm. plates to u.v. radiations for 2, 4, 6 and 8 min. from a 4 W. General Electric Germicidal lamp (dose incident 100 ergs/mm.^{2}/sec.). After heating and u.v. irradiation the samples were chilled immediately and used for transformation purpose as usual.

Table 1. *Transformation of nutritional markers in Aspergillus niger*

Donor strain—v35, DNA concentration 25 μg./ml.

Recipient strain*	No. of conidia treated† A, B & C	No. of transformants to wild type A	B	C	Transformants (%) A	B	C
2 *nic*$^-$	$1{\cdot}4 \times 10^6$	0	0	16	0	0	$1{\cdot}1 \times 10^{-3}$
3 *lys*$^-$	$3{\cdot}5 \times 10^6$	0	0	42	0	0	$1{\cdot}2 \times 10^{-3}$
4 *leu*$^-$	$2{\cdot}7 \times 10^6$	0	0	15	0	0	$5{\cdot}5 \times 10^{-4}$
14 *met*$^-$	$3{\cdot}8 \times 10^6$	0	0	40	0	0	$1{\cdot}0 \times 10^{-3}$
21 *cho*$^-$	$5{\cdot}5 \times 10^6$	0	0	162	0	0	$2{\cdot}9 \times 10^{-3}$
45 *arg*$^-$	$3{\cdot}1 \times 10^6$	0	0	35	0	0	$1{\cdot}1 \times 10^{-3}$

* All recipient strains derived through u.v. treatment of v35, for each strain, figure shows the isolate number and symbols denote the requirement as follows: *nic* = nicotinic acid; *lys* = lysine; *leu* = leucine; *met* = methionine; *cho* = choline; *arg* = arginine.

† A = conidia treated with its own DNA; B = conidia not treated with any DNA; C = conidia treated with donor DNA.

RESULTS

Transformation of nutritional markers

Table 1 gives the result of transformation to wild type in a number of deficient strains. Though the frequency of transformation is poor as compared with other transformable organisms, it is apparent that reversions had occurred in the presence of DNA, since controls did not show any revertants. The recipient strains were very stable, no spontaneous reverse mutations could be detected even after plating 1×10^8 organisms on Czapek Dox agar medium.

Table 2 shows that the frequency of transformation increased with the increase in the time of contact of recipient cells with the donor DNA. Germination of conidia started after 8 hr of incubation and by twentieth hr about 95% had germinated, with number of nuclei varying from two to six per conidium, the majority having two nuclei. In the DNA-treated samples germination was a little better than those without DNA treatment, perhaps due to some helping factors coming through DNA. Bucknall & Morton (1964), while making an attempt to transform *Penicillium chrysogenum*, observed an increase in spore yields and growth rate induced by DNA preparations; however, they were unable to observe the occurrence of transformation. In their

Table 2. *Transformation of 3 cyt⁻ to wild type*

Donor, v35; recipient, 3 *cyt*⁻ (or cytosineless mutant from v35 derived by u.v. irradiation).

Time given for transformation reaction	No. of conidia treated* A, B & C	No. of transformants to wild type			Transformants (%)		
		A	B	C	A	B	C
8	$3{\cdot}1 \times 10^6$	0	0	0	0	0	0
12		0	0	6	0	0	$1{\cdot}9 \times 10^{-4}$
16		0	0	48	0	0	$1{\cdot}5 \times 10^{-3}$
20		0	0	47	0	0	$1{\cdot}5 \times 10^{-3}$

* A = conidia treated with its own DNA; B = conidia not treated with any DNA; C = conidia treated with donor DNA.

opinion this stimulation of growth was possibly due to bridging some of the deficiency which might have been in the purine and pyrimidine metabolism of the recipient strain, by utilizing the purines and pyrimidines from the donor DNA. They also suggested the possibility of DNA preparations carrying some trace metals which stimulated growth. A question arises here that in DNA-treated samples there might be an increased probability of occurrence of spontaneous mutations to the transformant type, since these samples were better nourished, but this was not the case, since samples treated with the same DNA as the recipient did not give any revertants. It is possible that the germination helped the cells to become competent and allowed the integration of wild-type growth characters from the donor DNA molecule. In controls without DNA or with own DNA this did not happen since in the former case the samples lacked the donor DNA and in the latter did not carry any marker other than the recipient which could be identified after integration.

To attribute the transforming activity to the donor DNA a number of experiments, reported below, were done to show that transformation was actually taking place.

Transformation at different concentrations of DNA

Transformation experiments were done using different concentrations of donor DNA from v35 varying from 2 to 10 μg. DNA/ml. of transforming mixture. Figure 1 shows the dosage response data as a function of DNA concentration in transforming an argininеless strain (45 arg⁻) to wild type. An increase in the percentage of transformation was observed up to 6 μg. DNA/ml. Concentrations beyond that, apparently had very little or no effect on the increase of transformation frequency. This part of the curve could be referred to as the saturation region.

Effect of DNase on transforming DNA

$4{\cdot}0 \times 10^{-3}$ μg. of Mg-activated DNase solution/ml. was added to a number of tubes containing 5 ml. samples with 25 μg. donor DNA per ml. The tubes were incubated at 37°. After suitable intervals of time (Fig. 2), two tubes were taken and EDTA were added to each to a final concentration of 10^{-3} M. Spectrophotometer readings were taken from one and the other transformation experiments were carried out in the usual way. Transformation activity of the donor DNA was found to diminish with each successive increase of incubation time with DNase. Correspondingly an increase in the optical density of the DNA was observed showing the degradation of the DNA (Fig. 2).

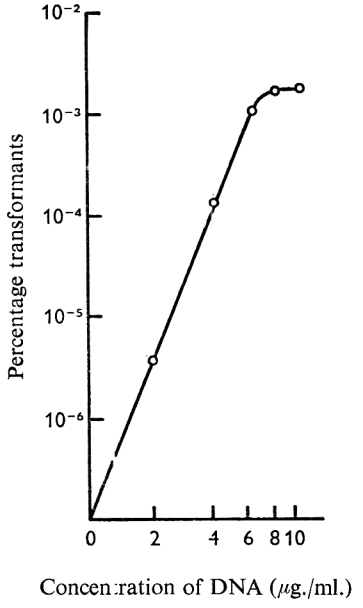

Fig. 1

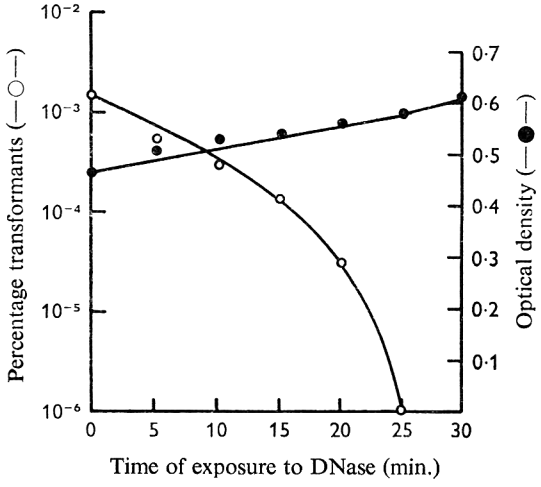

Fig. 2

Fig. 1. Percentage transformants as a function of concentration of transforming DNA. (Donor, v35; recipient, 45 *arg*$^-$.)

Fig. 2. Effect of DNase on transforming DNA. (Donor, v35; recipient, 45 *arg*$^-$.)

Effect of heat on transforming DNA

Figure 3 shows the effect of heat on transforming DNA. Transformation activity of DNA was found to diminish with the successive rises in temperature (especially at 80° and above). A corresponding increase in optical density showed that degradation of the DNA had occurred (Fig. 3).

Effect of u.v. irradiation on transforming DNA

Figure 4 shows the effect of u.v. irradiation on inactivation of transforming principle in *Aspergillus niger* (v35). There was a decrease in the transformation activity with successive increase in the time period of irradiation.

DISCUSSION

The results show that transformation process occurs in *Aspergillus niger*. The frequency of transformation is low as compared to the bacterial systems where transformation is known to occur. Transformation to prototrophy in bacteria has been observed by Spizizen (1958), Gwinn & Thorne (1964) and Leonard, Corley & Cole (1966). Shamoian *et al.* (1961) and Shockley & Tatum (1962) attempted the transformation of auxotrophic strains of *Neurospora crassa* to wild type, and the latter authors were of the opinion that the results provided suggestive but not convincing evidence of transformation in this strain. The present experiments show that there is

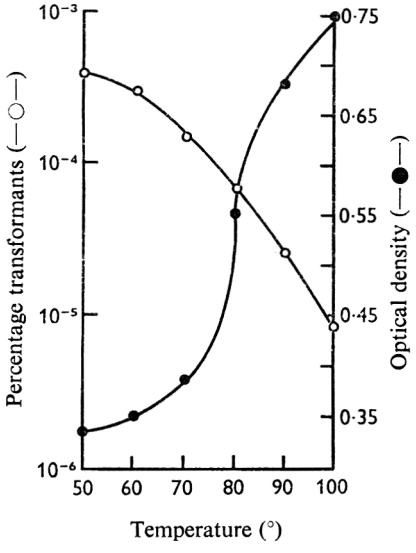

Fig. 3

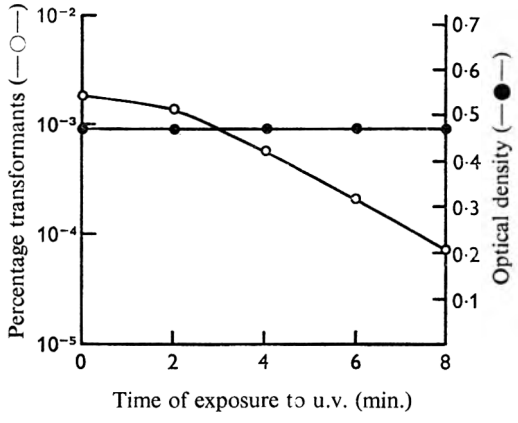

Fig. 4

Fig. 3. Heat inactivation of transforming DNA. (Donor, v35; recipient, 45 *arg*⁻.)

Fig. 4. Ultraviolet inactivation of transforming DNA. (Donor, v35; recipient, 45 *arg*⁻.)

a similarity with the findings of Hotchkiss (1951) and Marmur & Fluke (1955) in the response to concentration changes of donor DNA. The effect of heat, DNase and u.v. irradiation on donor DNA resulting in the inactivation of the transforming principle in *A. niger* is more or less similar to that reported by Lerman & Tolmach (1959) for Pneumococcus. The transformants scored in the present investigation were found to be stable.

The lower frequency of transformation reported here, as compared to bacterial system, might be attributed to a number of factors. The growth and multiplication of bacteria differ widely from those of the fungi and the environmental factors required for transformation are perhaps not similar to those applicable in the case of bacteria. Nothing is known as yet about the nature of competence in fungi, which depends on factors like structure of the recipient cell surface, production of DNase by the cell or the requirement of a specific nutrient to make the cells competent. Lower frequency of transformation could also be attributed to the method of extraction of DNA since crushing the cells in the presence of alumina possibly breaks the DNA into small

fragments thus making it biologically less active. Transformation which occurred in the present experiments could be deduced from two observations: (1) no reversions occurred in the controls, i.e. strains which were not treated with the donor DNA, or, were treated with their own DNA; (2) the transformation frequency diminished or totally disappeared when the transforming principle (the donor DNA) was subjected to treatment with heat, DNase or u.v. irradiation.

Our grateful thanks to Dr D. M. Bose former Director and Dr S. M. Sircar present Director, Bose Institute, for their continuous interest in the investigation.

REFERENCES

BUCKNALL, R. A. & MORTON, A. G. (1964). Attempted genetic transformation in *Penicillium chrysogenum*. *Nature, Lond.* **201**, 57.

CLUTTERBUCK, P. W., LOVELL, R. & RAISTRICK, H. (1932). XXVI. The formation from glucose by members of the *Pericillium chrysogenum* series of a pigment, an alkali-soluble protein and penicillin—the antibacterial substance of Fleming. *Biochem. J.* **26**, 1907.

GWINN, D. D. & THORNE, C. B. (1964). Transformation of *Bacillus licheniformis*. *J. Bact.* **87**, 519.

HOTCHKISS, R. D. (1951). Transfer of penicillin resistance in pneumococci by deoxyribonucleate derived from resistant cultures. *Cold Spring Harb. Symp. quant. Biol.* **16**, 457.

LEONARD, C. G., CORLEY, D. & COLE, R. M. (1966). Transformation of auxotrophic mutants of group H streptococci. *Science, N.Y.* **152**, 1255.

LERMAN, L. S. & TOLMACH, L. J. (1959). Genetic transformation. II. The significance of damage to the DNA molecule. *Biochim. biophys. Acta* **33**, 371.

MARMUR, J. & FLUKE, D. J. (1955). Uniformity of ionizing radiation on several transforming factors of pneumococcus. *Archs. Biochem. Biophys.* **57**, 506.

MISHRA, A. K. & NANDI, P. (1959). Ultraviolet induced biochemical mutations in *Aspergillus niger*. *Sci. Cult.* **25**, 81.

SHAMOIAN, C. A., CANZANELLI, A. & MELROSE, J. (1961). Back mutation of a *Neurospora crassa* mutant by a nucleic acid complex from wild strain. *Biochim. biophys. Acta* **47**, 208.

SHOCKLEY, T. E. & TATUM, E. L. (1962). A search for genetic transformation in *Neurospora crassa*. *Biochim. biophys. Acta* **61**, 567.

SPIZIZEN, J. (1958). Transformation of biochemically deficient strain of *Bacillus subtilis* by deoxyribonucleate. *Proc. natn. Acad. Sci. U.S.A.* **44**, 1072.

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Thermal Stability of Interspecies Neisseria DNA Duplexes

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SUMMARY

The thermal stability of interspecies DNA duplexes is markedly increased by raising the incubation temperature. When the DNA reassociation reactions are carried out at 75° in 0·12 M-phosphate buffer the thermal denaturation temperature of the reassociated product is almost identical to that of the native DNA, indicating that only DNA segments of very similar nucleotide sequence are associating. The genus *Neisseria* very clearly forms three groups based on the relatedness of their DNA to that of *N. meningitidis*; the 'pathogenic' Neisseria which have at least 80% of their nucleotide sequences similar; the 'non-pathogenic' Neisseria which share only 8 to 15%; and *N. catarrhalis* which shows no relatedness.

INTRODUCTION

The usefulness of DNA–DNA hybridization procedures in studying taxonomic relationships has been clearly demonstrated in many groups of bacteria (McCarthy & Bolton, 1963; Brenner, Martin & Hoyer, 1967; Kingsbury, 1967; Heberlein, De Ley & Tijtgat, 1967; Hoyer & McCullough, 1968; Johnson & Ordal, 1968). Recently, thermal stability has been used to investigate the extent of base pairing within reassociated interspecies polynucleotide sequences (Brenner & Cowie, 1967, 1968). The importance of incubation temperature in discriminating between distantly and closely related DNA sequences has been shown in several laboratories (Martin & Hoyer, 1966; Brenner *et al.* 1967; Brenner & Cowie, 1967, 1968; Johnson & Ordal, 1968). The experiments reported here utilized increased incubation temperature and studies of thermal stability to further assess previously determined relationships within the genus *Neisseria* (Kingsbury, 1967).

DNA relationships among the Neisseria are of particular interest since both pathogens and non-pathogens, as well as several tentatively assigned organisms, are included in this genus (Kingsbury & Ivler, to be published). Earlier work (Kingsbury, 1967) indicated that the Neisseria species form at least three distinct groups: the 'pathogenic' Neisseria, the 'non-pathogenic' Neisseria and the species *N. catarrhalis* and *N. caviae*. A similar view, based on genetic transformation studies in this genus, has been advanced by Henriksen & Bøvre (1968). The present study, using *N. meningitidis* as the reference organism, reaffirms this division of the genus *Neisseria* into three groups. Furthermore, the extensive duplex formation and the stability of reassociated nucleo-

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tide sequences shared between *N. meningitidis*, *N. gonorrhoeae* and strain Z (Slaterus, 1961) are indicative of extensive conservation of nucleotide sequences in the DNA of these pathogens.

METHODS

Organisms. The source of each strain used in this study and the procedures used for identification and cultivation have been described (Kingsbury, 1966, 1967).

Enzymes and radioisotopes. Ribonuclease and Pronase were purchased from Calbiochem, Los Angeles, California, U.S.A. The Pronase, in distilled water, was self-digested by incubation for 2 hr at 37° prior to use. Ribonuclease was heated at 90° for 10 min. to inactivate any deoxyribonuclease present as a contaminant. Adenine-8-^{14}C was purchased from Calbiochem. ^{32}P in the form of carrier-free H_2PO_4 was obtained from New England Nuclear Corporation, Boston, Massachusetts, U.S.A.

DNA extraction. Organisms were harvested from broth by centrifugation and washed in 150 ml of 0·15 M-NaCl + 0·1 M-EDTA, pH 8·0. Organisms were lysed and DNA prepared as previously described (Brenner & Cowie, 1968). The crude DNA preparations were repeatedly (3 to 4 times) precipitated with cold 95% ethanol and resuspended in SSC/100 (SSC = 0·15 M-NaCl + 0·015 M-sodium citrate; 3 × SSC is used to designate a threefold concentration). The DNA preparations were resuspended in a mixture of 0·1 M-NaCl, 0·05 M-EDTA and 0·5 M-tris buffer, and incubated with ribonuclease 25 μg./ml. at 37° for 60 min. Sodium lauryl sulphate was added to 0·5% and the DNA was incubated overnight at 37° with 50 μg/ml of Pronase. The concentration of sodium lauryl sulphate was increased to 1% and an equal volume of water-washed phenol was added. The mixture was shaken and centrifuged. The aqueous phase was collected and shaken twice with an equal volume of chloroform. The aqueous phase was then repeatedly (3 to 4 times) precipitated with 2 vol. of 2-ethoxyethanol. The DNA was finally resuspended in 0·12 M-PB (PB = a buffer solution consisting of equimolar Na_2HPO_4 and NaH_2PO_4, pH = 6·8).

DNA samples (except those unlabelled samples which were subsequently bound to nitrocellulose filters) were fragmented by mechanical shear at 50,000 p.s.i. to a molecular weight of approximately 2×10^5 (Britten & Kohne, 1966) and filtered through Metrical filter discs (0·45 μ pore size, Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.). Labelled single-stranded DNA fragments in 0·12 M-PB were further purified by passing them through a hydroxyapatite (Bio-Gel HT, Bio-rad Laboratories, Richmond, California, U.S.A.) column at 60° and discarding the material that bound to the column. (Single-stranded DNA does not bind to the column under these conditions.)

Preparation of labelled DNA. ^{14}C-labelled DNA was prepared as previously described (Kingsbury & Duncan, 1967). ^{32}P labelling was accomplished by growing the bacteria in Frantz broth containing 5 μC/ml. of carrier-free H_2PO_4 and 0·001 M-unlabelled phosphate.

Formation of DNA hybrids on nitrocellulose filters. The hybridization procedures used for nitrocellulose filter reactions have been described (Kingsbury, 1967). In the present studies only direct binding experiments were carried out. In each case 125 μg. of DNA was immobilized on a 25 mm. B-6 filter (Schleicher and Schuell Company, Keene, New Hampshire, U.S.A.) and incubated with 1 μg. of ^{14}C-labelled DNA fragments.

Thermal elution of reassociated DNA duplexes from hydroxyapatite. A modification of the technique of Miyazawa & Thomas (1965) for fractionating double-stranded

DNA bound to hydroxyapatite was used to determine the stability of reassociated Neisseria DNA (Britten & Kohne, 1966). Thermally denatured ^{32}P-labelled *N. meningitidis* DNA fragments (0·1 μg., containing 2000 to 5000 counts/min.) were incubated at 60° or 75° with an approximate 5000-fold excess of homologous or heterologous denatured, unlabelled DNA fragments in 1 ml. of 0·12 M-PB. The duration of incubation (12 hr) was chosen to insure maximal reassociation of the unlabelled DNA fragments. The 'zero-time' binding (the amount of labelled DNA bound to hydroxyapatite immediately after denaturation) was between 1 and 2%. The control values obtained for reassociation of labelled fragments with one another during the 12 hr incubation period were between 2 and 4%.

Following incubation, samples were quickly cooled and frozen until use. Each sample was subsequently thawed and passed through 10 ml. of hydroxyapatite equilibrated with 0·12 M-PB at the temperature at which the fragments had been incubated. The earlier hydroxyapatite experiments used water-jacketed columns. Later experiments employed a batch procedure for thermal elution which allowed six or more samples to be processed simultaneously. In the batch procedure reaction mixtures were added to 10 ml. of hydroxyapatite contained in test tubes in a circulating water bath. Elutions were accomplished by sedimenting the tubes in a heated centrifuge. The details of the batch procedure are given elsewhere (Brenner, Fanning, Rake & Johnson, private communication). Virtually identical results were obtained with either the column or batch procedure. Each sample was washed with 15 ml. portions of 0·12 M-PB until the eluted radioactivity (representing unreassociated DNA not bound to hydroxyapatite) was down to a background level (5–6 washes). The hydroxyapatite-bound DNA was then consecutively washed with 15 ml. portions of 0·12 M-PB at increasing 2·5° temperature increments to 100° and finally with 2–3, 15 ml. portions of 0·4 M-PB to elute any material remaining bound to the column. When the elution temperature was raised above the dissociation temperature of the reassociated nucleotide sequences, the resultant single-stranded DNA was eluted from the column.

The contents of the elution tubes were precipitated in 5% trichloracetic acid in the presence of approximately 100 μg. of yeast ribonucleic acid carrier. The precipitates were collected on membrane filters, dried, and placed in counting vials. Scintillation fluid was added (15 ml. per sample) and the samples were counted in either a Packard (Packard Instrument Company, Downers Gove, Illinois, U.S.A.) or a Nuclear Chicago (Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.) liquid scintillation spectrometer. Percentage reassociation was determined by dividing the counts present in the thermal elution by the total counts.

RESULTS

Effect of temperature on DNA–DNA reassociation. The effect of different incubation temperatures on interspecies DNA reassociation is shown in Table 1. When the incubation temperature was raised from 60° to 75°, the reassociation of homologous *Neisseria meningitidis* DNA dropped some 5–8% (the term reassociation is applied to both the homologous and heterologous reactions although the interspecies strands had never before been associated). The relative ability of *N. meningitidis* DNA fragments to reassociate with DNA from *N. gonorrhoeae* or strain z was only slightly diminished at the more stringent 75° incubation temperature. (Relative binding is used to compare the interspecies reactions with the homologous reaction under the identical

incubation conditions. In every case, the homologous *N. meningitidis* reaction is arbitrarily designated 100%.) Relatedness between *N. meningitidis* and *N. flava* was diminished more than twofold and between *N. meningitidis* and *N. sicca* was diminished more than fivefold when the criterion was changed to the high temperature of incubation. There was very little relatedness between *N. meningitidis* and *N. catarrhalis* at 60°. At 75°, no reaction was detectible between the DNA of these organisms.

As the ionic strength was raised, the effective temperature of incubation was lowered. Sodium ion concentration was similar in 0·12 M-PB and SSC. The three-fold increase in ionic strength from 0·12 M-PB to 3 × SSC resulted in a 5 to 6° decrease in effective incubation temperature (Marmur & Doty, 1962). Therefore, when the incubation is done at 67° in 3 × SSC, the extent of the reaction should be similar to that occurring at 60° in PB. The data presented in the last column of Table 1 show this to be true.

Table 1. *Relatedness of the DNA extracted from Neisseria species*

	Relative relatedness to labelled *N. meningitidis* DNA*				
	60° Incubation (0·12 M-PB)		75° Incubation (0·12 M-PB)		67° Incubation (3 × SSC)
Source of unlabelled DNA	%	Tm†	%	Tm	%
N. meningitidis	(100)‡	87	(100)	88	(100)
N. gonorrhoeae	78	85	76	86	80
Strain z	88	87	81	87	89
N. sicca	45	77	8	86	45
N. flava	35	75	15	86	30
N. catarrhalis	10	79	0	—	15
E. coli	0	—	0	—	0

* Average of 2 to 4 experimental values. The 60° and 75° incubations in 0·12 M-PB were carried out in free solution and assayed on hydroxyapatite. The reactions at 67° in 3 × SSC utilized unlabelled DNA immobilized on 25 mm. nitrocellulose filters.

† Tm = temperature at which 50% of the DNA is denatured.

‡ Homologous *N. meningitidis* reassociation was arbitrarily designated 100%. Relatedness is expressed as % relative to the homologous DNA reaction. The homologous reaction at 60° in PB gave approximately 85% reassociation; at 75° in PB, 75–80%; and 67° in 3 × SSC, approximately 60%.

Little or no difference was observable in the thermal elution midpoint (Tm) obtained from reaction products obtained at 60° or 75° involving homologous *Neisseria meningitidis* DNA fragments. Similarly, thermal elution mid-points from reactions involving the closely related DNA of *N. meningitidis*, *N. gonorrhoeae* and strain z were close to identical at both incubation temperatures. Alternatively, the marked decreases in duplex formation in *N. meningitidis–N. sicca* and *N. meningitidis–N. flava* reactions at 75° was accompanied by marked increases in the corresponding elution mid-points.

Relationships between Neisseria meningitidis and the other Neisseria species. It is clear from Table 1 that these Neisseria species fall into three main groups on the basis of DNA relatedness. First are the species normally associated with disease, *N. meningitidis*, *N. gonorrhoeae*, and strain z. The second group contains species generally considered 'non-pathogenic' represented by *N. sicca* and *N. flava*. The third group has little relationship to the other Neisseria tested and is represented in this study by *N. catarrhalis*.

The specificity of the DNA reassociation reaction was clearly increased at the higher incubation temperature. Figure 1 shows thermal elution profiles of interspecies DNA

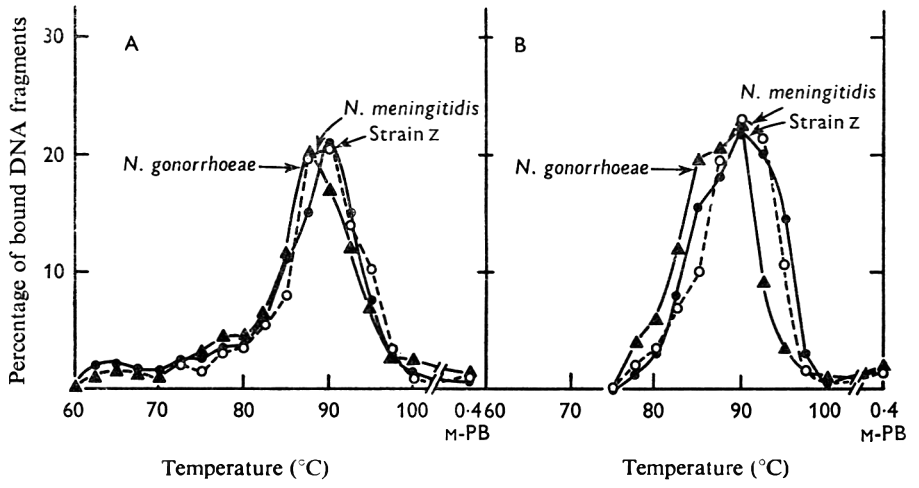

Fig. 1 (A). Thermal elution profiles of reassociated DNA duplexes formed between pathogenic Neisseria at 60°. 0·1 μg samples of single-stranded, ^{32}P-labelled *Neisseria menigitidis* DNA fragments were incubated with approximately 500 μg. of unlabelled single-stranded DNA from *N. meningitidis*, *N. gonorrhoeae* or strain z. (B). Thermal elution profiles of reassociated DNA duplexes formed between pathogenic Neisseria at 75°. Reactions are identical to those in (A) except for incubation temperature.

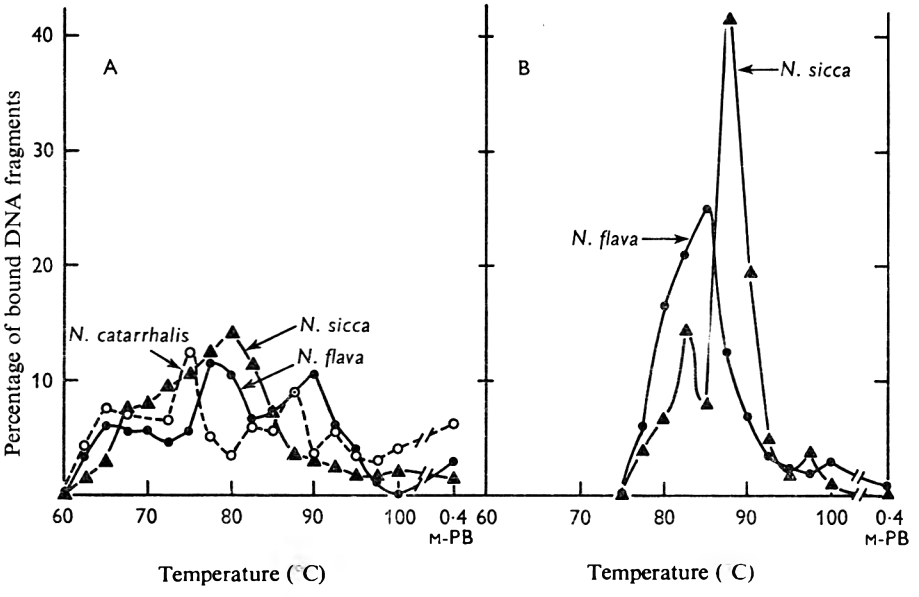

Fig. 2. (A) Thermal elution profiles of reassociated DNA duplexes formed between *Neisseria meningitidis* and non-pathogenic Neisseria species at 60°. 0·1 μg. samples of single-stranded, ^{32}P-labelled *N. meningitidis* DNA fragments were incubated with approximately 500 μg. of unlabelled, single-stranded DNA from *N. flava*, *N. sicca* and *N. catarrhalis*. (B) Thermal elution profiles of reassociated DNA duplexes formed between *Neisseria meningitidis* and non-pathogenic Neisseria species at 75°. Reactions are identical to those in (A) except for incubation temperature. No observable reaction took place between *N. meningitidis* and *N. catarrhalis* at 75° (see Table 1).

duplexes from group 1 when the incubations were carried out at 60° (Fig. 1*a*) and 75° (Fig. 1*b*) in 0·12 M-PB. The profiles are sharp and tend towards a Gaussian distribution at both incubation temperatures. In both cases, the Tm of these interspecies DNA duplexes was within one or two degrees of that exhibited by a homologous *N. meningitidis* reaction. (Tm = temperature of which 50% of the DNA is denatured.)

In contrast, Fig. 2 shows thermal elution profiles obtained from reactions between *N. meningitidis* and members of groups 2 and 3. The profiles from the 60° (Fig. 2*a*) incubation were very broad and the Tm was depressed as much as 13° below that of reassociated *N. meningitidis* DNA. The greater specificity of the duplexes formed at 75° (Fig. 2*b*) is shown by the comparative sharpness and the greatly increased thermal stability of profiles obtained from reactions carried out at the higher temperature. Where no interspecies polynucleotide sequences exist with a stability approaching that of *N. meningitidis* DNA, as is apparently the case with *N. catarrhalis*, no DNA duplexes were detectible by the more selective criterion.

To insure reaction specificity, a control DNA from *Escherichia coli* was included in each of these experiments. Although its base composition is similar to that of *N. meningitidis*, 50 mole % guanine+cytosine (G+C), no relationship was detected between the DNA of *N. meningitidis* and *E. coli*, at any of the three incubation temperatures used.

DISCUSSION

The optimal temperature for reassociation of denatured DNA free in solution is some 25° below its Tm (Marmur & Doty, 1962). The Tm for *Neisseria meningitidis* DNA is about 88° in 0·12 M-PB and about 93° in 3 × SSC. Therefore, reactions carried out at 60° in 0·12 M-PB and at 67° in 3 × SSC closely approximate optimal conditions for reassociation. A 75° incubation temperature for reactions in 0·12 M-PB constitutes a highly stringent criterion for reassociation in the Neisseria, as evidenced by a 5–8% decrease in homologous *N. meningitidis* DNA duplex formation. The use of stringent incubation conditions allows the formation of only those interspecies DNA duplexes that exhibit a high degree of thermal stability. It seems likely that these stable interspecies duplexes reflect extensive base sequence similarity between organisms. Similarly, duplexes that exhibit a Tm 10° to 25° below the Tm of homologous *N. meningitidis* DNA (Fig. 2) and which are not formed at 75° may be assumed to contain a significant proportion of unpaired nucleotide bases.

An alternative explanation for decreased stability in interspecies DNA duplexes is that the related sequences contain a preferentially high percentage of adenine (A)+thymine (T) base pairs. This explanation is considered unlikely because bacterial DNA usually shows a sharp and unimodal thermal melting transition indicative of a Gaussian distribution of G+C centred around the mean base composition. In the *N. meningitidis*–*N. sicca* reaction (Table 1), and in several interspecies enterobacterial DNA reactions (Brenner & Cowie, 1968; D. J. Brenner & S. Falkow, unpublished observations), at least 75% of the duplexes (corresponding to up to 30% of the genome) formed at 60° are lost in a 75° reaction. It is doubtful whether bacterial DNA with a mean 50 mole % G+C contains such a large fraction of A+T-rich sequences.

This argument does not rule out a combination of unpaired bases and preferentially high A+T containing duplexes as causative factors in reducing the Tm. It has been reported (Brenner & Cowie, 1968) that at least half of the diminished stability in

Escherichia coli–Salmonella typhimurium duplexes is attributable to unpaired bases. Preliminary results using labelled *E. coli* DNA fractionated according to base composition and reacted with *S. typhimurium* DNA give no indication of increased interspecies reaction when labelled A+T-rich fragments are used (D. J. Brenner, unpublished observation). This question remains to be answered directly for the Neisseria reactions.

The existence of three subgroups within the genus *Neisseria*, based on DNA relatedness, as suggested earlier (Kingsbury, 1967), is firmly supported by the present data on interspecies duplex stability. The pathogenic species tested are very closely related and form stable interspecies hybrids. Reciprocal experiments using labelled strain z DNA and unlabelled *N. meningitidis* also give an approximate value of 90% relative relatedness (67°, 3×SSC) (D. T. Kingsbury, unpublished observation). The coincidence of the reciprocal values rules out the possibility that strain z is a deletion mutant of *N. meningitidis*. Hoyer & McCullough (1968) noted even higher relatedness in Brucella species. It is tempting to speculate that the apparent conservation of nucleotide sequences seen in both of these pathogenic genera is the result of selection pressures resulting from obligate host–parasite interaction.

The degree of relatedness among the bulk of non-pathogenic Neisseria species is not nearly as high as that of the pathogens (Kingsbury, 1967). The stability patterns in non-pathogenic interspecies duplexes remain to be investigated. The low degree of relatedness and lack of stable interspecies duplexes between *Neisseria catarrhalis* and *N. meningitidis* is not surprising in view of the low G+C of *N. catarrhalis*. Catlin & Cunningham (1961) found *N. catarrhalis* strains with G+C percentages ranging from 40·7 to 44·6. These authors suggested that the *N. catarrhalis* strains formed a group unto themselves.

The recent work of Henriksen & Bøvre (1968), suggesting that *Neisseria meningitidis*, *N. gonorrhoeae*, *N. sicca* and *N. flava* may be variants of the same species, is not supported by DNA reassociation data.

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The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

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REFERENCES

BRENNER, D. J. & COWIE, D. B. (1967). Qualitative aspects of microbial DNA duplexes. *Yb. Carnegie Instn Wash.* **66**, 106.

BRENNER, D. J. & COWIE, D. B. (1968). Thermal stability of *Escherichia coli–Salmonella typhimurium* deoxyribonucleic acid duplexes. *J. Bact.* **95**, 2258.

BRENNER, D. J., MARTIN, M. A. & HOYER, B. H. (1957). Deoxyribonucleic acid homologies among some bacteria. *J. Bact.* **94**, 486.

BRITTEN, R. J. & KOHNE, D. E. (1966). Nucleotide sequence repetition in DNA. *Yb. Carnegie Instn Wash.* **65**, 78.

CATLIN, B. W. & CUNNINGHAM, L. S. (1961). Transforming activities and base contents of deoxyribonucleate preparations from various Neisseriae. *J. gen. Microbiol.* **26**, 303.

HEBERLEIN, G. T., DE LEY, J. & TIJTGAT, R. (1967). Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. *J. Bact.* **94**, 116.

HENRIKSEN, S. D. & BØVRE, K. (1968). The taxonomy of the genera *Moraxella* and *Neisseria*. *J. gen. Microbiol.* **51**, 387.

HOYER, B. H. & MCCULLOUGH, N. B. (1968). Polynucleotide homologies of *Brucella* deoxyribonucleic acids. *J. Bact.* **95**, 444.

JOHNSON, J. L. & ORDAL, E. J. (1968). Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. *J. Bact.* **95**, 893.

KINGSBURY, D. T. (1966). Bacteriocin production by strains of *Neisseria meningitidis*. *J. Bact.* **91**, 1696.

KINGSBURY, D. T. (1967). Deoxyribonucleic acid homologies among species of the genus *Neisseria*. *J. Bact.* **94**, 870.

KINGSBURY, D. T. & DUNCAN, J. F. (1967). Use of exogenous adenine to label the nucleic acids of wild-type *Neisseria meningitidis*. *J. Bact.* **94**, 1262.

MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. molec. Biol.* **5**, 109.

MARTIN, M. A. & HOYER, B. H. (1966). Thermal stabilities and species specificities of reannealed animal deoxyribonucleic acids. *Biochemistry* **5**, 2706.

MCCARTHY, B. J. & BOLTON, E. T. (1963). An approach to the measurement of genetic relatedness among organisms. *Proc. natn. Acad. Sci. U.S.A.* **50**, 156.

MIYAZAWA, Y. & THOMAS, C. A. (1965). Composition of short segments of DNA molecules. *J. molec. Biol.* **11**, 223.

SLATERUS, K. W. (1961). A serological typing of meningococci by means of micro-precipitation. *Antonie van Leeuwenhoek* **21**, 305.

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An Investigation into the Mode of Action of Actinonin

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SUMMARY

The mode of action of actinonin, a pseudo-peptide antibiotic has been investigated. When added to cultures of *Bacillus subtilis* 7198 and 3610 actinonin inhibits growth. Evidence is presented which shows that this inhibition of growth reflects a bacteriostatic rather than bactericidal effect and that the site of action of the antibiotic is associated with RNA synthesis.

INTRODUCTION

The antibiotic antinonin first obtained from the culture filtrates of a Streptomyces species classified as *Streptomyces* sp. CUTTER C/2 NCIB 8845 (Gordon, Kelly & Miller, 1962) has been shown to have the constitutional formula of the pseudo-peptide shown in Fig. 1 (Ollis, East, Gordon & Sutherland, 1964). As a natural product this structure is of interest since it is the first known naturally occurring derivative of L-prolinol and is also the first known naturally occurring hydroxamic acid of the type R—CO—NHOH. Actinonin shows some structural relationship to other polypeptide antibiotics but it

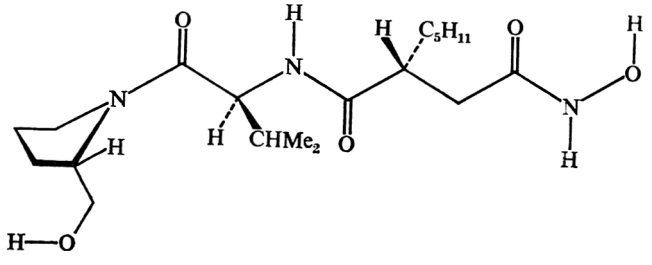

Fig. 1. The constitution of actinonin.

contains no D-α-amino acid residue. This absence of D-α-amino acid residues in association with antibiotic activity should be considered in relation to the idea put forward by Arnstein (1957) that amongst the polypeptide antibiotics the minimum requirement for biological activity is the presence of one amino acid residue with the D-configuration. Actinonin has been shown to be active against, in the main, Gram-positive bacteria, with some evidence of activity against Gram-negative bacteria, the acid-fast bacteria and a number, of phage strains (Gordon *et al.* 1962). This wide and in some instances unusual biological activity coupled with its chemical stability and low toxicity makes actinonin an interesting antibiotic. This work describes investigations directed towards an understanding of the mode of action of actinonin.

METHODS

Organisms and growth conditions. The organisms used in this investigation were *Bacillus subtilis* (NCIB 7198) and *B. subtilis* (NCIB 3610).

The bacteria were grown overnight at 30° with constant shaking in a defined medium of Davis mineral medium (Davis & Mingioli, 1960) supplemented with L-asparagine and L-glutamic acid (10 μg./ml.). Bacteria from this suspension were used to inoculate fresh medium and the growth of the bacteria was followed by measuring the extinction of the suspension in a Unicam SP 600 at 610 mμ. Any additions to the system were made at the end of the lag period of bacterial growth unless otherwise stated. Actinonin was always added as an aqueous solution and was stable under the experimental conditions.

Viable counts were made in triplicate on the defined medium plus agar (1·5%, w/v) using the surface-spreading method after serial dilution of the bacteria in saline to ensure that 100 to 200 bacteria were distributed on each plate.

Synthesis of cellular constituents. (1) *Net synthesis of cellular RNA, DNA and protein.* Suspensions of *Bacillus subtilis* 7198 were incubated at 30° with shaking in the defined medium in the presence and absence of actinonin. Samples (3 ml.) were taken at intervals and cooled rapidly. The bacteria were harvested by centrifugation and washed with a buffered salt solution. The bacterial pellet was extracted three times with 0·2 N-perchloric acid at 4° for 60 min. and the supernatant fluids from the extractions combined. This constituted the bacterial pool fraction. The nucleic acids were extracted from the pellet with 0·5 N-perchloric acid (3 ml.) at 70° for 15 min. Three extractions were required.

The RNA was estimated by the orcinol method (Schneider, 1957) and the DNA by the diphenylamine method of Burton (1956). The residual pellet was taken up in N-NaOH and the protein present estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). All estimations were performed in triplicate and gave an accuracy of $\pm 5\%$.

(2) *The production of bacteriophage infection centres. Bacillus subtilis* 3610 growing exponentially in nutrient broth was harvested when a titre of 2×10^8 bacteria/ml. was obtained and then infected with phage øE at a multiplicity of three. The mixture was shaken and incubated for 15 min. at 30° to allow the phage to become absorbed to the bacteria. The infected suspension was then centrifuged (5000 *g*) and the excess free phage particles removed with the supernatant fluid. The bacteria were resuspended in nutrient broth and diluted 4×10^5-fold with fresh growth medium. The latent period of the phage infection in this system is 70 to 80 min. Actinonin at concentrations sub-inhibitory to bacterial growth was added or removed at various times throughout the latent period. Samples were taken at the end of the latent period and assayed for total infective centres (plaque formers) by the procedure described by Adams (1959).

RESULTS

The effect of actinonin upon the viability of Bacillus subtilis 7198. The limiting growth inhibitory concentration for *B. subtilis* 7198 under these conditions was seen to be 2 mg./ml. At this concentration the effect was bacteriostatic rather than bactericidal. The results are shown in Table 1.

Resistance of Bacillus subtilis to repeated additions of actinonin. Bacteria grown in the presence of actinonin for 24 hr or more were resistant to further treatment with actinonin (Fig. 2) but had a slower growth rate than the untreated control bacteria. No information has been obtained from which the possible mechanism of this resistance could be ascertained.

Respiration and fermentation. Actinonin (at 100, 1000 and 3000 μg./ml.) had no effect on endogenous respiration, oxidation of glucose or anaerobic fermentation of glucose when added to washed suspensions of *Bacillus subtilis* 7198 in Warburg vessels. These studies were made by the standard manometric techniques (Umbreit, Burris & Stauffer, 1964).

Permeability and integrity of the cell membrane. Bacteria previously grown in a rich medium were suspended in buffer in the presence and absence of actinonin

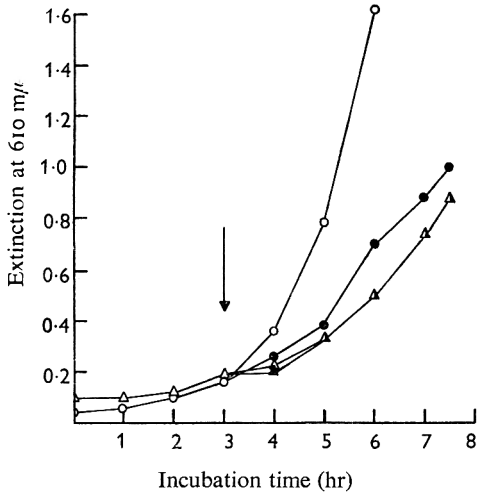

Fig. 2

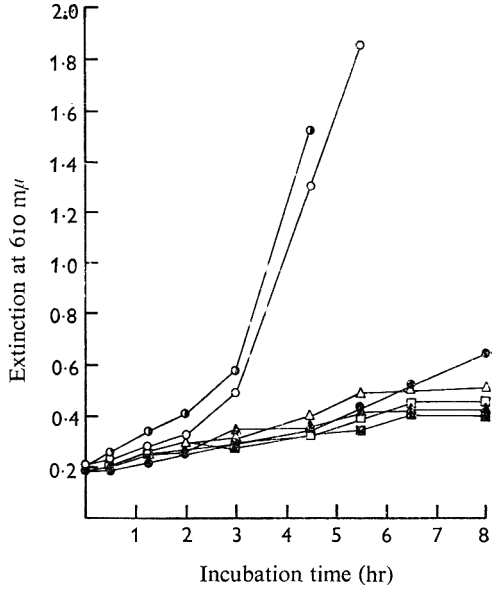

Fig. 3

Fig. 2. The resistance of 'actinonin pretreated' *Bacillus subtilis* 7198 to further treatment with actinonin. A suspension of *B. subtilis* 7198 was grown in defined medium containing actinonin (100 μg./ml.) for 24 hr. This culture became the 'pretreated' inoculum. Another suspension of *B. subtilis* 7198 was grown in defined medium alone for 24 hr—this culture became the control inoculum. Both types of inoculum were then used to inoculate fresh medium alone (○—○, control inoculum; and △—△, pretreated inoculum). After 3 hr incubation (at arrow) actinonin (100 μg./ml. final conc.) was added to one of the control inoculum cultures (●—●) and to one of the pretreated cultures (▲—▲). Growth was followed as described in the Methods section. When actinonin (100 μg./ml.) was added to the pretreated inoculum (▲—▲) growth of this sample and the control pretreated inoculum was identical (▲—▲).

Fig. 3. The effect of adding Mg^{2+} ions to a suspension of *Bacillus subtilis* 7198 growing in the presence of actinonin. A suspension of *B. subtilis* 7198 was incubated at 30° in the defined medium containing actinonin (100 μg./ml.) and 100 μg. Mg^{2+} ions/ml. (△—△); 200 μg. Mg^{2+}/ml. (▲—▲); 500 μg. Mg^{2+} ions/ml. (□—□) or 1000 μg. Mg^{2+} ions/ml. (■—■). Control suspensions of *B. subtilis* were incubated in the defined medium alone (○—○); defined medium with 1000 Mg^{2+} ions/ml. (◑—◑) and defined medium containing actinonin (100 μg./ml.) only (●—●). The growth was followed as described in the Methods section.

(100 and 1000 μg./ml.). The rate of release of amino acids, purines and pyrimidines from the bacteria into the medium was measured (Gale & Taylor, 1947). There was no evidence of increased permeability of the bacteria to these molecules in the presence of the antibiotic.

Chelation and antimetabolite studies. The addition of the metal ions Mg^{2+}, Mn^{2+}, Fe^{2+}, K^{+} and Ca^{2+} (0 to 1000 μg./ml.), the amino acids L-valine and L-isoleucine, D-alanine or L-aspartic acid (0 to 500 μg./ml.) to bacteria growing in the presence of actinonin failed to annul the growth inhibitory effect of the antibiotic. Figure 3 shows

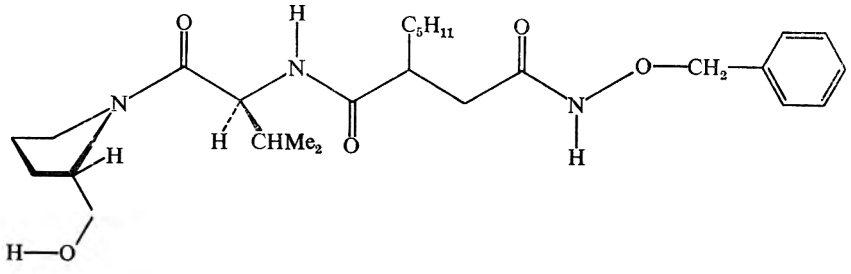

Fig. 4. The constitution of *O*-benzylactinonin.

Table 1. *Viability of Bacillus subtilis 7198 grown in the presence of increasing concentrations of actinonin*

B. subtilis (6×10^6 bacteria/5 ml.) was incubated at 30° for up to 4 hr in the defined medium. Actinonin in sterile distilled water was added in the concentrations shown at the beginning of the incubation period. Samples (0·1 ml.) were taken at the times indicated below and plated out in triplicate on to defined media as described in the Methods section and incubated for 24 hr at 30°. The colonies formed were then counted. Accuracy obtained was ±5%.

Actinonin added (mg./ml.)	Time (hr) 0	1	2	3
0	6×10^6	$7{\cdot}3 \times 10^6$	$1{\cdot}2 \times 10^7$	$3{\cdot}7 \times 10^7$
0·1		$6{\cdot}2 \times 10^6$	$8{\cdot}0 \times 10^6$	$2{\cdot}2 \times 10^7$
1·0		$5{\cdot}9 \times 10^6$	$6{\cdot}6 \times 10^6$	$1{\cdot}8 \times 10^7$
2·0		$6{\cdot}1 \times 10^6$	$6{\cdot}4 \times 10^6$	$6{\cdot}2 \times 10^6$
3·0		$6{\cdot}1 \times 10^6$	$6{\cdot}0 \times 10^6$	$5{\cdot}7 \times 10^6$

Table 2. *Growth inhibitory effect of actinonin and O-benzylactinonin on a culture of Bacillus subtilis 7198 growing in the test system*

Organisms were grown in defined medium in Monod flasks in presence of compound under test. The concentration of compounds varied from 100 to 2000 μg./ml. Growth was measured at 610 mμ. Inhibition was calculated as the percentage of the growth measured in the absence of any added compound.

Compound added	Concn. (μg./ml.)	% growth inhibition after 3 hr incubation in presence of test compound
Actinonin	100	44
	1000	50
O-Benzylactinonin	500	5
	1000	14
	2000	38

the results obtained using Mg^{2+} ions. Identical relationships were seen when the other additions were made to the system. Furthermore, the addition of a synthetic analogue of actinonin, *O*-benzylactinonin (kindly supplied by Mr J. P. Devlin) consistently and reproducibly produced an inhibition of bacterial growth. Results shown in Table 2. This analogue has the free hydroxamic acid residue blocked by the benzyl group (see Fig. 4).

Synthesis of cellular constituents. An investigation during the first 75 min. of growth after the addition of the antibiotic shows that the total synthesis of RNA and protein are inhibited (Table 3), but that DNA synthesis is not significantly affected. However, the initial site of action of the antibiotic appears to be associated with RNA synthesis since a significant reduction of total RNA is evident after 15 min. incubation, whereas a reduction in total protein is only evident after 30 min. incubation.

Table 3. *The effect of actinonin upon the growth and total synthesis of protein, RNA and DNA of Bacillus subtilis*

Six Monod flasks containing defined medium were inoculated with *B. subtilis* 7198 and incubated with shaking until the exponential growth phase was established (O.D. 0·405 at 610 mμ which gave a dry wt of 6·6 mg./3 ml. sample). To one set of three flasks actinonin was added in aqueous solution to give a final concentration of 1 mg./ml., to the other remaining three flasks an equal volume of sterile distilled water was added. The flasks were then returned to the water bath 37° and incubated with shaking. At intervals of 15 min. growth was measured (see Methods section) and samples (3 ml.) taken and the total protein, RNA and DNA estimated (see Methods section).

	Growth		Total protein (mg.)		Total RNA (mg.)		Total DNA (mg.)	
Time (min.)	−Actinonin	+Actinonin	−Actinonin	+Actinonin	−Actinonin	+Actinonin	−Actinonin	+Actinonin
0	0·41	0·41	3·30	3·30	1·05	1·05	0·15	0·15
15	0·52	0·52	3·84	3·80	1·32	1·20	0·15	0·14
30	0·64	0·63	4·50	4·29	1·56	1·22	0·15	0·15
45	0·77	0·71	5·43	5·01	1·87	1·31	0·17	0·16
60	0·92	0·75	6·45	5·60	2·28	1·37	0·29	0·28
75	1·10	0·89	7·29	5·63	2·43	1·42	0·31	0·29

Table 4. *The percentage inhibition of formation of infective centres caused by addition of actinonin to the Bacillus subtlis 3610/phage øE system*

Phage particles were allowed to adsorb and infect the bacteria: (A) After increasing time intervals actinonin was added to the system and the incubation continued (see Methods section). (B) Immediately after phage infection actinonin was added and removed by dilution at time intervals throughout the latent period (see Methods section). In both cases at the end of the latent period the bacterial suspensions were analysed for infective centre formation.

A. Time when actinonin added (min.)	% inhibition of infective centre production	B. Time when actinonin removed (min.)	% inhibition of infective centre production
0	98	0	—
10	98	10	57
20	97	20	67
30	98	30	57
40	98	40	57
50	—	50	57
60	95	60	79
70	—	70	74

Effect of actinonin upon the production of infective phage centres in Bacillus subtilis 3610 phage øE system. The addition of actinonin, only for the adsorptive period for the phage, showed that the antibiotic did not inhibit phage adsorption. Further studies during which actinonin was added: (*a*) at intervals throughout the adsorption period, and (*b*) for increasing time into the latent period before being removed from the system, demonstrated that actinonin affected some process which occurred during the first 10 min. of the latent period. Moreover, if the actinonin was not added until after an increasing time interval during the latent period and then left in the system for the rest of the latent period, the antibiotic affected some process which occurs late in the latent period. These results are shown in Table 4. When other antibiotics whose site of action have been reported were added to the system in such a manner and the results compared with those obtained for actinonin (Table 5), actinonin was shown to resemble actinomycin-D rather than chloramphenicol or 5-aminoacridine.

Table 5. (A) *Percentage inhibition of formation of infective centres caused by the addition of growth inhibitory substances at time intervals throughout the latent period after infection. Bacillus subtilis 3610/phage øE system*

Bacteria were infected with phage particles and after increasing time intervals growth inhibitory substances were added to the system and the incubation continued (see Methods section). At the end of the latent period the cell suspensions were analysed for infective centre formation.

Time when substance added (min.)	Growth inhibiting substance added			
	Actinomycin-D (1 μg./ml.)	5′-Aminoacridine (20 μg./ml.)	Chloramphenicol (100 μg./ml.)	Actinonin (100 μg./ml.)
0	99	100	97	98
10	99	92	91	98
20	97	95	80	97
30	95	98	46	98
40	96	87	50	98
50	97	74	—	—
60	95	46	43	95
70	50	34	54	71

(B) *Percentage inhibition of formation of infective centres caused by addition of growth inhibitory substances during adsorption of the phage and then removed at time intervals throughout the latent period. Bacillus subtilis 3610/phage øE system*

Bacteria were infected with phage particles and immediately the growth inhibitory substances were added (see Methods section). At time intervals throughout the latent period the inhibiting substances were removed from the infected cells by dilution and incubation continued. At the end of the latent period the cells were analysed for infective centre formation.

Time when substance removed (min.)	Growth inhibiting substance			
	Actinomycin-D (1 μg./ml.)	5′-Aminoacridine (20 μg./ml.)	Chloramphenicol (100 μg./ml.)	Actinonin (100 μg./ml.)
10	31	0	0	57
20	94	0	0	67
30	35	76	52	57
40	100	76	44	57
50	87	83	100	57
60	100	91	100	79
70	94	80	96	74

DISCUSSION

When the structure of actinonin with its free hydroxamic acid residue is considered the failure to reverse the inhibitory action of actinonin by the addition of metal ions to the system is both interesting and surprising. This is further emphasized by the reports that the growth inhibitory action of aspergillic acid, a cyclic hydroxamic acid, has been reversed by the addition of ferrous ions (Goth, 1945). Furthermore, as a hydroxamic acid derivative there is some structural similarity between actinonin, D-cycloserine and Hadacidin (Fig. 5). Thus from these structural considerations it appeared possible that the biological activity of actinonin could involve an anti-metabolite/essential metabolite relationship. However, the addition of the respective essential metabolites D-alanine and L-aspartic acid failed to annul the growth inhibitory effect of actinonin. Hogg, Biswas & Broquist (1965) reported that cyclic

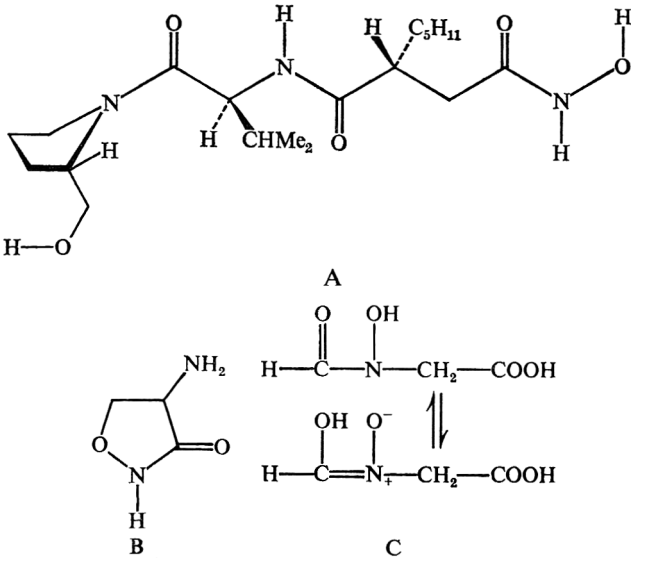

Fig. 5. The constitution of actinonin (A), D-cycloserine (B) and Hadacidin (C).

hydroxamic acids inhibited the growth of *Escherichia coli* by interfering with the biosynthesis of L-valine and L-isoleucine. They showed that the toxicity of cyclic hydroxamates could be counteracted by the addition of mixtures of L-valine and L-isoleucine or by L-valine and L-isoleucine separately. Again the addition of L-valine and L-isoleucine, together or individually, did not reverse the inhibitory effect of actinonin.

This inability to reverse the growth inhibitory action of actinonin, particularly when essential metal ions were added, suggested that the hydroxamic acid residue is not part of the active site of the actinonin molecule. This idea was supported when an analogue of actinonin, *O*-benzylactinonin, in which the hydroxamic acid residue had been chemically blocked proved to be biologically active. The growth inhibitory action of this compound proved to be very small but was reproducible. When further analogues become available the position of the hydroxamic acid residue with respect to the active site of the molecule will be investigated further.

The results presented here are compatible with the idea that the over-all growth inhibitory effect of actinonin is associated with RNA synthesis. This is supported by the inhibitory effect of the antibiotic on the total amount of RNA synthesized within the first 75 min. of incubation in the presence of actinonin. Within this time total RNA, and protein synthesis together with growth is seen to be inhibited but the initial inhibitory effect is associated with RNA synthesis. Moreover, the pattern of infective centre formation obtained when actinonin is added or removed from the phage øE/*Bacillus subtilis* 3610 system during the latent period demonstrates two possible sites of antibiotic action with respect to time within the latent period. This fact is also compatible with the site of action being associated with RNA rather than DNA or protein synthesis. The activity of actinonin also resembles the pattern obtained when actinomycin D, a known inhibitor of DNA directed RNA synthesis, is used in the system instead of actinonin and does not resemble the patterns obtained in the system when the protein synthesis inhibitor, chloramphenicol or 5-aminoacridine, which is known to inhibit viral DNA synthesis, is used. Thus these results indicate that actinonin inhibits bacterial growth by interfering with total cellular RNA synthesis. However, the actual site of action of actinonin cannot be deduced from these results.

I am grateful to Professor W. D. Ollis (University of Sheffield) and Dr J. J. Gordon (Chemical Defence Experimental Establishment, Porton) for the gift of actinonin which was used in this work and to Mr D. J. Trott for technical assistance.

REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*, p. 473. New York: Interscience Publishers Inc.

ARNSTEIN, H. R. V. (1957). The biosynthesis of penicillin and some other antibiotics. *Ann. Rep. Chem. Soc. Lond.* **54**, 339.

BURTON, K. (1956). A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribosenucleic acid. *Biochem. J.* **63**, 315.

DAVIS, B. D. & MINGIOLI, E. S. (1960). Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. *J. Bact.* **60**, 17.

GALE, E. F. & TAYLOR, E. S. (1947). The assimilation of amino acids by bacteria. 2. The action of tyrocidin and some detergent substances in releasing amino acids from the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 77.

GORDON, J. J., KELLY, B. K. & MILLER, G. A. (1962). Actinonin: an antibiotic substance produced by an actinomycete. *Nature, Lond.* **195**, 701.

GOTH, A. (1945). The antitubercular activity of aspergillic acid and its probable mode of action. *J. Lab. clin. Med.* **30**, 899.

HOGG, R. W., BISWAS, C. S. & BROQUIST, H. P. (1965). Interference with valine and isoleucine biosynthesis by cyclic hydroxamic acids. *J. Bact.* **90**, 1265.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

OLLIS, W. D., EAST, A. J., GORDON, J. J. & SUTHERLAND, I. O. (1964). The constitution of actinonin—structural and synthetic studies. *Institute of Applied Microbiology Symposium*, no. 6, p. 204. Chemistry of Microbial Products.

SCHNEIDER, W. C. (1957). Determination of nucleic acids in tissues by pentose analysis. *Meth. Enzymol.* **3**, 580.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1964). *Manometric Techniques*, 4th ed. Burgess Publishing Co.

J. gen. Microbiol. (1969), 55, 217–226
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Environmental Control of Glycogen and Lipid Content of *Mycobacterium phlei*

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SUMMARY

When the growth of surface cultures of *Mycobacterium phlei* was limited by nitrogen or sulphur, the organisms synthesized both glycogen and lipid as endogenous reserves. Equal weights of glycogen and lipid accumulated intracellularly which, combined, may account for 50 % of the cell dry weight. Both storage materials also accumulated when growth was inhibited by chloramphenicol or *p*-fluorophenylalanine in otherwise nutritionally adequate media. In the absence of exogenous carbon substrate, the glycogen and lipid reserves were utilized as energy and carbon for nitrogen incorporation and continued growth. Evidence is presented which suggests that glycogen may be the preferred endogenous reserve in *M. phlei*.

INTRODUCTION

In their pioneer work on the physiology of the mycobacteria, Stephenson & Whetham (1922, 1924) showed that, following depletion of glucose in growth media, *Mycobacterium phlei* utilized its stored lipids and the respiratory quotient, which had been 1·1 to 1·75 during growth, fell to 0·8. With the observation of the same phenomenon in *M. tuberculosis* (Andrejew, 1948), the generalization has been accepted that lipids serve as a reserve of carbon and energy in the mycobacteria. However, the demonstration of glycogen as a cell constituent in the avian (Chargaff & Moore, 1944) and human tubercle bacilli (Kent & Stacey, 1949), and in *M. phlei* (German, Jones & Nadarajah, 1961), suggests that the reserves may not be exclusively lipid. In addition, studies with *M. phlei* which have shown the simultaneous accumulation of equivalent amounts of lipid and carbohydrate during growth in nitrogen-limited media (Tepper, 1965) also suggest that both substances may be reserves in this organism.

There is ample evidence that micro-organisms are able to utilize more than one material for endogenous metabolism (see Dawes & Ribbons, 1964), but, usually in response to cultural or environmental conditions, a preferentiallly utilized reserve substrate is deposited. Different micro-organisms may store more than one reserve material. *Escherichia coli* (Dagley & Johnson, 1953) and *Rhodospirillum rubrum* (Stanier, Doudoroff, Kunisawa & Contopoulou, 1959) have been reported to accumulate either glycogen-like polysaccharide or lipid; however, the particular reserve

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material deposited within the cell depended on the chemical nature of the carbon substrate. Recently, Damoglou & Dawes (1967) were unable to confirm the accumulation of lipid in *E. coli* and found the lipid content to be essentially constant irrespective of the carbon source used for growth. Thus, the suggestion that both lipid and carbohydrate may be deposited as endogenous reserves in *Mycobacterium phlei* is of particular interest since these accumulations occur simultaneously under the same cultural conditions.

Storage material accumulation by micro-organisms has been extensively studied (for reviews see Herbert, 1961; Dawes & Ribbons, 1964) and it is known that conditions which restrict growth, but not the assimilation of the carbon source, favour accumulation of reserve substances. The present paper reports the effects of nutritional limitations and growth inhibitors on the accumulation of storage material by *Mycobacterium phlei*, and the extent to which lipid and carbohydrate, principally glycogen, serve as endogenous reserves in this organism.

METHODS

Cultivation. Mycobacterium phlei strain 72 was grown as a surface pellicle on media having the following basal composition per litre: potassium citrate, 2·0 g.; $MgSO_4.7H_2O$, 0·6 g.; $ZnSO_4.7H_2O$, 25 mg.; $FeCl_3.6H_2O$, 0·1 g.; $CoCl_2.6H_2O$, 2 mg.; $MnCl_2.4H_2O$, 1 mg.; $CaCl_2.2H_2O$, 20 mg.; anhydrous K_2HPO_4, 6·0 g.; final pH 7·0. Either L-asparagine.H_2O (3·0 or 12·0 g./l.) or ammonium chloride (2·15 or 8·6 g./l.) served as the sole source of nitrogen (N) in the growth media and represent 60 and 240 mg. N/100 ml. medium, respectively. Glycerol (60 g./l.) served as the major carbon source. The amounts of additional carbon supplied by asparagine were represented by molar equivalents of neutralized fumaric acid in the ammonium chloride medium. Media were dispensed in 100 ml. volumes in 250 ml. Erlenmeyer flasks and autoclaved for 15 min. at 121°. Cultures were incubated at 37° and 60 % relative humidity.

Medium exchange. Surface cultures were grown to the desired age and then medium exchanges were made according to the following procedure. The original medium was aseptically siphoned from the pellicle. The dropped pellicle was washed once by refloatation with basal medium, and then fresh medium of the desired composition was immediately added to refloat the pellicle. For these exchange experiments replicate cultures were used, some of which were removed for analysis at the time of exchange of medium while the remaining cultures were used, in duplicate, for exchange and subsequent incubations. The substrate concentrations used in the exchange experiments were as follows, per litre of basal medium: high nitrogen, 8·6 g. NH_4Cl; low nitrogen, 2·15 g. NH_4Cl; and high carbon, 60·0 g. glycerol. In the exchange studies where sulphates were omitted, the corresponding chloride salts were substituted in the basal medium.

Sampling. For estimation of surface growth, duplicate flasks were filtered through tared Sharkskin filter paper (Schleicher & Schuell), washed with water, and dried to constant weight. For analytical determinations, bacteria were harvested by centrifugation, washed, lyophilized and stored over phosphorus pentoxide in a vacuum desiccator until ready for use. All cell analyses were conducted on pooled samples from two or more pellicles.

Cell composition. Nitrogen content was determined by the micro-Kjeldahl digestion and colorimetric Nessler procedure described by Wilson & Knight (1952). Total carbohydrates were determined by the anthrone method of Seifter, Dayton, Novic & Muntwyler (1950). Glycogen was determined by the specific enzymic degradation and microdetermination method of Bueding & Hawkins (1964). Lipids were extracted according to the method of Winder & O'Hara (1962), evaporated under a stream of warm air, and weighed. Volatile and non-volatile fatty acids were isolated and separated by gas-chromatographic procedures by methods described by Saz & Lescure (1966). Volatile fatty acids and fatty acid esters were tentatively identified by comparison of relative retention times with known fatty acids.

RESULTS

Growth of Mycobacterium phlei

Under the conditions of these experiments, all cultures had formed a thin pellicle on the surface of the medium by the third day and thereafter growth consisted in the thickening of this pellicle. In the cultures used for composition analyses (Table 1), the third day was representative of the late exponential phase of growth and older cultures represented post-exponential growth. Unlike bacterial cultures which reach a maximum in incorporated nitrogen, post-exponential growth of pellicles of *Mycobacterium phlei* was characterized by a gradual increase in nitrogen content. This observation suggests that pellicles are composed of two types of bacterial population: a majority of organisms which have stopped growing and a lesser population, presumably in contact with the medium, which continues to divide.

Accumulation of storage materials. The relationship of storage material accumulation to the nitrogen concentration in the environment is shown in Table 1. In all media tested, storage materials did not increase during exponential growth and the lipid, carbohydrate and glycogen of *M. phlei* remained constant at values determined by the nature and amount of the nitrogen source.

Maximal lipid and carbohydrate contents occurred during post-exponential growth in nitrogen-limited media (Table 1). The highest contents were reached in the nitrogen-limited asparagine medium with lipid at 24·4 % and carbohydrate at 36·6 % of the dry wt of the bacteria; glycogen represented 64 % of the carbohydrate fraction. In both nitrogen-limited media the greatest increase in cell constituents was observed in the carbohydrate fraction. Maximal pellicle weights were produced during the period of maximal lipid and carbohydrate synthesis.

When the nitrogen concentration of the medium was increased fourfold, there were essentially no changes in the lipid and carbohydrate contents during growth (Table 1). Lipid contents differed for bacteria grown in the two media but remained constant at 12 % in the asparagine medium and 18 % in the ammonia medium; carbohydrate was approximately 12 % of the dry wt of bacteria from both media. Glycogen was present in these bacteria, but represented only 1·5 to 4·0 % of the pellicle weight.

Nitrogen concentrations of 120 mg. were also found to be nitrogen-limiting and resulted in constituent amounts intermediate to those at 60 and 240 mg. N. The composition of bacteria grown in media containing 480 mg. N. was identical to those grown on 240 mg. N.

Under the conditions of these experiments, the composition of *Mycobacterium*

phlei from nitrogen-excess media, throughout growth, resembled the composition of these bacteria during exponential growth in all media. Their lipid and carbohydrate content can be considered to be the minimal values essential for cell structure and metabolism which are produced during optimal pellicle growth. Contents of lipid and glycogen above these amounts apparently represent non-essential endogenous reserves.

Nature of the storage materials. In these analyses the major component of the carbohydrate fraction which accumulated during growth on nitrogen-limited media was glycogen. The possibility that lesser components of the carbohydrate fraction may also serve as endogenous reserves is presently under investigation. Glycogens have been isolated from *Mycobacterium phlei* and characterized; the results will be reported separately.

Table 1. *Yields of organisms and storage material content of Mycobacterium phlei grown on nitrogen-limited and nitrogen-excess media*

		Yield		Constituents		
Growth medium	Age (days)	Dry wt (mg./100 ml. culture)	Nitrogen (mg./100 ml. culture)	Lipid (mg./mg. cell N)	Carbohydrate (mg./mg. cell N)	Glycogen (mg./mg. cell N)
Nitrogen-limited (60 mg. N/100 ml. medium)						
Asparagine	0	1	< 0·1	1·7	2·3	0·9
	3	74	5·8	1·6	2·2	0·9
	5	257	19·8	1·8	2·5	1·0
	7	1010	38·4	6·4	9·0	6·1
	9	1325	39·8	8·0	12·2	8·1
Ammonia	0	1	< 0·1	1·4	0·9	0·2
	3	196	17·1	1·4	0·9	0·2
	5	711	39·8	3·4	4·5	2·4
	7	907	46·3	4·2	4·3	2·2
	9	877	43·0	4·5	4·1	2·2
Nitrogen-excess (240 mg. N/100 ml. medium)						
Asparagine	0	1	< 0·1	1·4	1·4	0·5
	3	78	6·5	1·4	1·4	0·5
	5	541	44·4	1·4	1·5	0·5
	7	1528	122·2	1·5	1·5	0·6
	9	2072	178·2	0·8	1·1	0·2
Ammonia	0	1	< 0·1	2·0	1·2	0·2
	3	100	9·1	2·1	1·2	0·2
	5	685	60·3	1·9	1·4	0·2
	7	1236	100·1	2·2	1·6	0·5
	9	1523	123·4	2·3	1·2	0·2

The fatty acids in lipid extracts from exponentially growing *Mycobacterium phlei* (15% lipid content) and after post-exponential accumulation (22% lipid content) were separated by gas-chromatographic procedures and quantitated. No single fatty acid accounted for the accumulation of lipid (Table 2). There was a general increase in all fatty acids from C_1 to C_{18}, with the greatest increases in the C_{14} to C_{18} acids. Experiments are in progress to determine whether the stored lipids occur as free acids, glycerides or complex lipids in *M. phlei*. Other lipids, especially the hydroxylated and branched chain fatty acids which were not determined in this study, may also accumulate.

Medium exchange experiments

In contrast to the gradual modifications in the nutritional environment which result during growth, the medium exchange procedure exposes bacteria of known composition, uniformly and abruptly to controlled environmental changes. This procedure was undertaken to define more accurately the factors which regulate storage material synthesis and to study the utilization of the lipid and glycogen endogenous reserves.

Table 2. *Fatty-acid content of Mycobacterium phlei at different values of lipid accumulation*

Fatty acids in lipid extracts of *Mycobacterium phlei* (20 mg. cell N) were separated by gas-chromatographic procedures. Contents are expressed as peak areas (cm.^{2}) of fatty acids in the original lipid extracts.

	Lipid content of cells	
Fatty acid	15 %	22 %
Acetic	34	52
Propionic	7	14
Isobutyric	4	11
Butyric	3	2
α-Methylbutyric	2	4
Caproic	4	26
Caprylic	312	463
Capric	96	123
Lauric	20	110
Myristic	228	1292
Myristoleic	30	165
Palmitic	1062	6726
Palmitoleic	153	2142
Stearic	224	1120
Oleic	392	5292
Linoleic	204	1326

Accumulation of storage materials. Table 3 records experiments in which pellicles of *Mycobacterium phlei* containing minimal amounts of lipid and glycogen were exchanged to modified environments. Cultures incubated on the initial growth medium, with and without exchange, served as controls. Exchange to the low nitrogen, high carbon medium resulted in an increase in the pellicle weight, but when the added nitrogen was utilized and nitrogen again became limiting, increases in the lipid, carbohydrate and glycogen occurred. The highest values of accumulation occurred when pellicles were incubated on a nitrogen-free, high-carbon medium. The increases in pellicle weight could be accounted for by the accumulation of lipid and carbohydrate. The increases in carbohydrate values were essentially due to glycogen accumulation. It is of particular importance that the rate and amount of glycogen accumulation exceeds that of lipid accumulation. At day 5 the combined lipid and carbohydrate fractions represented approximately 50 % of the pellicle wt of bacteria exchanged to the nitrogen-free high-carbon medium. When a high nitrogen carbon-free medium replaced the original growth medium, pellicle weights remained essentially constant, with slight decreases in the constituent amounts.

Effect of other methods of growth inhibition. In nitrogen-limited media, the depletion of exogenous nitrogen may stop protein synthesis but not protein turnover.

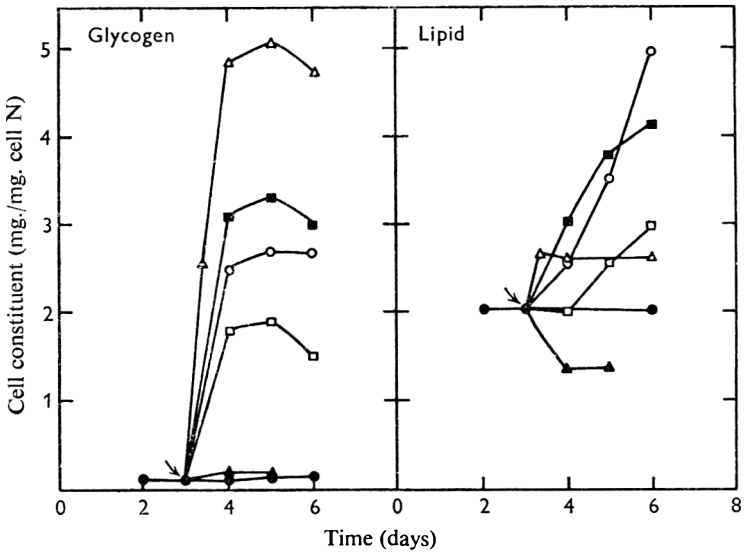

Fig. 1. Effect of sulphur deficiency and inhibitors of protein synthesis on the accumulation of glycogen and lipid in *Mycobacterium phlei*. Medium exchanges were made at the time indicated by the arrows. ●, Unchanged control, high nitrogen, high carbon medium; △, exchanged to sulphate-deficient, high-nitrogen, high-carbon medium; ○, exchanged to high-nitrogen, high-carbon medium containing chloramphenicol (100 μg./ml.); □, exchanged to high-nitrogen, high-carbon medium containing *p*-fluorophenylalanine (100 μg./ml.); ▲, exchanged to high-nitrogen, high-carbon medium containing 2,4-dinitrophenol (100 μg./ml.); ■, exchanged to no-nitrogen, high-carbon medium.

Table 3. *Effect of nitrogen and carbon concentration on the accumulation of storage materials in Mycobacterium phlei*

Replicate cultures of *Mycobacterium phlei* were grown as pellicles on the high-nitrogen high-carbon medium. Medium exchanges (see Methods) were made at day 3. Duplicate cultures were harvested for analysis at the time of medium exchange and following the additional incubation of cultures exchanged to modified media. Unchanged cultures served as normal growth controls. Results represent the average composition of the duplicate cultures.

		Yield		Constituents		
Medium	Age (days)	Dry wt (mg./100 ml. medium)	Nitrogen (mg./100 ml. medium)	Lipid (mg./mg. cell N)	Carbohydrate (mg./mg. cell N)	Glycogen (mg./mg. cell N)
Unchanged control						
High nitrogen, high carbon	3	241	21·7	1·6	1·4	0·2
	5	1125	97·9	1·8	1·6	0·3
Exchanged						
Low nitrogen, high carbon	5	715	43·6	3·0	4·1	2·3
No nitrogen, high carbon	5	396	19·8	4·2	5·7	3·7
High nitrogen, no carbon	5	249	23·2	1·5	1·2	0·1
High nitrogen, high carbon	5	1277	107·3	2·0	1·8	0·4
No nitrogen, no carbon	5	234	21·3	1·6	1·3	0·2

Chloramphenicol and *p*-fluorophenylalanine are substances which inhibit both protein synthesis and protein turnover more directly; when bacteria were incubated on the high nitrogen, high-carbon medium in the presence of chloramphenicol and *p*-fluorophenylalanine both glycogen and lipid accumulated (Fig. 1). Chloramphenicol inhibited growth, and the amounts of glycogen and lipid were comparable to accumulations in nitrogen-limited cultures. The rates and amounts of accumulation from *p*-fluorophenylalanine inhibition were lower than the nitrogen-deficient controls since the *p*-fluorophenylalanine concentration used (100 μg./ml.) resulted in only 40 % inhibition of growth. The addition of 2,4-dinitrophenol to high-nitrogen, high-carbon media resulted in complete inhibition of growth and glycogen accumulation and a decrease in the lipid content of the bacteria.

Sulphur-deficiency also provides a potential inhibitor of protein synthesis, as well as limiting co-factors of storage material syntheses such as glutathione, cysteine, and coenzyme A. Sulphur-limited *Escherichia coli* do not accumulate glycogen, presumably because of co-factor deficiencies (Holme & Palmstierna, 1956*b*), while *Aerobacter aerogenes* strains may accumulate glycogen to 50 to 100 % of values achieved in nitrogen-limited cultures (Segel, Cattaneo & Sigal, 1965). The reason for this difference is unknown. In *Mycobacterium phlei*, sulphur-deficient growth conditions stimulated glycogen accumulation to higher levels than those produced by nitrogen-deficiency (Fig. 1). Lipid also accumulated, but not to the same extent as the nitrogen-limited controls.

These results demonstrate that other methods of growth inhibition besides nitrogen-depletion can also result in storage material accumulation, provided the inhibition does not interfere with carbon-substrate utilization or the energy metabolism of the cell. These studies also suggest that the glycogen and lipid-synthesizing enzymes present at the time of addition of inhibitor were sufficient to participate in the subsequent accumulations.

Utilization of storage materials. Evidence for the utilization of stored lipid and glycogen for energy and as the source of carbon for the synthesis of nitrogenous cell constituents is shown in Table 4. When pellicles of *Mycobacterium phlei* containing stored lipid and glycogen were exchanged to a high-nitrogen medium without added carbon substrate, growth continued and the lipid and glycogen contents were decreased to minimal values. Pellicle weight did not decrease with the depletion of lipid and glycogen stores, but increased 5 % over the weight at the time of exchange. The accompanying 42·5 % increase in total pellicle nitrogen is highly significant. In the absence of exogenous carbon, only the stored lipid and glycogen could supply the energy and carbon for the incorporation of this nitrogen into cellular constituents. These observations show therefore that the stored lipids and glycogen can serve as a reserve of carbon, as well as energy, for the assimilation of exogenous nitrogen.

The results of the exchange to the low-nitrogen, high-carbon medium (Table 4) are also of particular significance because they show that, in *Mycobacterium phlei*, glycogen is a more labile storage material than lipid and is utilized before lipid. The replenishment of exogenous nitrogen on exchange to this medium allowed additional growth, as with the high-nitrogen medium, with a resultant decrease in carbohydrate and glycogen at day 4·5. When exogenous nitrogen again became limiting, glycogen was subsequently resynthesized. During this period, when glycogen was partially utilized and then resynthesized, the lipid content remained constant. Similarly,

exchange to the high-nitrogen, high-carbon medium depleted glycogen stores but only partially decreased the lipid content. This response suggests that glycogen synthesis is more readily regulated by the nutritional environment than lipid synthesis.

Table 4. *Effect of nitrogen and carbon concentration on the storage materials accumulated by Mycobacterium phlei*

Replicate cultures of *Mycobacterium phlei* were grown as pellicles on the low nitrogen, high carbon medium. Medium exchanges (see Methods) were made at day 4. Duplicate cultures were harvested for analysis at the time of medium exchange and following the additional incubation of cultures exchanged to modified media. Unchanged cultures served as normal growth controls. Results represent the average composition of the duplicate cultures.

		Yield		Constituents		
Medium	Age (days)	Dry wt (mg./ 100 ml. medium)	Nitrogen (mg./ 100 ml. medium)	Lipid (mg./mg. cell N)	Carbohydrate (mg./mg. cell N)	Glycogen (mg./mg. cell N)
Unchanged control						
Low nitrogen, high carbon	4	583	33·2	3·0	5·3	3·1
	5	871	43·6	3·8	6·3	3·8
Exchanged						
High nitrogen, no carbon	5	623	47·3	1·7	2·0	0·3
Low nitrogen, high carbon	4·5	859	52·9	3·0	3·7	1·7
	5	1123	61·8	3·8	4·8	2·5
High nitrogen, high carbon	5	1067	83·2	2·4	1·9	0·4
No nitrogen, no carbon	5	588	32·3	2·8	5·3	2·9
No nitrogen, high carbon	5	763	37·4	4·2	6·1	3·7

DISCUSSION

Studies of the lipids and polysaccharides of mycobacteria have mainly been concerned with the chemistry of the compounds found in these organisms, with little attention given to their physiological significance. This report has demonstrated that, in *Mycobacterium phlei*, the synthesis and accumulation of classes of lipid and polysaccharide are controlled by modifications in the nutritional environment and that these stored materials serve as endogenous reserves of carbon and energy in the absence of exogenous carbon substrate.

The lipid and carbohydrate content of *Mycobacterium phlei* were minimal during exponential growth in nitrogen-limited cultures but increased enormously during the post-exponential phase. The combined stored materials may accumulate to as much as 50% of the cell weight. Since storage materials do not accumulate during growth in nitrogen-excess media, it would appear that the synthesis of these reserves is largely controlled by the nitrogen concentration in the environment. In this respect, storage-material synthesis in *M. phlei* resembles synthesis of glycogen in *Escherichia coli* (Holme & Palmstierna, 1956*a*) and *Aerobacter aerogenes* (Segel *et al.* 1965), poly-β-hydroxybutyrate in *Bacillus megaterium* (Macrae & Wilkinson, 1958), and lipids in the yeasts *Rhodotorula gracilis*, *R. glutinis* and *Lipomyces starkeyi* (reviewed by Mulder, Deinema, Van Neen & Zevenhuizen, 1962). When grown on nitrogen-limiting media

these organisms only accumulate one reserve substance, but they may synthesize other reserves under different cultural and environmental conditions (see Dawes & Ribbons, 1964). In contrast, *M. phlei* has been found to synthesize and accumulate both lipid and glycogen when growth was inhibited by nitrogen depletion and by antibiotics.

The means by which growth inhibition controls storage material synthesis is uncertain. However, the data with *Mycobacterium phlei* are consistent with the hypothesis that conditions which favour accumulation of reserves appear to be conditions of 'uncoupled growth', conditions under which energy is produced at a rate faster than needed (Segel *et al.* 1965). The synthesis of storage materials may be considered a shunt mechanism by which some of this excess energy may be conserved intracellularly.

For these stored materials to be considered as endogenous reserves, their function as a source of energy and carbon must be demonstrated. In *Escherichia coli*, glycogen has been found to serve as a source of carbon for the synthesis of nitrogenous cell constituents (Holme & Palmstierna, 1956*c*). In *Mycobacterium phlei*, stored glycogen and lipid can be utilized as a carbon source for continued growth. When bacteria with stored glycogen and lipid are offered a nitrogen substrate in the absence of exogenous carbon, the depletion of glycogen and lipid is accompanied by a marked increase in incorporated nitrogen. Since no other carbon substrate was present, energy for nitrogen assimilation and the carbon skeletons for the synthesis of nitrogenous cell constituents could only be derived from these reserves. These growth experiments have also shown that, although glycogen and lipid stores are synthesized concurrently, glycogen may be utilized preferentially, and at least partially depleted, before lipid utilization is initiated. The regulatory mechanism which permits this differential utilization provides a perplexing problem for further investigation.

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REFERENCES

ANDREJEW, A. (1948). L'utilisation par oxydation de quelques substances azotées par le bacille de Koch. *Annls Inst. Pasteur* **74**, 464.

BUEDING, E. & HAWKINS, J. T. (1964). Enzymic degradation and microdetermination of glycogen. *Analyt. Biochem.* **7**, 26.

CHARGAFF, E. & MOORE, D. H. (1944). On bacterial glycogen: the isolation from avian tubercle bacilli of a polyglucosan of very high molecular weight. *J. biol. Chem.* **155**, 483.

DAGLEY, S. & JOHNSON, A. R. (1953). The relation between lipid and polysaccharide contents of *Bact. coli. Biochim. biophys. Acta* **11**, 158.

DAMOGLOU, A. P. & DAWES, E. A. (1967). Studies on the lipid content and phosphate requirement of glucose- and acetate-grown *Escherichia coli. Biochem. J.* **102**, 37P.

DAWES, E. A. & RIBBONS, D. W. (1964). Some aspects of the endogenous metabolism of bacteria. *Bact. Rev.* **28**, 126.

GERMAN, R. J., JONES, A. S. & NADARAJAH, M. (1961). Polysaccharides of *Mycobacterium phlei*. *Nature, Lond.* **189**, 1008.

HERBERT, D. (1961). The chemical composition of microorganisms as a function of their environment. *Symp. Soc. gen. Microbiol.* **11**, 391.

HOLME, T. & PALMSTIERNA, H. (1956*a*). Changes in glycogen and nitrogen-containing compounds in *Escherichia coli* B during growth in deficient media. I. Nitrogen and carbon starvation. *Acta chem. scand.* **10**, 578.

HOLME, T. & PALMSTIERNA, H. (1956*b*). Changes in glycogen and nitrogen-containing compounds in *Escherichia coli* B during growth in deficient media. II. Phosphorus and sulfur starvation. *Acta chem. scand.* **10**, 1553.

HOLME, T. & PALMSTIERNA, H. (1956*c*). On the glycogen in *Escherichia coli* B; its synthesis and breakdown and its specific labelling with ^{14}C. *Acta chem. scand.* **10**, 1557.

KENT, P. W. & STACEY, M. (1949). Studies on the glycogen of *M. tuberculosis* (human strain). *Biochim. biophys. Acta* **3**, 641.

MACRAE, R. M. & WILKINSON, J. F. (1958). The influence of cultural conditions on poly-β-hydroxybutyrate synthesis in *Bacillus megaterium*. *Proc. R. phys. Soc. Edinb.* A **27**, 73.

MULDER, E. G., DEINEMA, M. H., VAN NEEN, W. L. & ZEVENHUIZEN, L. P. T. M. (1962). Polysaccharides, lipids and poly-β-hydroxybutyrate in micro-organisms. *Rec. Trav. Chim.* **81**, 797.

SAZ, H. J. & LESCURE, O. L. (1966). Interrelationships between the carbohydrate and lipid metabolism of *Ascaris lumbricoides* egg and adult stages. *Comp. Biochem. Physiol.* **18**, 845.

SEGEL, I. H., CATTANEO, J. & SIGAL, N. (1965). The regulation of glycogen synthesis in *Aerobacter aerogenes*. In *Colloques internationaux de C.N.R.S., Mécanismes de régulation des activités cellulaires chez les microorganismes*, 1963. p. 337. Paris: Centre National de la Recherche Scientifique.

SEIFTER, S., DAYTON, S., NOVIC, B. & MUNTWYLER, E. (1950). The estimation of glycogen with anthrone reagent. *Archs Biochem.* **25**, 191.

STANIER, R. Y., DOUDOROFF, M., KUNISAWA, R. & CONTOPOULOU, R. (1959). The role of organic substrates in bacterial photosynthesis. *Proc. natn. Acad. Sci. U.S.A.* **45**, 1246.

STEPHENSON, M. & WHETHAM, M. D. (1922). Studies in the fat metabolism of the timothy grass bacillus. *Proc. R. Soc.* B **93**, 262.

STEPHENSON, M. & WHETHAM, M. D. (1924). Studies in the fat metabolism of the timothy grass bacillus. II The carbon balance-sheet and respiratory quotient. *Proc. R. Soc.* B **95**, 200.

TEPPER, B. S. (1965). Modification of cellular constituents during growth of *Mycobacterium phlei*. *Am. Rev. resp. Dis.* **92**, 75.

WILSON, P. W. & KNIGHT, S. G. (1952). *Experiments in Bacterial Physiology*, p. 55. Minneapolis: Burgess Publ. Co.

WINDER, F. G. & O'HARA, C. (1962). Effects of iron deficiency and of zinc deficiency on the composition of *Mycobacterium smegmatis*. *Biochem. J.* **82**, 98.

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Biochemical Patterns of Some Heterotrophic Marine Bacteria Grown in Defined Media

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SUMMARY

Three heterotrophic marine bacteria, isolated from the Tyrrhenian sea on the basis of their relative growth requirements for acetate and ammonium, were studied mainly in regard to their biochemical and nutritional characters. Data about the amino acid pools, protein amino acids, CO_2:O_2 ratios, enzyme activities and sensitivities to metal ions and antibiotics, are reported. The utility of an index obtained from the study of the oxygen uptake with various organic-C concentrations and the use of defined media for a more satisfactory discrimination of the various strains, is shown.

INTRODUCTION

In the marine ecosystem, bacteria play a very important role as consumers and producers of dissolved and particulate organic matter (Wood, 1965). The importance of marine bacteria was pointed out many years ago by ZoBell (1946) and more recently by Wood (1963, 1965) and Kriss (1963); but only in the last few years have marine microbiologists become interested in the more specific problems about the physiology of these micro-organisms. Recently, a considerable amount of work has been done by workers from various countries, because of the interest that marine microbiology has in many different fields (Scholes & Shewan, 1964). In general, the most recent work of physiological interest deals with the activity of enzyme systems in certain species isolated from the sea (MacLeod & Hori, 1960; Burton & Morita, 1963; Holmes & Halvorson, 1965; Shieh, 1965; Campbell, Hellebust & Watson, 1966; Gundersen, 1966), with their nutritional requirements (MacLeod, Onofrey & Norris, 1954; Tyler, Bielling & Pratt, 1960; Brown, 1960) and with their particular environments (Brown, 1964; MacLeod, 1965). This type of research assumes obviously the identification of isolated strains and in this field, too, significant results have been obtained (Shewan, Hobbs & Hodgkiss, 1960; Shewan, 1963; Hendrie, Hodgkiss & Shewan, 1964; Colwell, Citarella & Ryman, 1965; Pfister & Burkholder, 1965) without, however, reaching a definition of the problem (Wood, 1967). The present work reports the first results from a systematic study of heterotrophic bacteria of the Mediterranean basin with special reference to the Tyrrhenian sea. The main purpose has been the study of some biochemical characters of the micro-organisms, not only in the method of isolation (which has been achieved on the basis of relative specificity towards defined substrates such as acetate-C and ammonium-N) but also as useful criteria for their identification.

METHODS

Sampling. The sampling of the sea water was done along the Tyrrhenian coast, 20 miles out from Torvaianica (Rome), where the depth was 150 m.

The samples were taken by using ZoBell type bottles at the surface (0 m.) and at 25 and 100 m. depths; in the latter case stainless-steel bottles with internal glass container were used. The samples were kept between 10° and 15° and used in the laboratory within about 3 hr.

Isolation of bacteria. To 500 ml. of the sea water sample were added 5 ml. of aged natural sea water (ANSW) containing: 10 mg.-atom organic-C as $CH_3COONa.3H_2O$; 5 mg.-atom NH_4-N as $(NH_4)_2SO_4$ and 10 μg.-atom PO_4-P as K_2HPO_4. The sample was kept at 18° in a 1 l. flask and after 72 hr a suitable dilution from it was seeded on 1·5 % agar medium containing the same substrates as above. After a few days of incubation the colonies grown were examined and the strains isolated were maintained at 18° by weekly transfer on the same medium. Three of the strains isolated are reported on here; they were labelled as follows: TS/0-3, TS/25-1, TS/100-1 (TS = Tyrrhenian sea; 0, 25 and 100 = depth in metres; 3 and 1 = sample number). In the present paper the strains will be indicated by these initials, as our purpose was the study of peculiar physiological characters rather than a taxonomic classification of the species isolated. However, considering the morphological characters reported below and according to the scheme for the identification of Gram-negative rods proposed by Hendrie, Hodgkiss & Shewan (1964), TS/100-1 can be included in Pseudomonas group II, and TS/0-3 and TS/25-1 in Pseudomonas group IV.

Studies on nutritional requirements. The culture media were prepared in ANSW by adding the appropriate substrates and sterilizing at 115° for 15–20 min. These solutions were adjusted to pH 7·5 (glass electrode). After sterilization the pH value rose to pH 7·8–8·1. Heat-labile substrates were sterilized by filtration through Millipore filters (0·45 μ pore size).

The composition of various defined media used will be reported in the Results section, where the concentration of substrates will be also expressed in mg.-atom or in μg.-atom of C, N, P per litre according to results of oceanographic analysis (Riley & Skirrow, 1965).

Media were inoculated by adding 0·1 ml. of cultures grown for 72 hr in acetate + ammonium + phosphate medium. These inoculated cultures were incubated at 18° for 80–90 hr, without agitation, and the degree of growth was measured by extinction at 500 mμ in cuvettes of 2 cm. light-path in a PMQ II Zeiss spectrophotometer.

Morphological studies. The three micro-organisms were incubated at 18° for 60 hr with aeration in ANSW containing acetate-C, NH_4-N and PO_4-P. The examination of colonies was made after incubation for 72 hr on the acetate + ammonium + phosphate agar already described.

The observation of bacteria by an Orthomat Leitz phase-contrast microscope was preceded by treatment with a drop of 40 % formaldehyde to immobilize motile micro-organisms.

The electron-microscope observations were done with a Siemens Elmiskop 1 A using a drop of bacterial suspension placed on a microscope grid with Formvar membrane with drop of 1·5 % phosphotungstic acid (pH 7·0) added.

Qualitative amino acids chromatography. Eight-litre batches of bacterial cultures

incubated in acetate + ammonium + phosphate ANSW medium at 18° for 72 hr with aeration were used. The bacteria were centrifuged at low temperatures, washed three times with ANSW and disintegrated for 10 min. in distilled water at 0° by ultrasonic treatment in a MSE 60 W. disintegrator. The suspension was then heated at 100° for 10 min., cooled and a drop of 6 N-HCl added. After centrifugation the clear supernatant fluid (A = amino acid pool) was used for ion-exchange resin purification, while the precipitated material, washed three times with distilled water, was hydrolysed with 6 N-HCl at 110° for 24 hr in a sealed tube. This coloured solution, dried at reduced pressure, suspended in distilled water and filtered through Whatman no. 42 paper, was subsequently purified (B = protein amino acids).

Five ml. each of A and B solutions were placed, separately, on the tops of the two chromatographic columns (1 cm. diameter) containing Amberlite CG-120 200–400 mesh in the H^+ form (40 cm.); 100 ml. of freshly distilled water were passed through each column to remove impurities and then 150 ml. of 2 N-NH_4OH were used to elute the amino acids. The eluates, dried at reduced pressure, dissolved in 0·5 ml. distilled water and filtered through a micro-sintered-glass filter, were used for paper chromatography: 0·1 ml. was applied to Whatman no. 1 paper and the chromatograms developed according to the method of Mizzel & Simpson (1961).

Manometric measurements. In all experiments the micro-organisms were grown at 18°, with aeration, in 2 l. flasks containing: 1000 ml. ANSW, 20 mg.-atom total organic-C (acetate + pyruvate + maltose), 10 mg.-atom NH_4-N and 20 μg.-atom PO_4-P. After 50 hr the micro-organisms were centrifuged down, washed twice and suspended in 50 ml. of sterilized ANSW + PO_4-P and kept for 12 hr at 18° with aeration to deplete the endogenous respiration.

Before use the washed organisms were resuspended in fresh ANSW + PO_4-P so that, when diluted 1/10, the $E^{1\,cm.}_{500\,m\mu}$ value ranged between 0·4 and 0·5.

The oxygen uptake and CO_2 evolution were measured in a Warburg apparatus at 18° ± 0·1, over the entire experimental period, following the principle of Dickens & Simer (see Umbreit, Burris & Stauffer, 1957), but using a different technique. The pH value in the reaction vessels was about pH 7·8, at which value practically all the CO_2 was in the bicarbonate form (Riley & Skirrow, 1965). The amount of CO_2 normally present in sea water was determined in separate vessels and then subtracted from the other experimental results.

Crude-extract preparations. Aerated cultures grown at 18° for 50 hr in 2 l. ANSW containing acetate + ammonium + phosphate, as described, were centrifuged and the deposit washed twice with ANSW. The pellets suspended in 7 ml. ANSW + 3 ml. 0·2 M-tris buffer (pH 7·4) were treated in the MSE disintegrator for 5 min. at 0°. The debris was centrifuged down at 30,000 *g* for 7 min. and the opalescent supernatant fluid used as crude extract for enzyme assays.

Enzyme assays. The oxidation and reduction of nicotinamide adenine nucleotides with different substrates was done at room temperature by measuring the change in $E^{1\,cm.}_{340\,m\mu}$ in spectrophotometer cuvettes (Hilger-Gilford equipment). In a similar way fumarase activity was measured at 300 mμ by using 10–20 μmole fumarate and 1 ml. crude extract in 0·05 M-tris buffer (pH 7·4).

Inhibition by heavy metals and antibiotics. The media and inocula were prepared as described for nutritional requirements. The experiments with antibiotics were done in test-tubes containing 4 ml. 0·1 % nutrient broth (Difco) in ANSW sterilized at

115° for 20 min. Penicillin, tetracycline and streptomycin were added in a volume of 1 ml. of acidified medium at pH 4 to obtain a final pH of 7·2: 0·1 ml. inocula from serial dilutions of a culture aerated for 36 hr at 18° with $E^{1\,cm.}_{500\,m\mu} = 0{\cdot}1$ were obtained by dilution with the same medium.

Substrates and reagents. The amino acids and carbohydrates were purchased from British Drug Houses Ltd. or Mann Res. Lab. Inc., ketoacids and nucleotides were from Sigma or Boehringer, while all the other reagents were from Merck. The sea water for the media was taken in the sampling area and aged in the dark for about 30 days before being used.

RESULTS

The three strains isolated showed these common characters: straight or curved rods (Pl. 1, fig. 4–6), motile with a polar flagellum (Pl. 2, fig. 7–9), Gram-negative.

Colonial appearances (Pl. 1, fig. 1–3) on acetate + ammonium + phosphate agar were as follows.

Organism TS/0-3: colonies round with an entire margin (Pl. 1, fig. 1) of yellow colour due to a not diffusible pigment, transparent to transmitted light and opaque to incident light, with a delicate circular structure.

Organism TS/25-1: colonies round with an entire margin, colourless, transparent to transmitted light and opaque to incident light (Pl. 1, fig. 2).

Organism TS/100-1: colonies round with an entire margin, and a dark central core with internal scattered black dots (Pl. 1, fig. 3); on acetate + ammonium + phosphate slope a diffusible dark-brown pigment produced.

Nutritional requirements

Carbon sources. The results obtained with ammonium as nitrogen source and with different carbon substrates are given in Table 1, in which the data are expressed in arbitrary units of amount of growth related to the extinction. The results show good growth of all three isolates with the following organic-C compounds: starch, glycogen, trehalose, maltose, acetate, pyruvate, oxalacetate. None of the three micro-organisms utilized malic acid and α-ketoglutaric acid. Organisms TS/0-3 and TS/25-1 did not grow in the presence of the other intermediates of the Krebs cycle, whereas strain TS/100-1 grew in the presence of citrate, succinate or fumarate. TS/0-3 metabolized maltose but not glucose, TS/25-1 gave growth with all carbohydrates tested, and TS/100-1 utilized agar as a carbon source. The negative response to pentoses was typical of all strains, although TS/25-1 sometimes grew slightly.

Nitrogen sources. The data about the organic and inorganic nitrogen utilization in the presence or in absence of a carbon source are shown in Tables 2 and 3. The results show that in the presence of acetate-C the three organisms grew with glycine, alanine, valine, leucine, isoleucine and norleucine (leucines), proline, aspartic acid, glutamic acid, arginine or asparagine, but not with norvaline, glutamine or α-amino butyric acid.

With inorganic-N, organism TS/0-3 did not utilize NO_2-N and NO_3-N in the presence of acetate-C but, in common with the other strains, it multiplied in ANSW with gelatine added and liquified gelatine in nutrient broth. Less indicative were data obtained in absence of organic-C by reason of the poor growth reached with single amino acids as source of C and N.

General metabolic characteristics

Amino acids chromatography. A reference chromatogram and two typical chromatographic patterns of strain TS/25-1 are shown on Pl. 4, fig. 10–12.

By substituting acetate-C with glucose-C in the culture media, identical results were obtained with the same strain.

Table 1. *Growth of three heterotrophic marine bacteria on different carbon sources*

The organisms were grown in test-tubes at 18° in 10 ml. of ANSW containing: 10 mg.-atom/l. of organic-C; 5 mg.-atom/l. of NH_4-N and 20 μg. atom/l. of PO_4-P. The results are means of duplicate readings. Initial pH = 8·0; the final pH values are in parentheses.

	Organism		
Carbon sources	TS/0-3	TS/25-1	TS/100-1
Starch	2 (7·23)	4 (6·50)	4 (6·30)
Inulin	0	2 (7·28)	0
Glycogen	2 (7·31)	2 (7·27)	3 (6·50)
Agar	0	0	3 (5·80)
Sucrose	0	4 (6·0)	3 (6·60)
D(+)Cellobiose	0	3 (6·54)	3 (6·80)
D Trehalose	3 (7·22)	3 (6·60)	3 (6·80)
Lactose	0	3 (7·27)	2 (7·31)
Maltose	2 (7·28)	4 (7·00)	3 (6·57)
D(+)Glucose	0	4 (6·21)	3 (6·94)
D(+)Mannose	1 (7·27)	1 (7·40)	0
D(+)Galactose	0	3 (6·24)	4 (5·60)
D(−)Fructose	0	4 (6·25)	0
D(−)Mannitol	0	2 (6·21)	3 (6·74)
D(+)Xylose	0	1 (7·00)	0
D(−)Ribose	0	1 (7·20)	0
L(+)Arabinose	0	1 (7·20)	0
Acetate	2	3	2
Pyruvate	1	4	4
Citrate	0	0	4
α-Ketoglutarate	0	0	0
Succinate	0	0	4
Fumarate	0	0	2
DL-Malate	0	0	0
Oxalacetate	2	2	3

Key to the numbers, $E^{2\,cm.}_{500\,m\mu}$. 0–0·03 = 0: 0·03–0·1 = 1: 0·1–0·2 = 2: 0·2–0·3 = 3: > 0·3 = 4.

The amino acid composition of all three bacteria are given in Table 4. It will be noted that aspartic acid and glutamic acid were present in large amounts when acetate-C and NH_4-N were the only nutrients present in the medium. Strain TS/0-3 differed from the others in that histidine and arginine were absent; TS/25-1 had the largest number of amino acids in the pool.

Biochemical activity as measured in the Warburg apparatus

When nitrogen sources were absent, the consumption rate of oxygen by the starved micro-organisms was substantially different for acetate-C, pyruvate-C and maltose-C (Fig. 1, 2).

As described in Methods, when the micro-organisms were starved for 12 hr at 18°

Table 2. *Growth of three heterotrophic marine bacteria on different nitrogen sources in presence of acetate-C*

10 ml. of ANSW containing: 10 mg.-atom/l. of acetate-C; 5 mg.-atom/l. of NH_2-N or NO_3-N or NO_2-N or protein-N and 20 μg.-atom/l. of PO_4-P.

Nitrogen and carbon sources	Organism		
	TS/0-3	TS/25-1	TS/100-1
Acetate +			
Glycine	2	3	4
Alanine	1	2	3
Valine	1	1	1
Norvaline	0	0	0
Leucine	2	2	2
Isoleucine	2	2	3
Norleucine	0	2	1
Serine	0	2	3
Threonine	0	2	4
Phenylalanine	2	4	3
Tryptophan	2	2	0
Cysteine	0	0	2
Methionine	0	1	3
Proline	2	2	3
Aspartic acid	2	4	3
Glutamic acid	3	4	4
Histidine	0	2	3
Arginine	2	3	2
Glutamine	0	0	0
Asparagine	2	3	4
α-Aminobutyric	0	0	0
NH_4^-	2	4	2
NO_2^-	0	2	1
NO_3^-	0	3	2
Gelatine	4	4	4
Gelatine-liquef. + nutrient broth	+ (90 h)	+ (48 h)	+ (48 h)

Key and other conditions: see Table 1. DL amino acids have been used.

Table 3. *Growth of three heterotrophic marine bacteria on amino acids as nitrogen and carbon sources*

10 ml. of ANSW containing: 5 mg.-atom/l. of NH_2-N and 20 μg.-atom/l. PO_4-P

Nitrogen and carbon sources	Organism		
	TS/0-3	TS/25-1	TS/100-1
Alanine	0	1	2
Valine	0	1	0
Isoleucine	0	1	0
Serine	0	1	0
Threonine	0	1	0
Phenylalanine	0	2	0
Proline	2	0	2
Aspartic acid	0	0	2
Glutamic acid	2	0	1
Arginine	2	0	0
Asparagine	0	1	0

Key and other conditions: see Table 1. The other amino acids gave a negative response.

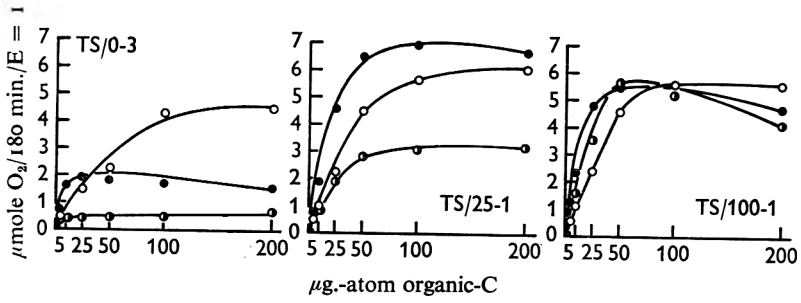

Fig. 1. Influence of various organic-C concentrations on the oxygen uptake of three heterotrophic marine bacteria. Each Warburg flask (19 ml.) contained: in the bottom compartment, 2 ml. of bacterial suspension of calculated extinction from 4·0 to 5·0, and 0 25 ml. of organic-C in ANSW; in the centre well, 200 µl. of 20 % KOH in distilled water. The final volume was made up to 3 ml. with ANSW. Endogenous oxygen has been subtracted. ●——●, Acetate-C; ○——○, pyruvate-C; ◑——◑, maltose-C.

Table 4. *Chromatographic amino acids composition of three heterotrophic marine bacteria*

First dimension: n-butanol + acetic acid + water, 25 + 6 + 25. Second dimension: methylethylketone + n-butanol + water, 2 + 2 + 1 by vol.

	Amino acid pools			Protein amino acids		
	TS/0-3	TS/25-1	TS/100-1	TS/0-3	TS/25-1	TS/100-1
Glycine	+	−	−	+	+	+ +
Alanine	+	+ +	+ +	+ +	+	+ +
Valine	−	+	−	+	+	+
Leucines*	+	+ +	+	+ +	+ +	+ +
Serine	−	+	−	+	+	+ +
Threonine	+	−	−	+	+	+ +
Phenylalanine	−	+	−	+	+	+
Methionine	+	+ +	+	+	+ +	+ +
Proline	+	+	+	+	+	+
Aspartic acid	+	+ +	+ + +	+ + +	+ + +	+ + +
Glutamic acid	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Histidine	−	+	−	−	+	+
Lysine	−	+ +	+	+	+ +	+ +
Arginine	−	−	+ +	−	+ +	+ +
Tyrosine	−	+	−	+	+	+
Number of unknowns	0	1	0	3	0	0

Key: −, absence; +, present in small amounts; + +, moderate amounts; + + +, large amounts.
* Leucines = leucine, isoleucine, norleucine. These amino acids do not appear well separated, sharing the same tone of blue colour in presence of cyclohexylamine according to Mizzel & Simpson (1961). Therefore it was not possible to profit by the differences in the colours as for the other amino acids (e.g. glutamic acid, aspartic acid, glycine, arginine, and so on).

with aeration, the endogenous oxygen uptake was very low, showing that the bacteria had exhausted their reserves; when an organic-C source was added, oxygen uptake increased rapidly. Acetate-C and pyruvate-C always caused more oxygen to be taken up than maltose (Fig. 1), but for organism TS/100-1 this happened only at high carbon-source concentration. Other experiments showed that when NH_4-N and NO_3-N were added to the Warburg vessels the oxygen uptake was the same, while with NH_2-N (as glycine) more oxygen was taken up.

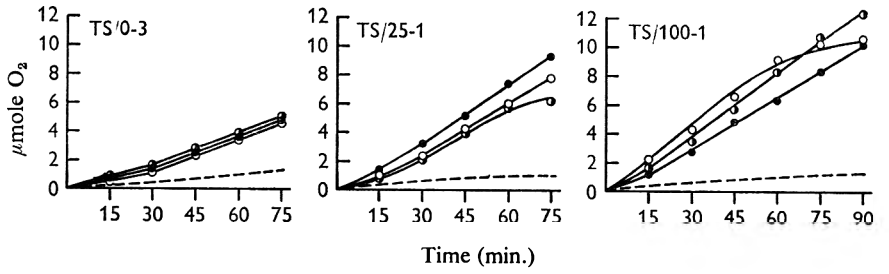

Fig. 2. Oxygen consumption rates, at fixed organic-C concentration, of three heterotrophic marine bacteria. Each Warburg flask (19 ml.) contained: in the bottom compartment, 2 ml. of bacterial suspension ($E^{1\,cm.}_{500m\mu}$ = 3·5) and 25 μg.-atom of organic-C in 0·25 ml. of ANSW; in the centre well, 200 μl. of 20 % KOH in distilled water. The final volume was made up to 3 ml. with ANSW. Endogenous oxygen has been subtracted. ●——●, Acetate-C; ○——○, pyruvate-C; ◑——◑, maltose-C; – – –, endogenous oxygen.

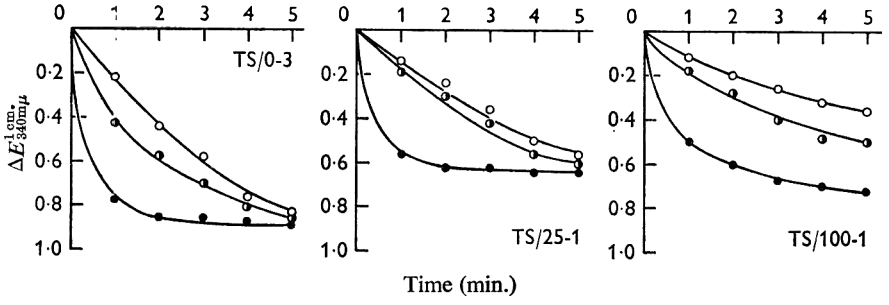

Fig. 3. Enzyme activity on $NADH_2$, $NADH_2$ + oxalacetate and $NADH_2$ + pyruvate, by cell-free extracts of three heterotrophic marine bacteria. Each spectrophotometer cuvette contained: 1 ml. of crude extract; 1·5–3 μmole of $NADH_2$; 10 μmole of substrate and 0·05 M tris buffer at pH 7·4 in a total volume of 3·5 ml. with distilled water. ○——○, $NADH_2$; ◑——◑, $NADH_2$ + pyruvate, ●——● $NADH_2$ + oxalacetate.

Table 5. *Ratio CO_2:O_2 and carbon sources for three heterotrophic marine bacteria*

For CO_2 evolution each Warburg flask (7·5 ml.) contained: in the bottom compartment, 50 μl. of KOH collected from the centre well of the flasks used for O_2 uptake, and 0·45 ml. of distilled water. In the side arm, 0·5 ml. of 3 N-HCl. After equilibrium, the HCl was transferred to the bottom compartment and CO_2 evolved after 20 min. was recorded. For O_2 uptake, see Fig. 2.

Carbon sources	TS/0-3		TS/25-1		TS/100-1	
	S.v.*	M.v.†	S.v.	M.v.	S.v.	M.v.
Acetate	1·88 2·42 1·46	1·92 ± 0·27	1·08 1·06 1·27	1·13 ± 0·07	0·78 0·90 1·08	0·92 ± 0·09
Pyruvate	2·14 2·35 1·96	2·15 ± 0·11	1·48 1·64 1·91	1·67 ± 0·28	1·51 1·31 1·27	1·37 ± 0·07
Maltose	0·78 0·9c 1·08	0·92 ± 0·09	1·25 1·24 1·40	1·30 ± 0·16	0·71 0·67 0·59	0·65 ± 0·01

* Single value. † Mean value ± standard error.

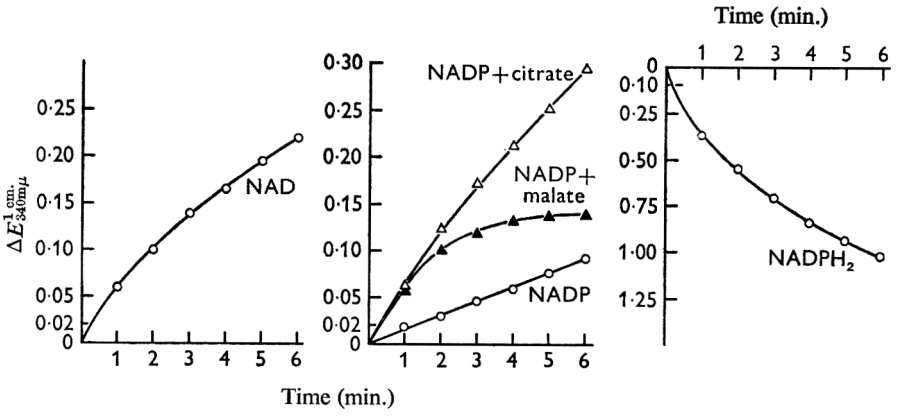

Fig. 4. Enzyme activity on NAD, NADP, $NADH_2$ NADP+malate and NADP+citrate, by cell-free extract of strain TS/25-1. Experimental conditions as in Fig. 3.

Table 6. *Enzyme activities in sonicated cell-free extracts of three heterotrophic marine bacteria*

Each cuvette contained in 3·5 ml.: 1 ml. of crude extract, 10 μmole of substrate, 1·5–3 μmole of nucleotide in 0·05 M-tris buffer at pH 7·4.

	Micro-organisms		
Coenzymes and substrates	TS/0-3	TS/25-1	TS/100-1
$NADH_2$	+ + +	+ + +	+ + +
$NADPH_2$	+ + +	+ + +	+
NAD	+ +	+ +	+ +
NADP	–	+	+
$NADH_2$ + pyruvate	+ + +	+ + +	+ + +
$NADH_2$ + oxalacetate	+ + +	+ + +	+ + +
NAD + malate	+ +	+ + +	–
NAD + α-ketoglutarate	–	–	–
NADP + citrate	–	+ + +	–
NADP + malate	–	+ +	–
NADP + α-ketoglutarate	–	–	–
Fumarate	–	+	–

Key: + + +, high activity; + +, moderate activity; +, low activity; –, absence of activity.

The values for CO_2:O_2 ratios of the three organic-C sources (Table 5) were obtained from the experiments illustrated in Fig. 2. These results show that the ratios were higher for pyruvate-C and lower for maltose-C for organisms TS/0-3 and TS/100-1, but, for TS/25-1 this latter value was intermediate between acetate-C and pyruvate-C.

Enzyme activities in crude extract experiments. In Fig. 3 the rates of change in extinction (ΔE) at 340 mμ obtained with cell-free extracts of the three bacteria are shown. The results show that when oxalacetate or pyruvate were added to sonicated soluble fractions, the rate of $NADH_2$ oxidation was greater than in the controls. Data about the NAD and NADP reduction and $NADPH_2$ oxidation by cell-free extracts of organism TS/25-1 are given in Fig. 4, which shows the enzyme activity

towards citrate or malate. The results of all experiments on enzyme activity in crude extracts are summarized in Table 6.

Effect of some inhibitors. From the data presented in Table 7 it can be seen that organism TS/0-3 was the most sensitive to inhibition by heavy metal ions; in general, Cu^{2+} and Zn^{2+} were the most inhibitory ions. None of the metals tested stimulated the growth of the three organisms under the experimental conditions adopted. As shown in Fig. 5, the three organisms were in general fairly resistant to antibiotics under the described conditions. Organisms TS/0-3 and TS/25-1 were sensitive to penicillin when the inoculum was small; with larger inocula all the organisms showed a clear resistance; especially TS/25-1 and TS/100-1 towards penicillin, and TS/0-3 towards tetracycline.

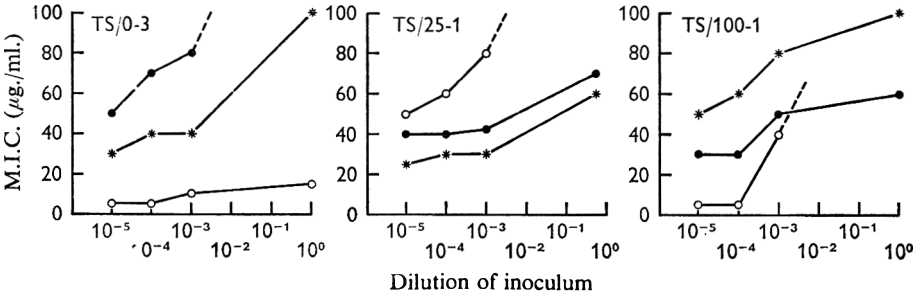

Fig. 5. Minimum inhibitory concentration (M.I.C.) of penicillin, tetracycline and streptomycin, on three heterotrophic marine bacteria, at various inoculum densities. Experimental conditions, see methods. The values represent the mean of duplicate readings. ○——○, penicillin; ●——●, tetracycline; ✳——✳, streptomycin.

Table 7. *Effect of heavy metal ions on growth of three heterotrophic marine bacteria*

		Micro-organisms		
Metal ions	μg.-atom/l.	TS/0-3	TS/25-1	TS/100-1
		2	3	2
Cu^{2+}	0·1	2	3	2
Cu^{2+}	1·0	1	3	1
Cu^{2+}	10	0	2	0
Fe^{3+}	0·1	2	3	2
Fe^{3+}	1·0	2	3	2
Fe^{3+}	10	2	3	2
Mn^{2+}	0·1	1	3	2
Mn^{2+}	1·0	2	3	2
Mn^{2+}	10	1	3	2
Zn^{2+}	0·1	1	3	2
Zn^{2+}	1·0	1	3	2
Zn^{2+}	10	0	2	1
MoO_4^{2-}	0·1	1	3	2
MoO_4^{2-}	1·0	1	3	1
MoO_4^{2-}	10	1	3	2
Co^{2+}	0·1	1	3	2
Co^{2+}	1·0	1	3	2
Co^{2+}	10	1	3	1

Key and other conditions: see Table 1.

DISCUSSION

The conditions used in the isolations of the bacteria were based on the principle of selection under minimal nutrient conditions (acetate + ammonium + phosphate) at the temperature of the sea water in the sampling area (15 to 18°). In this way all the strains isolated shared the relative specificity of a nutritional character. Although the specificity towards a substrate might depend upon the physiological conditions of the micro organism at time of sampling, and the use of such a principle for strain isolation might be questionable (Hungate, 1962; Hayaishi, 1955), the bacteria isolated in this way have kept, during the time of the research (16 months), the property of multiplying on acetate-C and ammonium-N. This property has proved to be stable. Future work will test whether this principle might be of use in grouping different strains of marine bacteria according to their relative specificity towards defined substrates.

Morphological characters. The photographs given were chosen as the most typical; but very often, especially for organisms TS/0-3 and TS/100-1, very different shapes were observed. In fact, curved forms were frequently observed and this could have been considered as characteristic of the genus *Vibrio*. However, in accord with the Hendrie scheme, based on behaviour of micro-organisms towards glucose-containing medium and the pteridine 0/129, this possibility was excluded and the organisms recognized as belonging to the genus *Pseudomonas*. However, further morphological observations, made on different strains successively isolated, showed that the vibrio shape was attributable to a stage in division in which the bacteria showed an inflexion before dividing in two. These data are in agreement with the criticism made by Rhodes (1965) and by Wood (1967) on the morphological characters of bacteria used in taxonomic studies. One character, on the contrary, which has shown a noticeable constancy, at least in the experimental conditions used, is the shape and pigmentation of the colonies on acetate + ammonium agar: organism TS/0-3 yellow: TS/25-1 colourless; TS/100-1 black-brown, although the appearance of colourless variants was occasionally noted.

Nutritional characters. Results obtained from study of nutritional requirements were distinctive under the experimental conditions used. Organism TS/100-1 possessed characters quite different from the other two organisms in that it used agar, citrate, succinate, fumarate and, with the exception of lactose, produced acid from the carbohydrates. Organism TS/0-3 had the singular character of using maltose but not glucose and it did not use NO_3-N and NO_2-N. An interesting property common to the three organisms was the non-utilization of malate, α-ketoglutarate or glutamine. Apart from the consideration of substrate permeability, the non-utilization of glutamine could be due to the lack of glutaminase, which hydrolyses glutamine to glutamic acid and ammonia (Cohen & Sallach, 1961; Roberts, 1960).

Hence clear differences in cultural behaviour exist among the three microscopically similar organisms.

General biochemical characters. This little-known aspect of marine bacteria was studied to compile biochemical properties of eventual general interest and to try to relate some positive or negative characters common to the three organisms with their relative specificity to acetate and ammonium. Obviously the data given here are not sufficient to clarify the metabolic activities of the organisms for the various substrates, but their utility in bringing out the differences is evident. Chromatography of pools

of amino acids has been used sometimes for taxonomic studies (Holden, 1962). Taking into account the experimental conditions used for growth and extraction, some significant differences were found in the amino acid pools of the three strains (Table 4). These differences cannot be easily interpreted biochemically, but should be useful. when a sufficient variety of marine bacteria have been examined, to judge whether the composition of amino acid pools is helpful in their classification.

The Warburg experiments clearly indicate differences among the three strains in the rates of oxygen uptake from various carbon sources, particularly with acetate. Starved cells were used because endogenous respiration was then very low; with not-starved cells, endogenous respiration was so high that the differences in the oxygen uptake with different substrates were less marked.

Assuming that the micro-organisms did not multiply in the Warburg flasks, Fig. 1 may be used to calculate a substrate affinity constant resembling Michaelis-Menten's kinetic constant for the enzymes. Half-maximum reaction rates correspond, for the three strains with acetate-C, to values (in molar units) of: TS/0-3, $K_{acet.} = 1{\cdot}6 \times 10^{-3}$: TS/25-1, $K_{acet.} = 5{\cdot}6 \times 10^{-3}$; TS/100-1, $K_{acet.} = 2{\cdot}7 \times 10^{-3}$. The reproducibility of these values was indicated by other experiments made with similar strictly controlled experimental conditions: for pyruvate (25 μg./Warburg vessel) the oxygen uptakes, over 60 min., were respectively: 58–66–55 μl. for TS/0-3; 116–134–123 μl. for TS/25-1; 165–198–170 μl. for TS/100-1, where the values are the means of duplicate tests in three independent experiments.

In spite of the approximate nature of these values it appears that differences in the substrate affinity are sufficient to differentiate the three strains despite their specificity for the same substrate. The ratios $CO_2:O_2$ obtained with the same substrates are also significantly different for the three micro-organisms, which presumably reflects differences in the metabolism of these substrates under starved conditions. Values below 1 indicate a prevalence of oxidative processes and agree very well with the pH values for various strains shown in Table 1. The $CO_2:O_2$ ratio is thus important in marine bacterial respiration, but it is not yet clear whether such values will be useful in taxonomic studies as proposed by Katznelson & Robinson (1956) for halophilic strains.

The redox potential of these systems did not correlate with $CO_2:O_2$ ratios. With TS/100-1 strain, for example, the initial value of E_h was +330 mV. at pH 8·0 (slightly different from the value of +322 mV. at 20° and at pH 8·15 for the sea water in the sampling area at 0 m. Of course, the E_h of this bacterial system changed during the experiments and after 24 hr it was +160 mV. with little differences for acetate, pyruvate and maltose.

Experiments on cell-free extracts have been recently employed by some authors (De Vries & Stouthamer, 1967; Joyner & Baldwin, 1966) to get indications on the taxonomy and ecology of many non-marine micro-organisms, but the significance of these researches depends strictly on the use of well-defined experimental conditions. The synthesis of many enzymes in bacteria represent an adaptation phenonemon (Daron, 1967) and the nutritional conditions influence the enzyme synthesis of the tricarboxylic acid cycle (Hanson & Cox, 1967). Despite the limited purification of the extracts and the possible differences in the optimal conditions required for the similar enzyme activities in various strains, the experiments reported here with cell-free extracts have shown their utility by demonstrating the presence of some enzyme activities which were not recognizable with Warburg experiments. For example, in

preliminary experiments, suspensions of sonicated marine bacteria did not take up any oxygen with pyruvate, citrate or succinate, whereas by direct spectrophotometry these enzyme activities were indicated. Presumably some enzyme systems of the terminal electron transport mechanisms were inactivated by ultrasonics or removed by centrifugation.

A particular substrate and pyridine nucleotides evoked enzyme activities related to Krebs cycle in the TS/25-1 extract. The apparent absence of comparable activities in extracts of TS/0-3 and TS/100-1 was probably because the majority of these enzymes do not link to NAD or NADP and require an artificial acceptor for the measurement of activity. As already emphasized by Ochoa & Stern (1952) and Barret & Kallio (1953), such experiments as these do not prove that the Krebs cycle is really the route used *in vivo* for terminal carbohydrate metabolism. The results of nutritional and cell-free extract experiments indicate that the non-utilization of citrate, succinate, fumarate and malate by TS/25-1 as sole carbon source in the presence of ammonium nitrogen could be attributable to inducible permeation systems (Kogut & Podoski, 1953; Barret & Kallio, 1953).

Growth inhibitors. Antibiotic sensitivity has been recently adopted by Pfister & Burkholder (1965) as a discriminating character among marine strains and Shewan (1963) used pteridine 0/129 as a differential test of Gram-negative asporogenous marine rods.

The experiments with metal ions reported in Table 7 show a significant difference between strain TS/25-1, which is practically insensitive, and strain TS/0-3, which is the most sensitive. The experiments with antibiotics were carried out in a complex medium, instead of a defined one as generally used with non-marine strains. Only penicillin showed a clear difference in sensitivity between TS/25-1 and the other two bacteria; this strain was, however, sensitive when the inoculum was low.

All three strains showed apparent resistance to tetracycline and streptomycin, but the ease with which these drugs, especially tetracycline, form complexes with Ca^{2+} and Mg^{2+} that lower antibacterial activity (Laskin, 1967), suggests that these two antibiotics are of little use in sea-water media which contain Mg^{2+} and Ca^{2+} ions.

Finally, a common characteristic of the three strains was their incapability of multiplying with malic acid or α-ketoglutaric acid. This property was not shown by some terrestrial strains of pseudomonas, classified, according Hendrie *et al.* (1964), into groups I (three strains), II (two strains) and IV (one strain), all of which grew very well after 24 hr at 37° with ammonium nitrogen and malic acid or α-ketoglutaric acid. This interesting nutritional characteristic of these marine bacteria has been confirmed by the isolation of eight other strains which behave similarly. Hence the use of particular defined media for the isolation of marine bacteria sharing characteristic biochemical properties is important and may in future assist the identification of newly isolated strains.

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REFERENCES

BARRET, J. T. & KALLIO, R. E. (1953). Terminal respiration in *Pseudomonas fluorescens*: component enzymes of the tricarboxylic acid cycle. *J. Bact.* **66**, 517.

BROWN, A. D. (1960). Some properties of a Gram-negative heterotrophic marine bacterium. *J. gen. Microb.* **23**, 471.

BROWN, A. D. (1964). Aspect of bacterial response to the ionic environment. *Bact. Rev.* **28**, 296.

BURTON, S. D. & MORITA, R. Y. (1963). Denaturation and renaturation of malic dehydrogenase in a cell-free extract from a marine psycrophyle. *J. Bact.* **86**, 1019.

CAMPBELL, A. E., HELLEBUST, J. A. & WATSON, S. W. (1966). Reductive pentose phosphate cycle in *Nitrocystis oceanus*. *J. Bact.* **91**, 1178.

COHEN, P. P. & SALLACH, H. J. (1961). Nitrogen metabolism of amino acids. In *Metabolic Pathways*. Ed. by D M. Greenberg. Vol. 2. New York: Academic Press Inc.

COLWELL, R. R., CITARELLA, R. V. & RYMAN, I. (1965). Deoxyribonucleic acid base composition and Adansonian analysis of heterotrophic, aerobic Pseudomonas. *J. Bact.* **90**, 1148.

DARON, H. H. (1967). Occurrence of isocitrate lyase in a thermophilic Bacillus species. *J. Bact.* **93**, 703.

DE VRIES, W. & STOUTHAMER, H. (1967). Pathway of glucose fermentation in relation to the taxonomy of Bifidobacteria. *J. Bact.* **93**, 574.

GUNDERSEN, K. (1966). The growth and respiration on *Nitrocystis oceanus* at different partial pressure of oxygen. *J. gen. Microb.* **42**, 387.

HANSON, R. S & COX, D. P. (1967). Effect of different nutritional conditions of the synthesis of tricarboxylic acid cycle enzymes. *J. Bact.* **93**, 1777.

HAYAISHI, O. (1955). Special techniques for bacterial enzymes. Enrichment culture and adaptive enzymes. *Meth. Enzymol.* **1**, 126.

HENDRIE, M. S., HODGKISS, W., & SHEWAN, J. M. (1964). Considerations on organisms of the Achromobacter–Alcaligenes group. *Annls Inst. Pasteur, Lille.* **15**, 43.

HOLDEN, J. T. (1962). The composition of microbial amino acid pools. In *Amino Acid Pools*, Ed. by J. T. Holden. Amsterdam: Elsevier Publ. Co.

HOLMES, P. K & HALVORSON, H. O. (1965). Purification of a salt requiring enzyme from an obligately halophilic bacterium. *J. Bact.* **90**, 312.

HUNGATE, R. E. (1962). Ecology of bacteria. In *The Bacteria*. Ed. by I. C. Gunsalus and R. Y. Stanier. Vol. 4. New York: Academic Press Inc.

JOYNER, A. E. JUN. & BALDWIN, R. L. (1966). Enzymatic studies of pure cultures of rumen microorganisms. *J. Bact.* **92**, 1321.

KATZNELSON, H. & ROBINSON, J. (1956). Observations on the respiratory activity of certain obligately halophilic bacteria with high salt requirements. *J. Bact.* **71**, 244.

KRISS, A. E. (1963). *Marine Microbiology. Deep-Sea.* Translation by J. M. Shewan and Z. Kabata. Edinburgh: Oliver Boyd.

KOGUT, M. & PODOSKI, E. P. (1953). Oxidative pathways in a fluorescent Pseudomonas. *Biochem. J.* **55**, 800.

LASKIN, A. I. (1967). Tetracyclines. In *Antibiotics, Mechanism of Action*, Ed. by D. Gottlieb and P. D. Shaw. Vol. 1, Jena: Springer-Verlag.

MACLEOD, R. A. (1965). The question of the existence of specific marine bacteria. *Bact. Rev.* **29**, 9.

MACLEOD, R. A. & HORI, A. (1960). Nutrition and metabolism of marine bacteria. VIII. Tricarboxylic acid cycle enzymes in a marine bacterium and their response to added salt. *J. Bact.* **80**, 464.

MACLEOD, R. A., ONOFREY, E. & NORRIS, M. E. (1954). Nutrition and metabolism of marine bacteria. I. Survey of nutritional requirements. *J. Bact.* **68**, 680.

MIZZEL, M. & SIMPSON, B. S. (1961). Paper chromatographic separation of amino acids. A solvent to replace phenol. *J. Chromat.* **5**, 157.

OCHOA, S. & STERN, J. R. (1952). Carbohydrate metabolism. *A. Rev. Biochem.* **21**, 547.

PFISTER, R. M. & BURKHOLDER, P. R. (1965). Numerical taxonomy of some bacteria isolated from antarctic and tropical sea-waters. *J. Bact.* **90**, 863.

RHODES, M. E. (1965). Flagellation as criterion for the classification of bacteria. *Bact. Rev.* **29**, 442.

RILEY, J. P. & SKIRROW, G. (1965). *Chemical Oceanography*, vol. 1. New York: Academic Press.

ROBERTS, E. (1960). Glutaminase. In *The Enzymes*. Ed. by P. D. Boyer, H. Lardy and K. Myrback. Vol. 4. New York: Academic Press.

SCHOLES, R. B. & SHEWAN, J. M. (1964). The present status of some aspects of marine microbiology. In *Advances in Marine Biology*. Ed. by F. S. Russel. Vol. 2. New York: Academic Press.

SHEWAN, J. M. (1963). The differentiation of certain genera of Gram-negative bacteria frequently encountered in the marine environment. *Symposium on Marine Microbiology*. Springfield, Ill.: C. Thomas.

SHEWAN, J. M., HOBBS, G. & HODGKISS, W. (1960). A determinative scheme for the identification of certain genera of Gram-negative bacteria with special reference to the Pseudomonadaceae. *J. appl. Bact.* **23**, 379.

SHIEH, H. S. (1965). Further studies on the oxidation of betaine by a marine bacterium: *Achromobacter cholinophagum*. *Can. J. Microb.* **12**, 299.

TYLER, M. E., BIELLING, M. G. & PRATT, D. B. (1960). Mineral requirements and other characters of selected marine bacteria. *J. gen. Microb.* **23**, 153.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Technique*. Minneapolis: Burgess Publ. Co.

WOOD, E. J. F. (1963). Heterotrophic microorganisms in the oceans. *Oceanogr. Mar. Biol. Ann. Rev.* **1**, 197.

WOOD, E. J. F. (1965). *Marine Microbial Ecology*. London: Chapman & Hall.

WOOD, E. J. F. (1967). *Microbiology of Oceans and Estuaries*. Amsterdam: Elsevier, Publ. Co.

ZOBELL, C. E. (1946). *Marine Microbiology*. Waltham, Mass.: Chronica Botanica Press.

EXPLANATION OF PLATES

PLATE 1

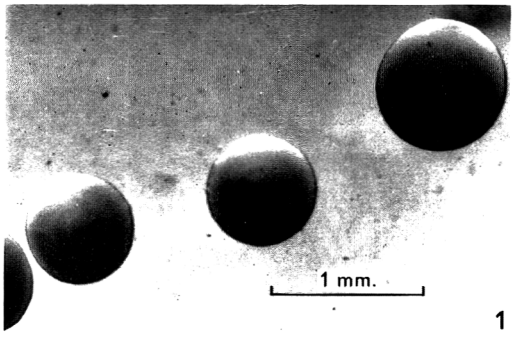
Fig. 1. Micrograph of colonies of strain TS/0-3.
Fig. 2. Micrograph of colonies of strain TS/25-1.
Fig. 3. Micrograph of one colony of strain TS/100-1.
Fig. 4. Phase-contrast micrograph of strain TS/0-3. × 1500.
Fig. 5. Phase-contrast micrograph of strain TS/25-1. × 1500.
Fig. 6. Phase-contrast micrograph of strain TS/100-1. × 1500.

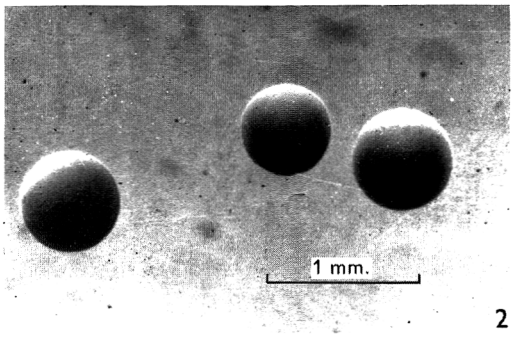
PLATE 2

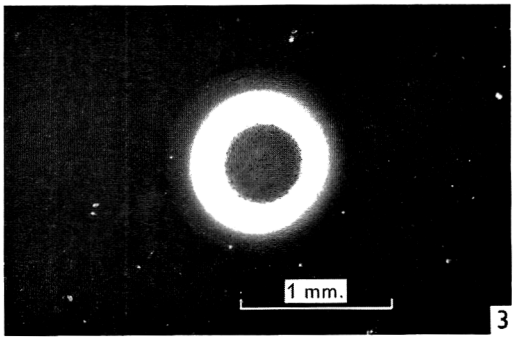
Fig. 7. Electron micrograph of strain TS/0-3.
Fig. 8. Electron micrograph of strain TS/25-1.
Fig. 9. Electron micrograph of strain TS/100-1.

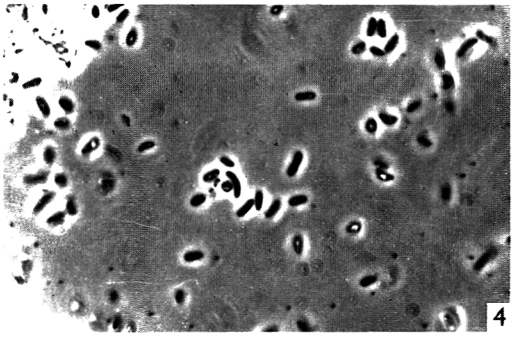
PLATE 3

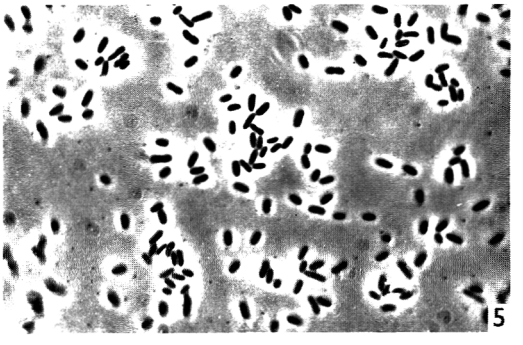
Fig. 10. Amino acids standard chromatogram.
Fig. 11. Amino acids pool chromatogram of strain TS/25-1.
Fig. 12. Protein amino acids chromatogram of strain TS/25-1.

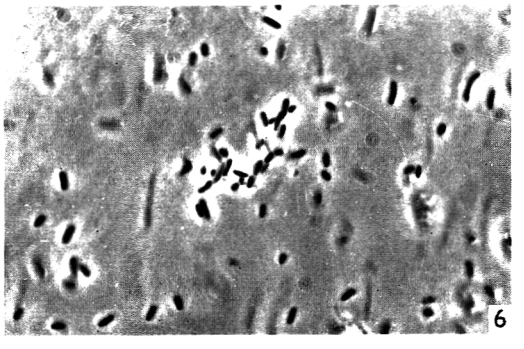












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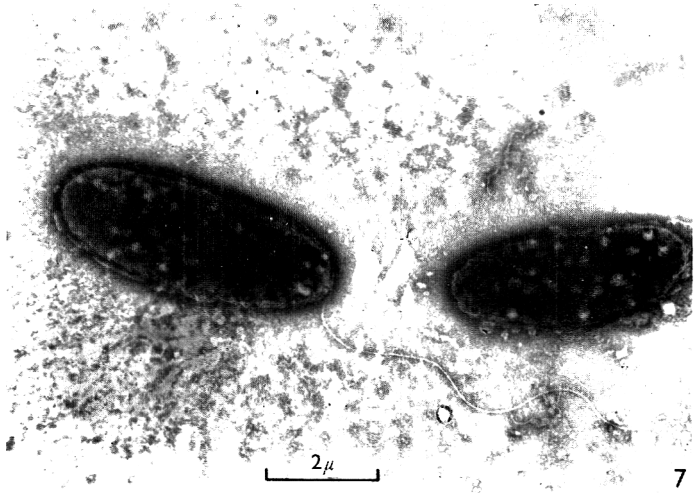

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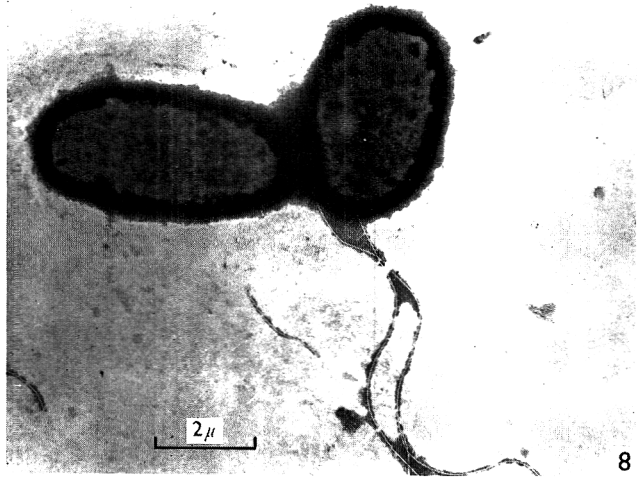

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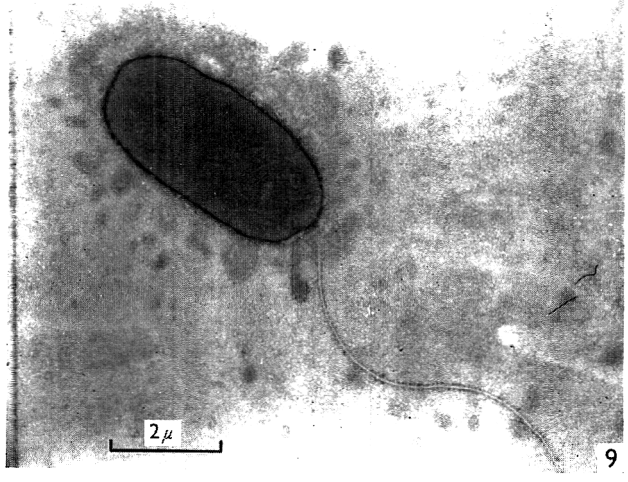

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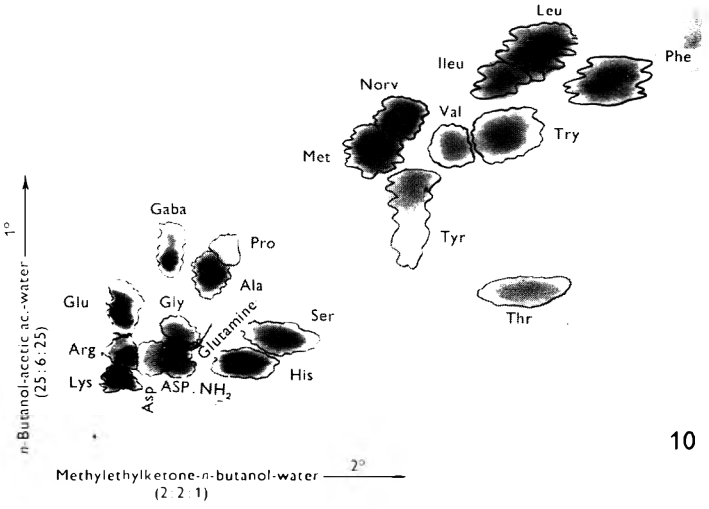
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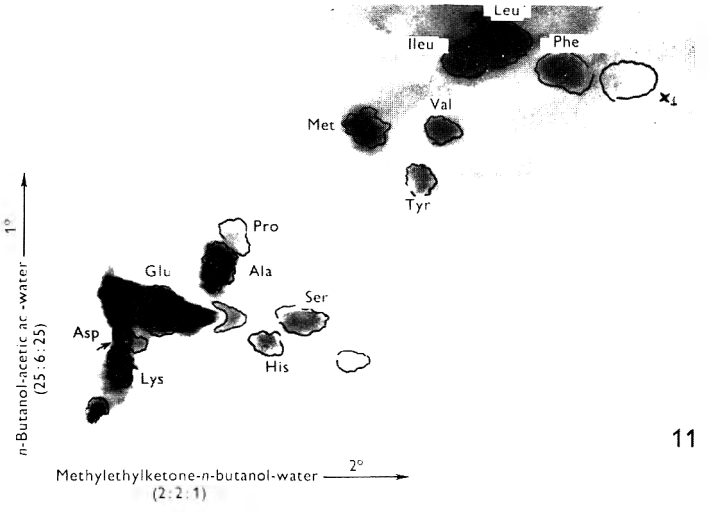
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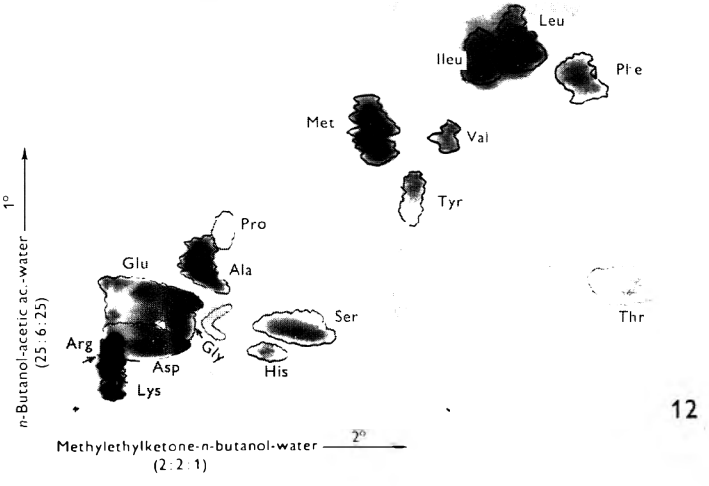
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Electron and Phase-contrast Microscopy of Spores in Two Species of the Genus *Mycotypha* (Mucorales)

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SUMMARY

Capitella of *Mycotypha africana* and *M. microspora* regularly produce sterigmata of two lengths. Spores of *M. africana* are dimorphic and this is probably also the case in *M. microspora*. A membrane which separates from the spore, particularly on germination, is interpreted as evidence that the spore of *M. africana* is a sporangiole and it is suggested that the genus *Mycotypha* should probably be classified in Thamnidiaceae.

INTRODUCTION

Fenner (1932) described the asexual stage of a mucoraceous fungus *Mycotypha microspora* Fenner and classified it in Mucoraceae, as defined by Gaumann (1928). Since conidia are the only spores known to be produced in the asexual stage, Hesseltine (1955) suggested that *M. microspora* should probably be included in Cunninghamellaceae. Novak & Backus (1963) described a second species, *M. africana* Novak & Backus, reported to produce zygospores which are typically mucoraceous in form. In both species, asexual spores are produced on sterigmata which develop from the capitellum of the conidiophore. Spores are dry and readily detached at maturity. Liberated spores of *M. microspora* are described as varying from ovoid to spherical (Fenner, 1932) and those of *M. africana* as oblong–elliptic and globose to oval (Novak & Backus, 1963).

There are clearly two forms of spore in *Mycotypha africana* and there appear to be two spore forms in *M. microspora* also. The aim of this paper is to describe features of interest associated with spore dimorphism in the genus *Mycotypha* as revealed by electron and phase-contrast microscopy and, in the light of these findings, to discuss the possible re-classification of this genus as a member of Thamnidiaceae.

METHODS

The strain of *Mycotypha africana* used was obtained from the Centraalbureau voor Schimmelcultures, Baarn, and *M. microspora* from Birkbeck College, London. They were grown on malt and potato-glucose agars either at 25° or room temperature. Germinating spores of *M. africana* were obtained by incubating them at 37° for about 10 hr.

Single-stage carbon replicas were prepared by the method used previously (Young, 1968). Electron micrographs were taken on Zeiss E.M. 9 and Hitachi 11A microscopes. Anoptral phase-contrast and light photographs were taken with a Reichert Zetopan microscope.

RESULTS

A capitellum of *Mycotypha microspora* subjected directly to an intense beam of electrons may split longitudinally along the surface exposed to the beam, unfold and flatten, thus exposing the inner surface (Pl. 2, fig. 17). Apertures of two sizes can be seen 'large' and 'small' which alternate if traced in a shallow helix around the capitellum (Fenner's shallow primary spiral). Fenner (1932) observed the helical arrangement of conidia on the capitellum and noted that those on one turn alternate with those on the turn directly above and below it. On a collapsed capitellum they appear to be arranged in diagonal rows. If the apertures are traced diagonally, a row of 'large' holes alternates with a row of 'small' ones. To consider only the larger apertures, it can be seen that they alternate in the manner described by Fenner. This is also true of the smaller apertures considered in isolation from the larger ones.

Persistent sterigmata protrude from the external surface of the capitellum. These are cone-shaped, either 'long' or 'short', and alternate as described above. Thus, sterigmata are dimorphic. At the region of attachment of the spore stalk to the sterigma is an annular constriction (Pl. 2, fig. 17). This is a weak zone and the mature spore is readily detached here. An approximately circular scar can be observed at the apex of a sterigma and at the base of a detached spore (Pl. 2, fig. 14, 16). Between the sterigmata are minute spines which are just visible with the light microscope in *M. africana* (Pl. 3, fig. 21) and clearly shown in replicas (Pl. 1, fig. 1, 4).

Detached spores of *Mycotypha microspora*, as seen in silhouette, are smooth. Three shapes are common in profile; oval with a stalk, approximately rectangular with or without a stalk and spherical (Pl. 1, fig. 10). In replicas the spherical spore tends to lie with the stalk either upwards (Pl. 2, fig. 14) or downwards (Pl. 2, fig. 15). The oval spore tends to lie with the stalk horizontal (Pl. 2, fig. 12). A spore in replica invariably shows a single large indentation which may be due to drying out during processing. Spores are probably observed in a collapsed state and may not actually be biconcave. Thus those which appear rectangular in profile could represent collapsed spheres (Pl. 2, fig. 13).

The surface of the spore as seen in replica is granular or roughened and no pattern of surface structure is evident. This contrasts with other described members of Cunninghamellaceae. The pyramidal conidial spines of *Cunninghamella elegans* Lendner have raised, angular bases and conidia of *Phascolomyces articulosus* Boedijn are covered with numerous flattened projections. However, ultrastructural spore morphology of *Mycotypha microspora*, as seen in profile and replica, closely resembles that of *Cokeromyces poitrasii* Benjamin in Thamnidiaceae.

Detached spores of *Mycotypha africana* are either oval (Pl. 2, fig. 18) or spherical (Pl. 2, fig. 11). Liberated spores of *M. microspora* tend to be either oval (Pl. 2, fig. 12) or spherical (Pl. 2, fig. 16). However, this difference is not so sharply defined as in *M. africana*. The oval spores of *M. africana* are separable from those of *M. microspora* since the latter bear relatively prominent stalks whilst the former do not. Further, there can often be distinguished towards the base of the spore in *M. africana* a roughly circular, raised zone (Pl. 2, fig. 18, 19). This distinctive mark has not been observed on the oval spores of *M. microspora*.

The smaller spherical spores can be distinguished on a similar basis. The stalk in spores of both species is relatively short. However, there can often be observed either

one or two raised zones on the spores of *Mycotypha africana* (Pl. 2, fig. 11) which are not evident on those of *M. microspora* (Pl. 2, fig. 15). A further difference between the two species involves the appearance of liberated spores observed in a mass. Those of *M. africana* often fail to separate and can be seen as chains in which oval spores alternate with spherical ones (Pl. 1, fig. 6, 7). Groups of two or three spores are common. Liberated spores of *M. microspora* do not appear to adhere in chains. Detached spores of *M. africana* and *M. microspora* are compared diagrammatically in Fig. 1.

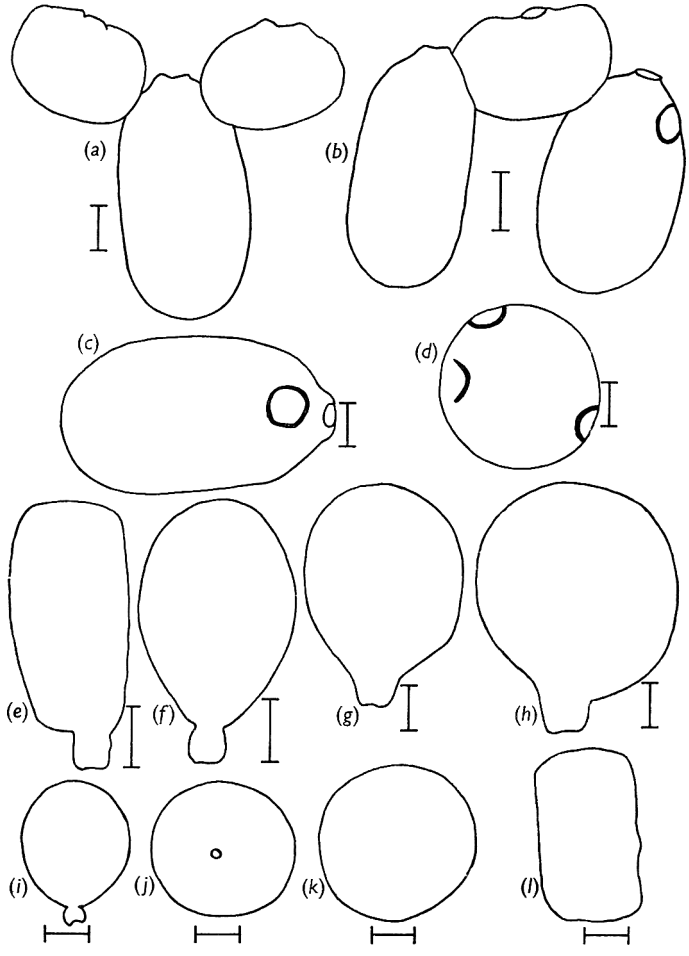

Fig. 1. The scale line indicates 1 μ *a–d, Mycotypha africana*: outlines of spores traced from photographs of replicas; they are clearly dimorphic. *e–l, M. microspora*: spore outlines for comparison with those of *M. africana*; there is a greater range of form although they tend to be either oval or spherical.

It seems highly probable that raised zones on the spores of *Mycotypha africana* correspond to areas of contact between adjacent spores on the capitellum and in detached chains. The positions of these areas support this view (Fig. 1 *a* to *d*). Spherical spores attached to the capitellum (Pl. 3, fig. 20) fit between oval ones and contact the

latter on the basal curve of the oval. The apparent absence of raised zones on the spores of *M. microspora* could be explained by assuming that relatively long stalks on the oval spores hold them out of contact with the spherical spores. This could also account for the absence of spore chains since spores which are not initially in contact are unlikely to adhere together in a special sequence on liberation.

On the basis of light microscopy, the capitellum of *Mycotypha africana* has been described as 'minutely punctate' and 'covered with very small spirally arranged

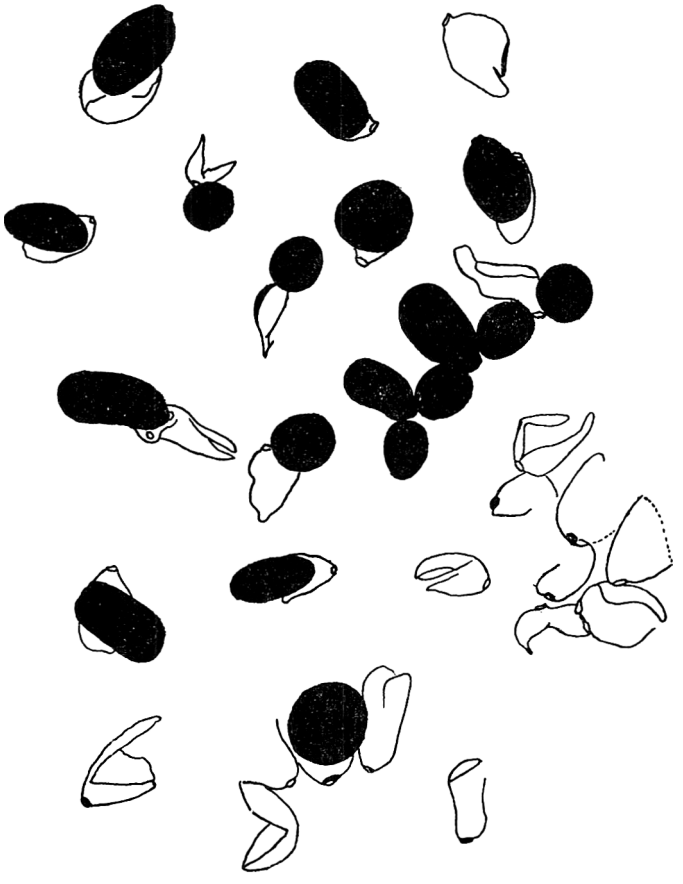

Fig. 2. *Mycotypha africana*: all × 1300. A semi-diagrammatic representation of spores associated with separable outer membranes, traced from phase-contrast photographs. The relatively large size of some of the spores probably indicates that they were at a later stage in germination than the smaller ones.

protuberances which bear the spores' (Novak & Backus, 1963). At high magnification under the light microscope it is possible to see the alternation of 'large' and 'small' holes at the apices of 'long' and 'short' sterigmata respectively (Pl. 3, fig. 21). Either a shallow transverse helix or steep diagonal helices can be traced. Along one side of the capitellum illustrated, both 'long' and 'short' sterigmata can be seen. Replicas of the surface of the capitellum reveal a regular pattern of 'long' and 'short' sterigmata similar to that of *M. microspora* (Pl. 1, fig. 1). Sterigmata are therefore dimorphic in *M. africana*.

Oval spores are probably borne on the 'long' sterigmata and spherical spores on the 'short' ones. The evidence for this view is largely indirect because spores are closely packed on the capitellum (Pl. 3, fig. 20) and it is usually only possible to view sterigmata after spore liberation. However, in part of the capitellum illustrated (Pl. 3, fig. 22) two diagonal rows of spores remain attached. Alongside each row is a row of three or four 'large' holes, assumed to be apertures of 'long' sterigmata. At the edge of this capitellum (labelled A), the peripheral spore from each diagonal row looks spherical and clearly alternates with oval spores. Thus, by inference, spherical spores are attached to 'short' sterigmata. At points indicated by arrows (Pl. 3, fig. 22), oval spores viewed obliquely appear to be attached to 'long' sterigmata. This view is further supported by evidence from electron micrographs of attached spores in profile (Pl. 1, fig. 3, 5), replica (Pl. 1, fig. 2) and from preliminary observations of ultrathin sections of the capitellum.

Spores of *Mycotypha africana* germinate readily on malt or potato-dextrose agar at 37°. Within 10 to 12 hr spores swell considerably. Both forms of spore have a relatively loose outer membrane which is shed as the germinating spore expands. Empty membranes are readily demonstrated by means of phase-contrast microscopy and appear to be common in preparations of spores allowed to germinate for approximately 10 to 12 hr. They have also been observed in preparations of ungerminated spores and in ultrathin sections. The base of a ruptured membrane remains intact and appears as a refractive circle. The circle is the region of attachment to the sterigma. The apex of the membrane is often ruptured and, in oval spores where this may be clearly observed, a longitudinal fissure runs towards the base. Ruptured membranes are illustrated (Pl. 3, fig. 23 to 32; Fig. 2).

DISCUSSION

Sterigmata are dimorphic in the two described Mycotypha species. Basidial dimorphism is a well-known phenomenon in Coprinus species (Buller, 1924), where long basidia constitute the first generation and short basidia the second. It seems likely therefore that a parallel system has evolved in the genus *Mycotypha*, where 'long' sterigmata develop first, followed by a second generation of 'short' sterigmata. This view is largely theoretical. However, in the young capitella illustrated (Pl. 1, fig. 4, 8, 9), 'short' sterigmata are barely developed whereas the 'long' sterigmata are relatively well developed. This evidence appears to support the idea that 'short' sterigmata are second generation.

In contrast to Coprinus, spores in *Mycotypha africana* (and probably *M. microspora*) are dimorphic. In the absence of experimental evidence, a discussion of the probable significance of this phenomenon can only be speculative. One possibility is that the production of spherical and oval spores, linked with 'short' and 'long' sterigmata, may result in making full use of the space available for spore production. Spores are packed closely together on the capitellum (Pl. 3, fig. 20). Secondly, it is possible that each form of spore has a different function and this aspect is under investigation.

The separable outer membrane of the spore is likely to be homologous with the sporangiole wall of such species as *Cokeromyces recurvatus* Poitras or *Choanephora trispora* (Thaxt.) Sinha. Thus, the probability is that the 'conidium' of *Mycotypha africana* is a single-spored sporangiole. Benjamin (1960, plate 2*f*), demonstrated

spores of *Cokeromyces poitrasii* associated with broken membranes which he interpreted as sporangiole membranes. This species produces single-spored sporangioles. The apparent similarity in form of the ruptured sporangiolar membrane to that in *M. africana* is remarkable. Further, as seen in replica, spore surfaces are similar and their capitella bear persistent sterigmata when spores are detached. Zygospores are formed between opposed progametangia in both species. It is generally considered that Cunninghamellaceae are fungi in which the conidium represents the final stage in reduction of a single-spored sporangiole since spore and sporangial walls cannot be distinguished as separate structures. The essential difference between Cunninghamellaceae and Thamnidiaceae is that in the former only conidia are produced in the asexual stage, whereas in the latter, sporangia and/or sporangioles are produced. It seems reasonable to interpret the separable outer wall of the liberated spore in *M. africana* as that of a sporangiole, in which case this species should probably be removed from Cunninghamellaceae and classified in Thamnidiaceae.

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REFERENCES

BENJAMIN, R. K. (1960). Two new members of the Mucorales. *Aliso* **4**, 523.

BULLER, A. H. R. (1924). *Researches on Fungi*, vol. 3, Hafner.

FENNER, E. A. (1932). *Mycotypha microspora*, a new genus of the Mucoraceae. *Mycologia* **24**, 187.

GAUMANN, E. A. (1928). *Comparative Morphology of Fungi*. Translated and revised by C. W. Dodge. London: McGraw-Hill.

HESSELTINE, C. W. (1955). Genera of Mucorales with notes on their synonymy. *Mycologia* **47**, 344.

NOVAK, R. O. & BACKUS, M. P. (1963). A new species of Mycotypha with a zygosporic stage. *Mycologia* **55**, 790.

YOUNG, T. W. K. (1968). Electron microscopic study of the asexual structures in Mucorales. *Proc. Linn. Soc. Lond.* **179**, 1.

EXPLANATION OF PLATES

All magnifications are approximate.

PLATE I

Fig. 1. *Mycotypha africana*. Replica of part of a capitellum showing dimorphic sterigmata. × 4500.

Fig. 2. *M. africana*. Replica of two oval spores attached to 'long' sterigmata. × 7000.

Fig. 3. *M. africana*. Silhouette of an oval spore attached to a 'long' sterigma. × 8750.

Fig. 4. *M. microspora*. Replica of part of a young capitellum. 'Short' sterigmata are not well developed. × 7000.

Fig. 5. *M. africana*. Silhouette of a spherical spore apparently attached to a 'short' sterigma. × 8750.

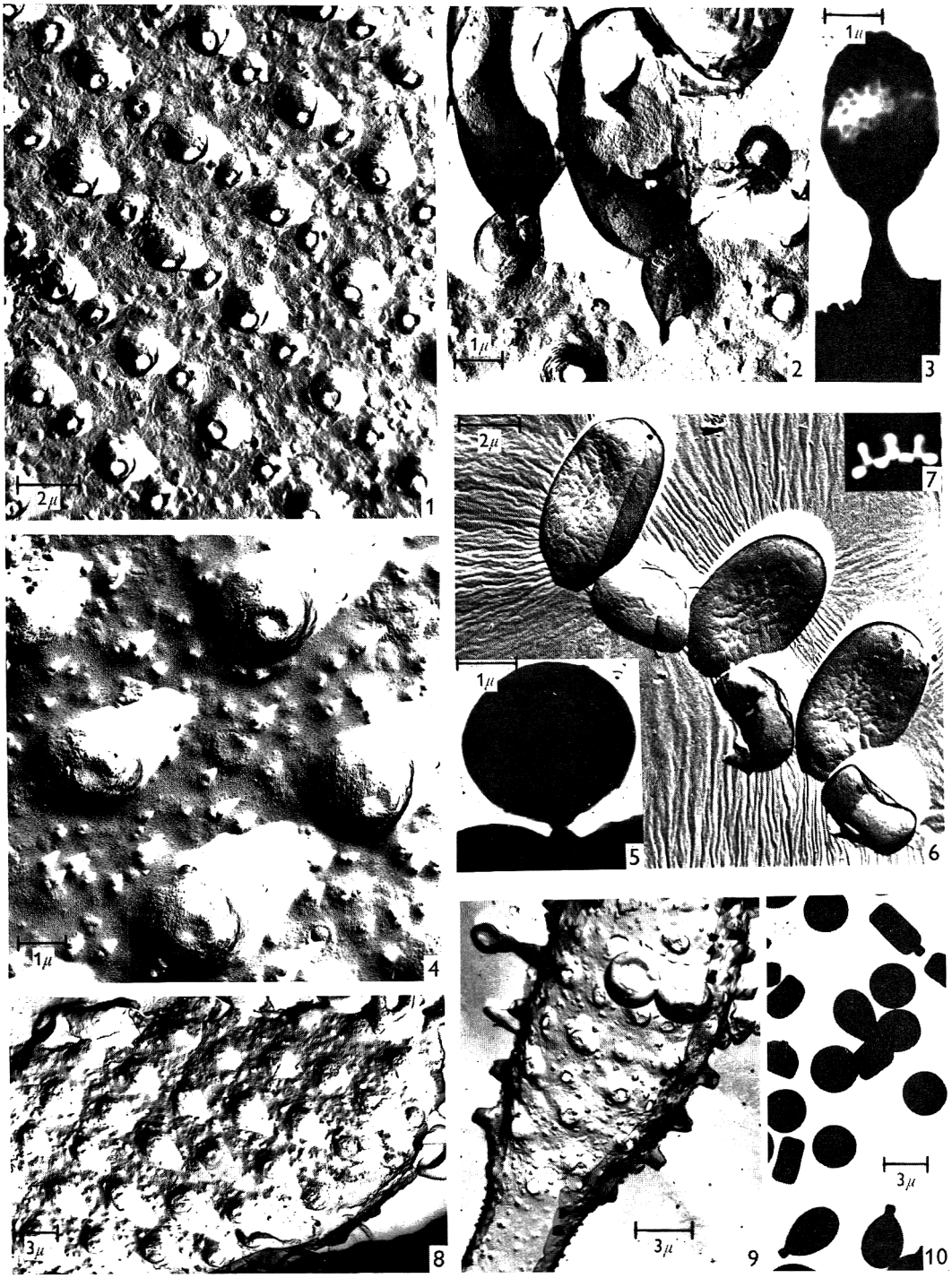
Fig. 6. *M. africana*. Replica of a chain of spores. × 4500.

Fig. 7. *M. africana*. Phase-contrast photograph of a chain of spores. × 1000.

Fig. 8. *M. microspora*. Replica of the young capitellum illustrated in fig. 4. Helical rows of 'long' sterigmata are evident. 'Short' sterigmata are barely perceptible at this magnification. × 2200.

Fig. 9. *M. africana*. Replica of a young capitellum. An apparently oval spore is attached to a 'long' sterigma. × 3000.

Fig. 10. *M. microspora*. Some variety of spore form as seen in silhouette. × 2200.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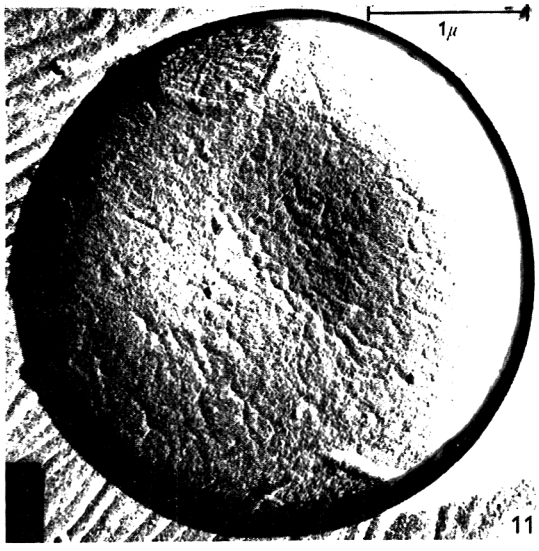
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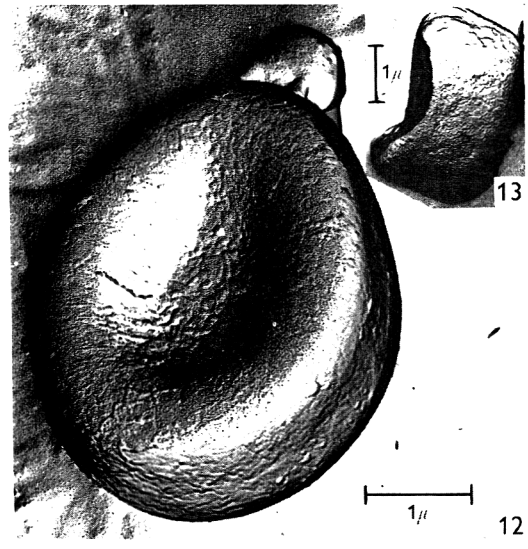
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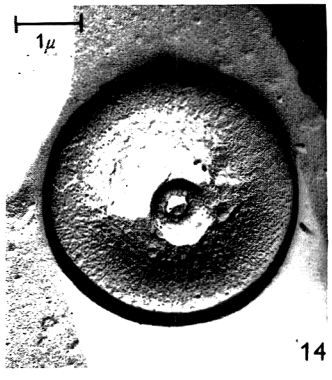
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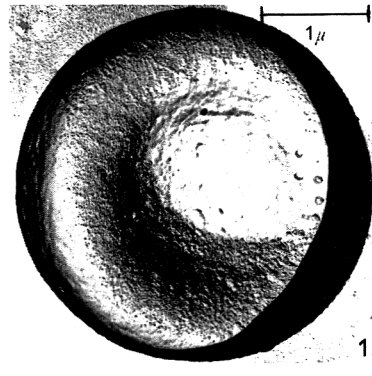
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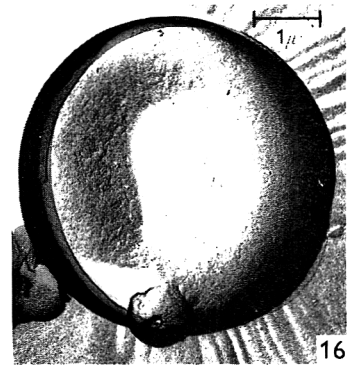
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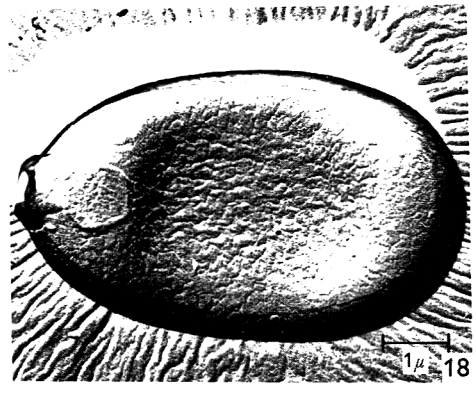
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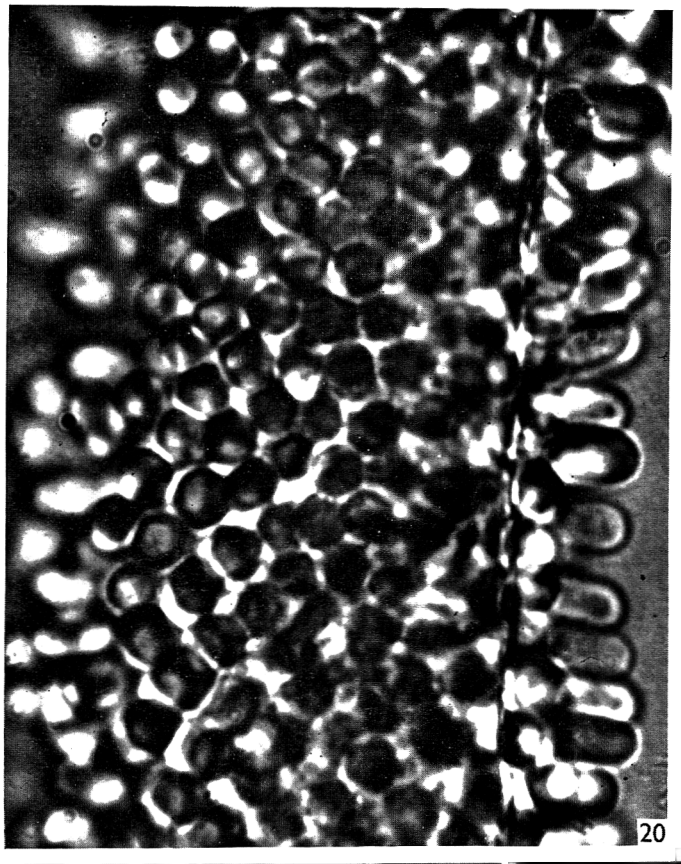
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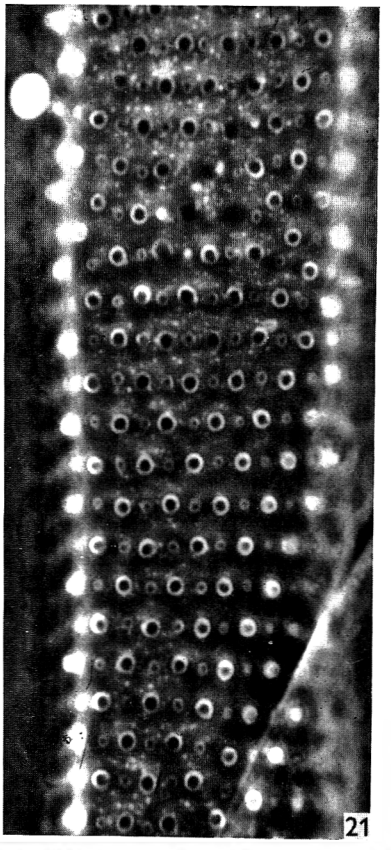
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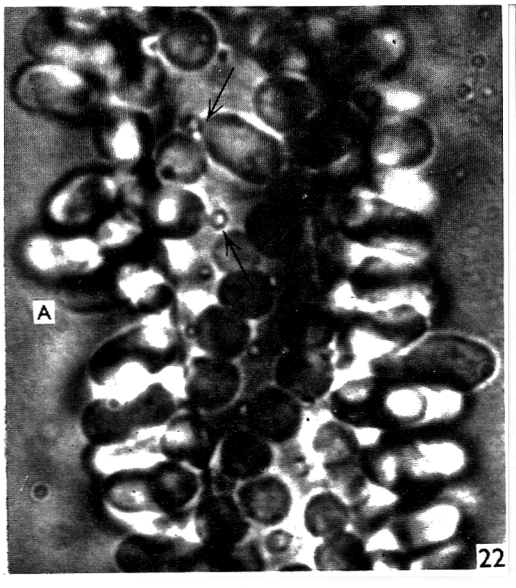
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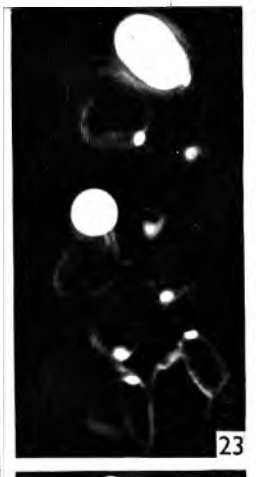
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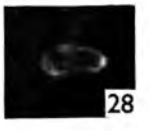
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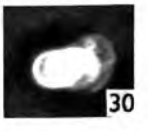
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PLATE 2

Fig. 11. *M. africana*. Replica of a spherical spore bearing two raised areas. × 21,000.

Fig. 12. *M. microspora*. Replica of an oval spore. × 14,250.

Fig. 13. *M. microspora*. Replica of a spore. This corresponds to approximately rectangular spores seen in profile and is probably a collapsed spherical spore. × 7500.

Fig. 14. *M. microspora*. Replica of a spherical spore with the stalk uppermost. × 8750.

Fig. 15. *M. microspora*. Replica of a spherical spore with the stalk downwards. × 14,250.

Fig. 16. *M. microspora*. Replica of a spherical spore with the stalk almost horizontal. × 8750.

Fig. 17. *M. microspora*. The internal surface of part of a capitellum as seen in silhouette. The arrow indicates the annular constriction at the junction of a spore stalk and a sterigma which is still attached to the outer surface of the capitellum. × 2200.

Fig. 18. *M. africana*. Replica of an oval spore. A raised area is visible towards the base of the spore. × 8750.

Fig. 19. *M. africana*. Replica of a raised area towards the base of an oval spore. Note that the spore stalk is relatively short in comparison with that on the oval spore of *M. microspora* in fig. 12. × 24,500.

PLATE 3

Fig. 20 and 22 were taken with ordinary light, the remainder by means of anoptral phase contrast. All of *M. africana* and × 2000.

Fig. 20. Spores closely packed on the capitellum. Oval spores project well beyond the spherical ones.

Fig. 21. A capitellum from which spores have become detached. Between the apertures of dimorphic sterigmata are minute spines.

Fig. 22. Two rows of spores attached to the capitellum alternate with two rows of 'large' holes. These spores appear to be spherical. Arrows indicate two oval spores associated with 'large' holes.

Fig. 23 to 32. Empty membranes, in some cases associated with spores. Fig. 23 to 27 and 31 were observed in cultures of spores allowed to germinate for approximately 11 hr, the remainder in cultures 2 days or more in age.

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Proteolytic Enzymes from Extremely Halophilic Bacteria

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(*Accepted for publication* 1 *October* 1968)

SUMMARY

Halobacterium strains produce a truly extracellular proteinase which degrades gelatine and casein. It has a pH optimum of about 8 and depends upon divalent cations and a high concentration of NaCl or KCl for activity and stability. Proteolytic enzymes were also found in cell homogenates obtained by ultra sonic treatment. A caseinolytic enzyme, probably different from the extracellular one, is associated with particles which sediment upon ultracentrifugation. A soluble peptidase of lower molecular weight is also present in the extract. Both enzymes are dependent upon divalent cations and a high concentration of NaCl or KCl for activity. In contrast to other halophilic enzymes, the proteolytic enzymes of *Halobacterium salinarium* are more active in the presence of NaCl than KCl at equimolar concentrations.

INTRODUCTION

Bacteria of the genus *Halobacterium* are widely distributed in brines of high salt concentration. They are thus conspicuous in solar evaporation ponds of salt works, where they often become the dominating type of organisms when the salt concentration rises above 25 %. The extremely halophilic bacteria are best cultivated in complex media containing peptones and yeast extract to which at least 15 % (w/v) NaCl has been added. Best growth is obtained in media containing about 25 % salt, and when bacteria are transferred to solutions with a salt concentration of less than about 10 % they lyse rapidly.

The unusual properties of halophilic bacteria make them interesting objects of study for both microbiologists and biochemists, and Larsen (1967) summarized the result of a large number of investigations on them. Special methods are often necessary in studies of their metabolism and conventional procedures are unsuitable for the purification and assay of 'halophilic' enzymes.

The extreme halophiles do not generally grow well on carbohydrates but they have a well-developed enzyme apparatus for metabolizing amino acids. In order to utilize the proteins of dead organisms in salt brines the bacteria must form proteolytic enzymes. Gibbons (1957) found that 45 out of 49 tested strains were gelatinolytic, and most of these strains also degraded casein. However, a closer study of the properties of the proteolytic enzymes of such strains has apparently not been made. It is known that the bacteria have an intracellular concentration of NaCl + KCl, which is approximately the same as the NaCl concentration of the growth medium. The halophilic bacteria's enzymes are thus active at salt concentrations which inhibit or even denature many enzymes of non-halophilic organisms. This must be reflected in marked differences in the composition and properties of the protein molecules. Ingram (1947) has

suggested that the halophilic enzymes are smaller than most other enzymes, which would make them more resistant to being salted out. However, no halophilic enzymes have yet been studied in sufficient detail to give substantial support to this hypothesis.

METHODS

Bacterial strains. Most experiments were made with a number of Halobacterium strains obtained from Professor Helge Larsen, Trondheim. Some comparative studies were also made with halophilic bacteria isolated directly from Mediterranean Sea salt or salted fish at this laboratory.

Growth conditions. The bacteria were grown in medium 73, which was developed in this laboratory: $MgSO_4.7H_2O$, 10 g; KCl, 5 g; $CaCl_2.H_2O$, 0·2 g; Mediterranean Sea salt (mainly NaCl), 250 g; gelatine (Oxoid), 10 g; yeast extract (Difco), 1 g; distilled water to 1 l.; pH 7. Some commercial preparations of gelatine were unsuitable because of low solubility in the salt solution. This medium gave good growth and better production of proteolytic enzymes than other media described. The bacteria were grown in Erlenmeyer or Fernbach flasks incubated at 37° on a rotary shaker at 180 oscillations/min. When the bacteria were grown on solid media, 1·5 % (w/v) agar was added.

Measurements of growth. Cell densities of samples from liquid cultures were measured in tubes of 16 mm diameter in a Beckman Colorimeter Model C, using a red filter.

Determination of enzyme activities. Hydrolysis of casein was measured by incubating 1 ml. of enzyme solution (usually containing 25 % (w/v) NaCl) with 1 ml. of a 1 % (w/v) solution of casein (Merck) in 0·1 M-tris which was adjusted to pH 8 with HCl and contained 15 % (w/v) NaCl. The reaction was carried out at 37° and terminated, usually after 60 min, by the addition of 3 ml. of 5 % (w/v) trichloroacetic acid (TCA). After filtration, the extinction of the filtrate was read at 280 mμ against a reaction blank for each sample.

The hydrolysis of peptides was determined at 37° by the ninhydrin procedure of Matheson & Tattrie (1964) with 0·1 % L-leucyl-glycine as substrate, in the presence of 25 % (w/v) NaCl; incubation time 60 min.

RESULTS

Studies of enzyme formation. The amount of TCA-precipitable gelatine was decreased roughly in proportion to the rate of growth when *Halobacterium salinarium* 1 was cultivated in medium 73. Figure 1 shows that there was very little protein left in the medium by the time the culture reached the stationary phase of growth.

Proteolysis was also demonstrated by growing the bacteria on the same medium solidified with agar. The agar plates were inoculated in the middle with a loop so that one colony was obtained. The enzyme activity was then determined by measuring the clear zones around the colonies. Marked differences in proteolytic activities of different strains were observed by this method. The highest activity was obtained with a colourless mutant strain, *Halobacterium salinarium* 1 M, and a vacuolated strain designated *H. salinarium* 5. Relatively small zones were obtained with the wild-type strain *H. salinarium* 1 and almost no activity could be detected after growth of

H. cutirubrum, strain CCNRLO 9. A number of coccoid halophilic bacteria of the Sarcina type and several strains freshly isolated from sea salt and salted fish were found not to be proteolytic with this method.

The formation of extracellular proteinase in liquid cultures was studied quantitatively by removing samples and determining their activity against casein. Figure 1 shows the progress of growth and enzyme formation by strain *H. salinarium* 1 M in medium 73. Merthiolate (0·01 % w/v) was without effect on the proteolytic activity and was therefore added to the samples to stop growth. There were only small differences in caseinolytic activity between samples which had been centrifuged free from bacteria and of samples taken directly from the culture. Furthermore, dense suspensions of growing bacteria which had been washed and suspended in a salt solution of similar

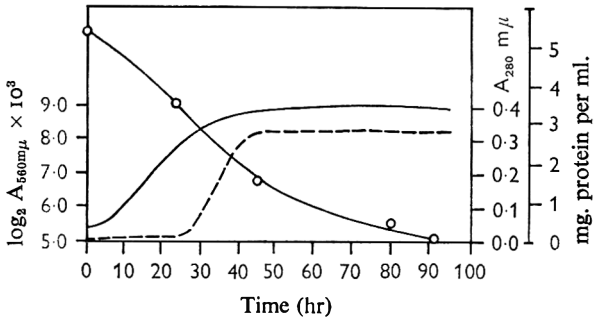

Fig. 1

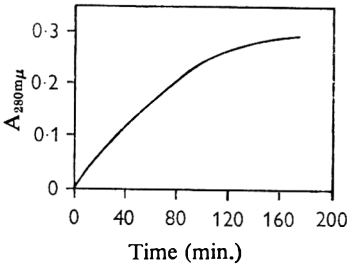

Fig. 2

Fig. 1. Gelatinolytic and caseinolytic ativity of *H. salinarum* 1 cultivated in medium 73. ——, Growth of the bacteria ($A_{280m\mu}$); O——O, the amount of TCA-precipitable gelatine in the cell-free medium as determined gravimetrically (mg. protein/ml.); - - - -, the caseinolytic activity of the medium ($\log_2 A_{560m\mu} \times 10^3$).

Fig. 2. Hydrolysis of casein by cell-free medium after growth of *H. salinarium* 1 M.

composition as medium 73 were without detectable activity. Hence the enzyme was truly extracellular and there was very little caseinolytic enzyme associated with the surface of the cells. As will be shown later, a measurable caseinolytic activity was associated with particles obtained by ultrasonic treatment, but this has different properties from the extracellular proteinase. Furthermore, the cell-bound proteinase had such a low activity against casein under the conditions of our assay that no measurable increase in activity was detected when culture samples were treated ultrasonically before assay.

Figure 2 shows that cell-free culture samples degraded casein to a rather limited degree. The pH optimum for the reaction was about 8 in 0·05 mM-phosphate buffer containing 20 % (w/v) NaCl. There was only slight activity at pH 6·5.

It was necessary to concentrate cell-free culture samples several times by ultrafiltration before gel filtration experiments could be made. When such enzyme solutions were filtered through a column with Sephadex G-100 equilibrated with 0·1 M-tris-HCl, pH 8·0, and containing the salts of medium 73, all the proteolytic activity was obtained in a single peak about 0·5 void volumes after the initial unretarded high molecular weight material.

Cell-bound proteolytic activities. Halobacteria were washed twice in a solution

of (25 % w/v) NaCl and 0·1 % (w/v) $CaCl_2$, suspended as a dense suspension in the same solution, and completely disintegrated by treatment in a Raytheon 250 W, 10 kc., sonic oscillator for 30 min. A low but measurable caseinolytic activity was found in such homogenates, but about 90 % of this activity was associated with particles (cell envelope particles?) which sedimented during centrifugation at 105,000 *g* for 1 hr. Attempts to dissociate the enzyme from these particles by treatment with various detergents have so far been unsuccessful. The pH optimum for hydrolysis of

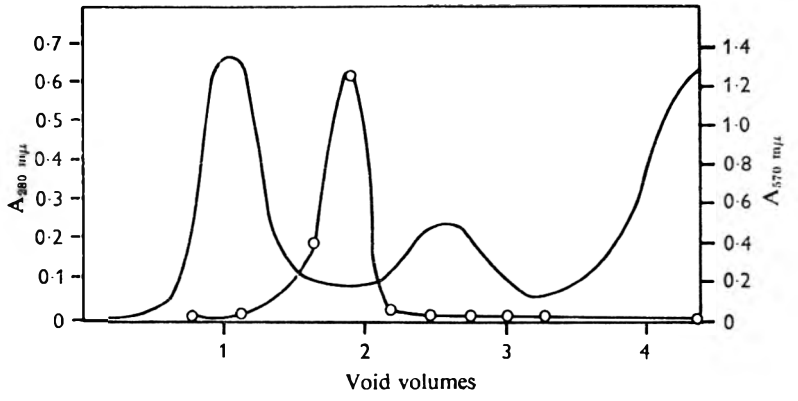

Fig. 3. Gel filtration of cell extract of *H. salinarium* 1 on Sephadex G-100 in the presence of of 25 % NaCl. ——, Material distribution. O——O, Peptidase activity measured against L-leucylglycine.

casein by cell homogenates was significantly lower, about 7, than the value obtained for the extracellular enzyme. Furthermore, the cell-bound proteinase was completely inhibited by addition of 0·04 mg. poly-L-proline per ml., a compound which inhibits some proteolytic enzymes, (Sela & Katchalski 1959). No such inhibition was observed with the extracellular enzyme, and none of the enzymes were inhibited by soybean trypsin inhibitor or chicken egg-white trypsin inhibitor when 0·01 mg. of these were added per ml.

The halophilic bacteria also contained a peptidase which cleaved L-leucylglycine. This enzyme was not particle-bound and the gel filtration diagram shown in Fig. 3 indicates that it had a lower molecular weight than other soluble enzymes in cell extracts.

Influence of NaCl, KCl and divalent ions on the enzymes. Dialysis overnight at +4° of cell-free culture samples or cell homogenates against solutions of 25 % (w/v) NaCl did not lower the enzyme activities. However, dialysis against a neutral NaCl solution containing 0·01 M-EDTA caused complete inactivation of both the extracellular and the cell-bound proteinase and the peptidase. Attempts to obtain reactivation by dialysis against solutions containing 25 % (w/v) NaCl and either 0·02 M-$MgSO_4$, $ZnSO_4$ or $MnSO_4$ were unsuccessful with the extracellular proteinase and the peptidase: 70–90 % of the original activity of the particle-bound proteinase could be reactivated by Zn^{2+} or Mn^{2+} but not by Mg^{2+}. These results indicate that the enzymes are dependent on divalent metal ions for stability and/or activity but the ions are rather firmly bound to the enzyme proteins.

All enzymes of the halophilic bacteria appear to be rapidly and irreversibly denatured in ordinary buffers which do not contain large concentrations of NaCl or

KCl, (Larsen, 1967). The proteolytic enzymes are no exceptions to this rule and we have not been able to reactivate them by dialysis against solutions containing the salts of medium 73. The influence of varied salt concentrations on the assay was also tested. Cell-free culture samples and cell homogenates were dialysed against solutions containing different concentrations of NaCl or KCl, then assayed at concentrations similar to those of the dialysis solutions. As shown in Fig. 4 the caseinolytic and the peptidase activities were constantly higher in the presence of NaCl than KCl.

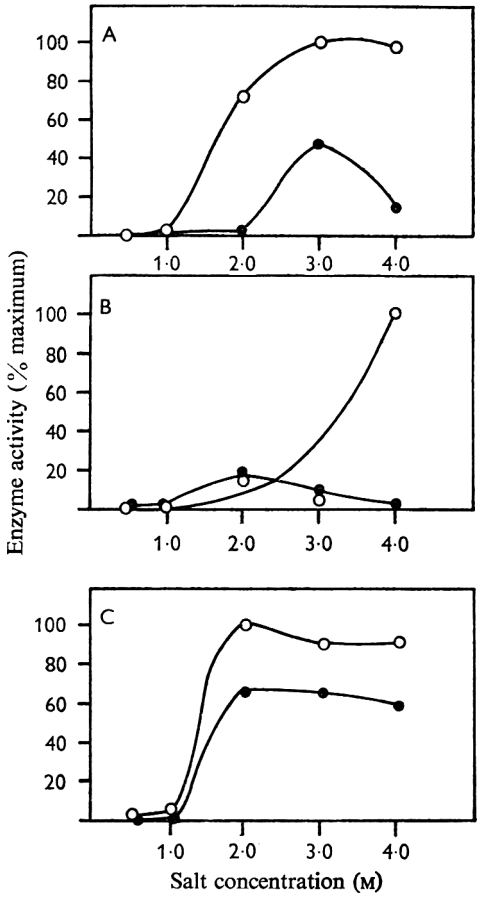

Fig. 4. Effects of NaCl and KCl on the enzyme activities. To facilitate comparisons the highest activity value for each enzyme has been designated arbitrarily as 100% in each experiment. A, Extracellular caseinolytic activity. B, Cell-bound caseinolytic activity. C, Cell-bound peptidase activiy. O——O, NaCl; ●——●, KCl.

DISCUSSION

One would expect the extremely halophilic bacteria to be proteolytic because they occur in protein-rich salt brines and they cause salted fish to spoil. Our results show that some Halobacterium strains do form a true extracellular proteinase, but that different strains vary in proteolytic activity. The low activity of *Halobacterium salinarium* 1 and *N. cutirubrum* may depend on the long time during which these strains

have been kept in culture collections, because proteolysis was a distinguishing characteristic of *H. cutirubrum* when it was originally isolated by Lochhead (1934) and Anderson (1954).

Christian (1956) and Brown & Gibbons (1955) have concluded that potassium ions play an important role in the metabolism of halophilic bacteria by activating certain enzymes. Several enzymes are more active in the presence of KCl than NaCl (Baxter & Gibbons, 1956; Baxter, 1959). An exception is the cytochrome oxidase of *H. salinarium*, which is activated equally well by either KCl or NaCl. The proteolytic enzymes studied by us have a higher activity with NaCl than with KCl. In the case of the extracellular proteinase this is not surprising in view of its occurrence in environments where NaCl dominates.

More experimental work is needed before anything can be said about the physiological function and properties of the cell-bound proteolytic enzymes. It is not impossible that the particle-associated proteinase might be a precursor of the extracellular enzyme, but it may also play a role in intracellular protein turnover.

Our finding that the proteolytic enzymes of the extreme halophiles are irreversibly denatured in ordinary buffers make their purification and characterization difficult. Holmes & Halvorsen (1965) have recently managed to purify a couple of halophilic enzymes in the absence of high salt concentrations during the fractionation, but only a small fraction of the original enzyme activities was recovered in the pure enzyme proteins. Recent developments in separation techniques offer some promise in solving the special problems of the purification of halophilic enzymes, but they still present an interesting challenge to the biochemist.

We are much indebted to Professor Helge Larsen for introducing us into the biology of the extremely halophilic bacteria and for helpful discussions and suggestions. This work has been supported by grants from Magnus Bergwalls Stiftelse, Stockholm.

REFERENCES

ANDERSON, H. (1954). The reddening of salted hides and fish. *Appl. Microbiol.* **2**, 64.

BAXTER, R. M (1959). An interpretation of the effects of salts on the lactic dehydrogenase of *Halobacterium salinarium*. *Can. J. Microbiol.* **5**, 47.

BAXTER, R. M. & GIBBONS, N. E. (1956). Effects of sodium and potassium chloride on certain enzymes of *Micrococcus halodenitrificans* and *Pseudomonas salinaria*. *Can. J. Microbiol.* **1**, 486.

BROWN, H. J. & GIBBONS, N. E. (1955). The effect of magnesium potassium and iron on the growth and morphology of red halophilic bacteria. *Can. J. Microbiol.* **1**, 486.

CHRISTIAN, J. H. B. (1956). *The physiological basis of salt tolerance in halophilic bacteria.* Doctoral thesis, University of Cambridge.

GIBBONS, N. E. (1957). The effect of salt concentration on the biochemical reactions of some halophilic bacteria. *Can. J. Microbiol.* **3**, 249.

HOLMES, P. K. & HALVORSEN, H. O. (1965). Purification of a salt requiring enzyme of *Halobacterium salinarium*. *J. Bact.* **90**, 316.

INGRAM, M. (1947). A theory relating the action of salts on bacterial respiration to their influence on the solubility of proteins. *Proc. R. Soc.* B **134**, 181.

LARSEN, H. (1967). Biochemical aspects of extreme halophilism. In *Advances in Microbiol Physiology*. Ed. by Rose A. H. and J., F. Silkinson, Vol. 1, p. 97. London and New York: Academic Press.

LOCHHEAD, A. G. (1934). Bacteriological studies on the red discoloration of salted hides. *Can. J. Res.* **10**, 275.

MATHESON, A T. & TATTRIE, B. L. (1964). A modified Yemm and Cocking ninhydrin reagent for peptidase assay. *Can. J. Biochem.* **42**, 95.

SELA, M. & KATCHALSKI, E. (1959). Biological properties of polyamino acids. *Adv. Protein Chem.* **14**, 392.

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Ethanol-soluble Intermediates and Products of Acetate Metabolism by *Euglena gracilis* var. *bacillaris*

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(*Accepted for publication* 3 *October* 1968)

SUMMARY

During utilization of [^{14}C]acetate by non-photosynthetic *Euglena gracilis* var. *bacillaris*, ^{14}C was regularly found in trehalose, phosphate esters, glutamate, malate, fumarate, succinate, aspartate, alanine, γ-aminobutyrate, and six unidentified ethanol-soluble compounds. Of the unidentified substances only two, an anthrone-positive material and an ether-soluble substance, were quantitatively important. Small amounts of radioactive laminaribiose, laminaritriose, and what were probably higher oligosaccharides of the laminarin series were found by a more sensitive procedure, as well as several additional labelled ninhydrin-positive substances. The kinetics of labelling were consistent with oxidation of acetate via the tricarboxylic acid cycle and assimilation via the glyoxylate cycle and 'reversed' glycolysis. There was no indication of any qualitative difference between the intermediates of acetate metabolism and those of oxidation of endogenous reserves. Rapid fluctuations in levels of labelled intermediates were observed, some of which coincided with shoulders or plateaux in the radioactivity of the ethanol-soluble fraction as a whole.

INTRODUCTION

Tracer studies (Marzullo & Danforth, 1964*b*) of oxidative assimilation of acetate by non-photosynthetic *Euglena gracilis* var. *bacillaris* indicated that the primary stage of acetate metabolism results in almost quantitative conversion of acetate to two products, CO_2 and the reserve polysaccharide, paramylon. Six hours after addition of [^{14}C]acetate, these two products accounted for some 88% of the acetate carbon consumed, and much of the other 12% was in the form of ethanol-soluble compounds believed to be, at least in part, intermediates on the pathways to paramylon and CO_2. Moreover, paramylon and CO_2 have been found to be produced from acetate in constant proportions; 58% of the acetate carbon is converted to paramylon and 42% to CO_2 (Wilson & Danforth, 1958; Danforth, 1961). Thus, 'primary acetate metabolism' in Euglena is a highly stereotyped process, producing two major products in constant proportions.

In the absence of exogenous substrates, intracellular reserves, particularly carbohydrates, are oxidized at an appreciable rate, and this 'reserve oxidation' has been found to continue, at nearly the same rate, during the utilization of exogenous acetate (Danforth & Wilson, 1961). Although the pathways of reserve oxidation and primary acetate metabolism almost certainly have many enzymic steps in common, there seems to be no competition and little or no pooling of intermediates between the two

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processes. If exchange of intermediates occurred, the apparent carbon balance of primary acetate metabolism as estimated by chemical methods should differ from that estimated by tracer techniques; in fact, the two estimates agree (Danforth, 1961).

In kinetic studies of utilization of [^{14}C]acetate (Marzullo & Danforth, 1964*a*), Euglena cells were separated into fractions soluble and insoluble in 60% (v/v) ethanol. The ethanol-insoluble ^{14}C was almost entirely in paramylon; the composition of the soluble fraction was not further investigated at that time. It was found that after the addition of radioactive acetate, radioactivity appeared in the soluble fraction in two distinct steps. The first phase ('soluble I') behaved as would be expected of a pool of precursors of paramylon, rising rapidly at first and reaching a plateau at 30 to 60 min. This was followed by a second rise in ethanol-soluble radioactivity ('soluble II') beginning at 40 to 90 min. and reaching a plateau at 2 to 3 hr. Soluble II could not consist of precursors of paramylon, since it began to appear only after paramylon synthesis was occurring at near maximal rates.

The present experiments were undertaken to identify the ethanol-soluble intermediates and products of acetate metabolism.

METHODS

The non-photosynthetic strain of *Euglena gracilis* var. *bacillaris*, and the general methods of growth and harvesting of cells were as in previous experiments (Danforth, 1953; Danforth & Wilson, 1957; Marzullo & Danforth, 1964*a*, *b*). Organisms for tracer experiments were grown at 25° in 2000 ml. Erlenmeyer flasks containing 1000 ml. of a medium containing (w/v) 0·25% Bacto-Tryptone (Difco) and 0·3% sodium acetate $3H_2O$. To obtain a highly concentrated ethanol extract of unlabelled euglenas, they were grown in a 20 l. carboy containing 13 l. of the same medium, aerated by bubbling.

The experimental medium consisted of 0·067 M-sodium phosphate buffer, pH 7·0. In the main experiment reported here, 10^7 washed euglenas were suspended in 2 ml. of this medium in a 50 ml. Erlenmeyer flask. At zero time, 21 μmole of sodium [2-^{14}C]acetate (Volk Radiochemical Co., 5 mc./mmole) were added. The flask was incubated on a shaker at 25°. At intervals, 0·1 ml. samples were transferred to 12 ml. conical centrifuge tubes containing 0·15 ml. absolute ethanol. The tubes were shaken, stoppered, and stored at $-5°$. On the following day, each sample tube was centrifuged and 0·2 ml. of the supernatant fluid (ethanol-soluble fraction) was transferred to an empty tube. The precipitates (ethanol-insoluble fractions) were washed twice by resuspension in 0·3 ml. portions of 60% (v/v) ethanol and suspended in 1·0 ml. 60% (v/v) ethanol. Except that [2-^{14}C]acetate instead of uniformly labelled acetate was used, the procedure was essentially the same as that used in earlier studies (Marzullo & Danforth, 1964*a*).

The ethanol-soluble fractions were dried over KOH in an evacuated desiccator and redissolved twice in 0·1 ml. 50% (v/v) acetic acid, drying each time to drive off any remaining [^{14}C]acetate. The dried fractions were taken up in 3 μl. water. Samples (1·0 μl.) of these solutions were applied to chromatograph paper, at a rate which gave spots less than 2 mm. in diameter.

Several experiments of this type were done, differing slightly in concentrations of Euglena and of [^{14}C]acetate. Results were generally similar; the experiment reported is the one which resulted in the greatest resolution on paper chromatographs.

Primary chromatographic separation of the ethanol-soluble fractions was on

Whatman no. 1 filter paper, using ethyl acetate + acetic acid + water (9:2:2, by vol.) as a descending solvent. The paper was cut in the form of a symmetrical trapezoid, with solvent applied to the shorter of the two parallel edges. This geometry gave more compact spots, with less 'tailing', than the conventional rectangular geometry. The total distance of solvent flow was just under 18 in. Radioautographs were prepared using X-ray film (Kodak No-Screen) applied directly to the dried chromatogram and exposed for 10 days. Using the radioautographs for reference, the chromatographs were cut into 'horizontal' strips, so that each strip contained a series of spots representing a single compound (or several unresolved compounds) at successive sampling intervals. Both sides of each strip were scanned with a Geiger–Mueller chromatograph scanning counter which provided a plot of count-rate against distance, and which included a scaler, allowing the total number of counts under each peak to be observed and recorded. We thank Dr W. E. Kisieleski for the use of this instrument, which was designed and built at Argonne National Laboratory. The radioactivity of each spot was taken to be the sum of the counts for that spot from the two sides of the paper. The radioactivity of the soluble fraction was taken to be the sum of counts for all spots at a single sampling time.

Samples (0·1 ml.) of the ethanol-insoluble fractions were dried directly on planchets and counted with a conventional gas flow Geiger–Mueller counter and scaler. Because of the difference in counting method, the data cannot be used to compare absolute amounts of ^{14}C in the soluble and insoluble fractions; the counts of the insoluble fraction were used only to provide a record of the kinetic behaviour of this fraction.

Thin-layer chromatography, both of ethanol extracts and of individual spots eluted from paper chromatograms, was used as a supplementary procedure for identifying labelled components. Glass plates, 20 × 20 cm., spread with Silica Gel G (Research Specialities Co.) were developed two-dimensionally, using phenol + H_2O (75 + 25, w/w) in one direction and *n*-propanol + conc. NH_4OH (7 + 3, v/v) in the second. Reducing sugars were detected by spraying with freshly mixed 0·1 N-$AgNO_3$ + 5 N-NH_4OH (1 + 1, v/v) and heating 10 min. at 110°. Carbohydrates were detected by spraying with anthrone-saturated 10% (v/v) H_2SO_4 and heating 5 min. at 100°, sugar phosphates with the anthrone spray or by the method of Hanes & Isherwood (1949), and amino acids by spraying with 0·1% (w/v) ninhydrin in *n*-butanol and heating 10 min. at 100°.

Tentative identification of individual spots was based on comparison with authentic compounds chromatographed beside the ethanol extracts in the one-dimensional paper chromatographic system. Authentic compounds screened in this way were: acetyl coenzyme A, alanine, α-ketoglutarate, aspartate, β-hydroxybutyrate, cellobiose, citrate, fructose, fructose-1-phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, fumarate, γ-aminobutyrate, glucosamine, glucose, glucose-1-phosphate, glucose-6-phosphate, glucuronic acid, glutamate, glycerol, glyoxylate, laminaribiose, laminaritriose, malate, maltose, 3-phosphoglycerate, proline, pyruvate, ribose, ribose-5-phosphate, succinate, trehalose, uridine diphosphoglucose, and xylose. Laminaribiose and laminaritriose were gifts from Dr B. J. D. Meeuse and Dr B. A. Stone. Other compounds and enzymes were obtained commercially. These tentative results were confirmed by co-chromatography of eluted spots and known substances on paper and in two dimensions on thin layers. Colour reactions, and enzymic treatment of eluates followed by re-chromatography, were also used in some cases. The compounds identified, and the means of identification of each, are listed in Table 1.

It is possible that some unidentified spots may correspond to substances tested in the tentative identification procedure, since we found that the migration rates of a number of pure compounds differed from those of the same compounds in the presence of Euglena extract.

Table 1. *Identification of spots on paper chromatograms of ethanol-soluble Euglena fractions*

Spots are numbered consecutively, from origin toward solvent front.

Spot no.	Identity	Means of identification, comments
0	Unknown	At origin
1+2	Phosphate esters	Acid phosphatase* yields glucose plus other products; aldolase* yields two new products
3	Trehalose	Co-chromatography; hydrolysed to glucose by trehalase (identity of glucose confirmed with glucose oxidase*); hydrolysed, as was authentic trehalose, by the acid phosphatase used in our experiments
3′	Unknown	—
4	Aspartate	Co-chromatography; ninhydrin positive
5	Unknown	—
6	Glutamate	Co-chromatography; reacts with glutamate carboxylase* to γ-aminobutyrate; ninhydrin positive
7	Unknown	Anthrone positive
7′	Glucose	Probably a hydrolysis artifact; found only in extracts which had been stored for considerable periods
8	Alanine	Co-chromatography; ninhydrin positive
9	Malate	Co-chromatography
10	Unknown	—
11	γ-Amino-butyrate	Co-chromatography; ninhydrin positive
12	Unknown	—
13	Fumarate	Co-chromatography
14	Succinate	Co-chromatography
15	Unknown	—
16	Unknown	—
17	Unknown	Ether soluble

* Acid phosphatase (wheat germ) and glucose oxidase (fungal) were purchased from Sigma Chemical Co., glutamic decarboxylase from Nutritional Biochemical Co., and aldolase from Biochemica Boehringer. Samples of trehalase were gifts from Dr E. P. Hill and Mr B. Trivedi.

RESULTS

General characteristics of the oxidative assimilation process. Figure 1 shows the time course of incorporation of ^{14}C derived from acetate into the ethanol-soluble and ethanol-insoluble fractions of Euglena. By comparison with similar experiments (Marzullo & Danforth, 1964*a*), it may be concluded that the first major rise in soluble radioactivity, approaching a plateau at 20 to 30 min., corresponds to 'soluble I' and the rapid rise between 30 and 40 min. to 'soluble II'. The small shoulder at 2 to 8 min. has been noted in most experiments of this type; its significance is unknown. The acetate in the medium was depleted at about 100 min.

Gross composition of the ethanol-soluble fraction. Of the 20 radioactive spots detected by radioautography on paper chromatographs of the ethanol-soluble fractions (Table 1), only 16 were sufficiently active to give significant counts with the chromatograph scanner. Of these eight were identified as single known substances, while no. 1 and 2 (which usually overlapped) were found to contain mixtures of phosphorylated com-

pounds, including fructose diphosphate and at least one phosphorylated derivative of glucose. Of the unidentified spots, only no. 7 (an anthrone positive substance whose chromatographic behaviour resembled that of monosaccharides but which was not identical with any of the known sugars tested) and no. 17 (the only component which entered the ether phase when the ethanol-soluble fraction was partitioned between equal volumes of water and ether) were major components. None of the other unidentified spots ever contained more than 2 % of the total ethanol-soluble radioactivity; the sum of the activities in all such spots was never more than 8 % (usually about 4 %) of the total.

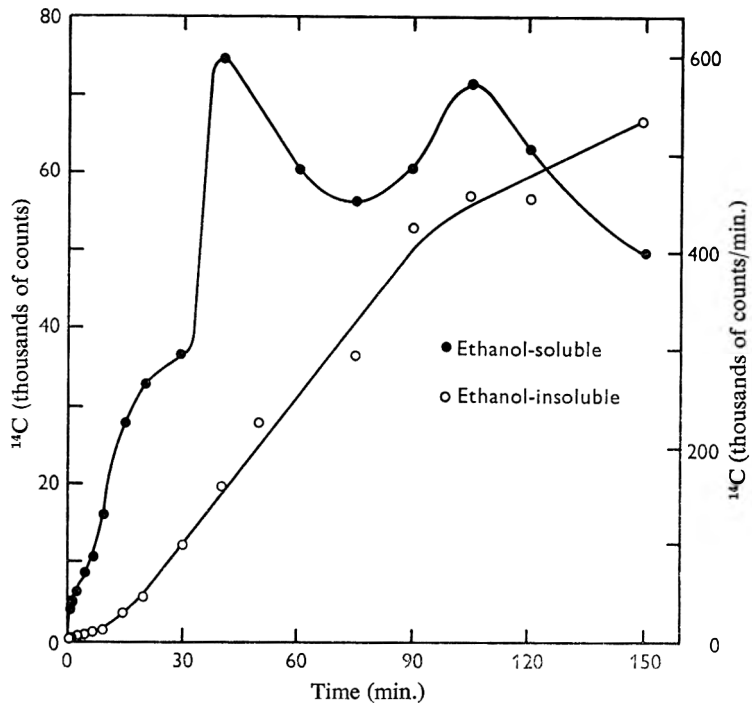

Fig. 1. Labelling of ethanol-soluble and ethanol-insoluble fractions of Euglena as functions of time after addition of [2-^{14}C]acetate. Left ordinate, total counts in soluble fraction; right ordinate, counts per min. in insoluble fraction. The numerical values of the counts for the two fractions are not comparable (see 'Methods'). In Figs. 1–5, points have been connected by smooth curves for clarity. Such curves are not intended to imply any information additional to that represented by the points themselves.

In a separate experiment, an ethanol extract was prepared from a batch of Euglena labelled with acetate of very high specific activity. This extract was combined with a very concentrated extract from unlabelled Euglena and chromatographed two-dimensionally on thin layers of silica gel. Mixtures of known amino acids or carbohydrates were added to some of these plates. Radioautographs were made, and the plates were then treated with anthrone reagent or ninhydrin. In addition to the substances listed in Table 1, these chromatographs showed small amounts of labelled laminaribiose, laminaritriose, and further anthrone-positive substances whose chromatographic behaviour suggested that they were higher oligosaccharides of the

laminarin series. Several weakly-labelled ninhydrin-positive substances were present in addition to the amino acids listed in Table 1; these were not further identified.

Table 2 shows the distribution of ^{14}C among the components of the ethanol-soluble fraction at different times during the experiment shown in Fig. 1. The intervals were chosen to coincide with the plateau of soluble I, the plateau of soluble II, and the period of endogenous metabolism after depletion of acetate. The most noteworthy feature of these data is the similarity of the composition of the ethanol-soluble fraction in all three phases. Although the proportions of individual compounds varied somewhat (see also Fig. 3), every constituent which was found at all was present at all three stages. Spot no. 0, containing one or more substances which did not move from the origin, first appeared at 20 min.; all other spots were already present at 1 min., the earliest sampling time.

Table 2. *Distribution of ^{14}C among the components of the ethanol soluble fraction*

The activity of each component is expressed as percentage of the total ethanol-soluble activity. The highest and lowest values within each time period are given for each compound.

Labelled material	Percentage of total during interval: 15–30 min.	40–75 min.	120–125 min.
Trehalose	31–32	28–32	36
Phosphate esters (no. 1 + no. 2)	22–28	19–23	13
Glutamate	18–23	15–21	7·4–9·2
No. 7	4·0–9·1	10–13	15–16
Malate	2·8–3·3	2·4–2·7	3·5
Fumarate	2·9–4·5	1·9–2·7	1·9–2·0
Succinate	1·9–2·3	1·7–2·3	2·2
Aspartate	2·2–3·4	1·3–1·5	0·8
Alanine	0·8–1·0	0·7–0·8	0·6
γ-Aminobutyrate	2·8–4·2	4·2–5·1	3·4–3·6
No. 17	0·6–7·3	2·1–7·4	8·8
No. 3′	1·7–2·0	1·6–1·7	1·5–1·9
No. 5	1·3–1·7	1·2–1·3	0·9–1·1
No. 7′ (glucose?)	0·6–1·1	0·8–0·9	1·2–1·3
No. 0	0–0·4	0·2–0·6	1·2

Kinetic data. Plots of the percentage of the total soluble radioactivity found in individual components give some indication of the sequence of the metabolic pathways involved. In such plots, the first compounds to be formed from radioactive acetate should start at high values and decline. Compounds further along the pathway should rise to a maximum and then fall. In general, the earlier the maximum, the earlier the position of the compound in the reaction sequence. Figure 2 shows a few examples of such plots for the first 30 min. of the present experiment. Four groups of components were recognized: (*a*) substances with maxima at or before 1 min. (fumarate, succinate, malate, aspartate, alanine, γ-aminobutyrate); (*b*) substances with maxima at 5 to 10 min. (glutamate and phosphate esters); (*c*) trehalose, with a maximum at about 15 min.; (*d*) compounds which have not yet reached a maximum at 30 min. (compound no. 7 and probably compound no. 17; spots corresponding to compound no. 17 were much distorted by irregularities in the solvent front, and this distortion produced large counting errors; for this reason, it is uncertain how much significance should be attributed to the apparent maxima and minima on the curves for this

substance). Figure 3 shows examples of similar plots for the entire duration of the experiment. Trehalose reached a second maximum, higher than the first, at 90 min., approximately the time that the supply of radioactive substrate was used up. Glutamate showed a fairly strong secondary maximum at about 40 min. and γ-aminobutyrate at about 75 min. Compound no. 7, and possibly compound no. 17 continued to increase even after exhaustion of the substrate.

When the early time course of labelling of individual compounds is examined, many of the curves show plateaux or even temporary decreases of labelling superimposed on the general upward trend (Fig. 4). Most such irregularities occurred between 2 and 8 min. or between 20 and 30 min., coinciding with the shoulder and the plateau in the total label of the soluble fraction.

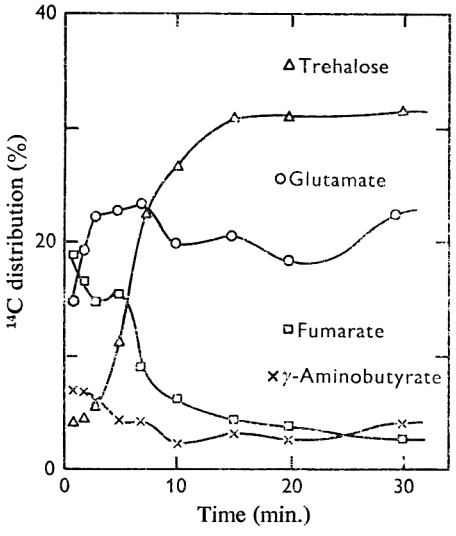

Fig. 2

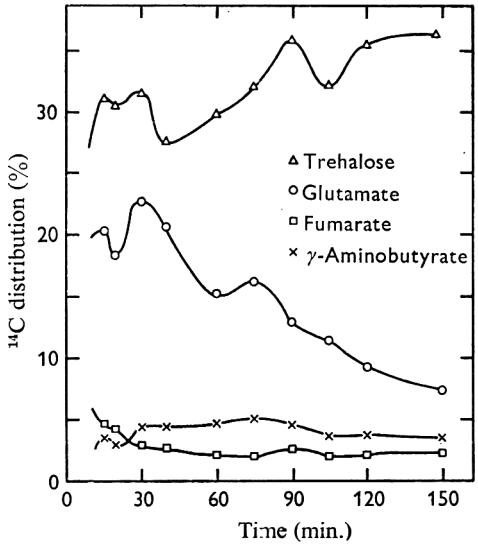

Fig. 3

Fig. 2. Distribution ^{14}C among ethanol-soluble components during the first 30 min. after addition of [^{14}C]acetate. Activities are expressed as percentages of total activity in the soluble fraction.

Fig. 3. Distribution of ^{14}C among ethanol-soluble components between 15 and 150 min. after addition to [^{14}C]acetate. Activities are expressed as percentages of total activity in the soluble fraction.

Between 30 and 100 min., the total amount of radioactivity in the ethanol-soluble fraction fluctuated widely, although results of earlier experiments indicate that oxidative assimilation of acetate should be occurring at a steady rate during this period (Danforth, 1961). We consider it most unlikely that these fluctuations represented errors in sampling or counting procedures. Had errors of such magnitude occurred, they would be expected to distort the portion of the labelling curve between zero and 30 min., yet this portion of the curve is relatively smooth, and of the same form as in previous experiments (Marzullo & Danforth, 1964*a*). No single component, or group of related components, accounted for the peaks and valleys of the ethanol-soluble fraction between 30 and 100 min. (Fig. 5). Almost all the soluble components increased

at the time of the 40 min. peak, though not in equal proportions. Trehalose, compound no. 7, and malate increased greatly during the peak at 100 min., while other components showed little or no rise at this time.

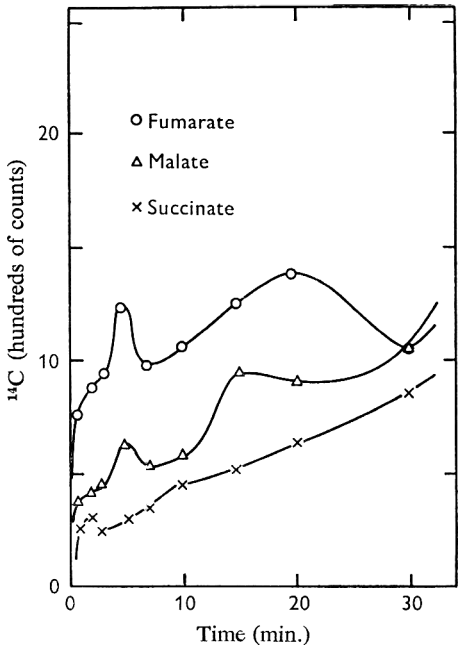

Fig. 4

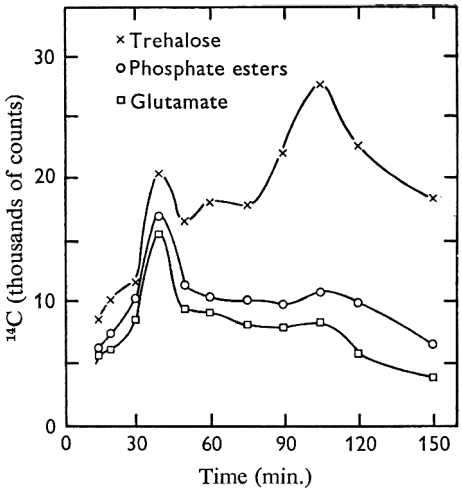

Fig. 5

Fig. 4. Activities of ethanol-soluble components during the first 30 min. after addition of [^{14}C]acetate.

Fig. 5. Activities of ethanol-soluble components between 15 and 150 min. after addition of [^{14}C]acetate.

DISCUSSION

For the most part, the ethanol-soluble intermediates found were those which would have been predicted on the basis of the known or probable pathways of acetate metabolism in phytoflagellates, i.e., intermediates in the tricarboxylic acid and glyoxylate cycles and of glycolysis, or compounds closely related to such intermediates. Likewise, the sequence of labelling of the several groups of compounds is consistent with the operation of these pathways. The presence of trehalose as a major metabolite has not to our knowledge been previously reported in Euglena or any other phytoflagellate. The present results suggest that, as in fungi, it serves as a readily mobilized reserve of carbohydrate and energy. It is likely that trehalose constitutes a portion of the 'labile reserve' postulated by Danforth & Wilson (1961) from kinetic studies, but the amounts of trehalose present are too small to account fully for this labile reserve (Marzullo & Danforth, 1964*a*).

The time-course of labelling of the unidentified compound no. 7 suggests that it may also be an end-product of assimilation, perhaps a reserve substance.

From what is so far known of the metabolism of paramylon, it is likely that the laminarin series of polysaccharides are intermediates in paramylon breakdown

(Maréchal & Goldemberg, 1964; Goldemberg, Maréchal & De Souza, 1966). However, since laminaribiose has been found to stimulate paramylon synthetase (Maréchal & Goldemberg, 1964), these oligosaccharides may also play at least an indirect role in paramylon synthesis.

The amount of glutamate formed from labelled acetate was surprisingly large considering that no exogenous nitrogen source was provided. Moreover, the kinetic behaviour of glutamate (Fig. 2) is that of a much later intermediate than either the Krebs cycle acids or its presumed product, γ-aminobutyrate. Slow mixing of a very large pool of glutamate might account for these kinetics. γ-Aminobutyrate occurs in the extracellular medium of Euglena cultures (McCalla, 1963) and in Chlorella (Warburg, 1958). The rapidity with which it is formed from acetate (Fig. 2) suggests that it is more than an incidental by-product of acetate metabolism. Glutamate, γ-aminobutyrate, and succinic semialdehyde provide a potential by-pass for the α-ketoglutarate dehydrogenase reaction in the Krebs cycle. In brain, as much as 10% of the Krebs cycle activity may go by way of this by-pass (Elliott, 1965).

The soluble components labelled in the first 30 min. almost certainly consist mainly of intermediates and products of primary acetate metabolism (Marzullo & Danforth, 1964*a*), while the intermediates of paramylon breakdown should have begun to show appreciable labelling at about 30 min. (Marzullo & Danforth, 1964*a*; Danforth & Wilson, 1961). After exhaustion of acetate at about 100 min., the intermediates of primary acetate metabolism should gradually disappear. The qualitative identity of the soluble compounds during all three of these periods (Table 2) is direct evidence that primary acetate metabolism and reserve oxidation have many intermediates in common. The lack of interaction between the two processes is thus probably a result of spatial compartmentation.

The reasons for the fluctuations in proportions and absolute amounts of radioactive intermediates (Fig. 4, 5) are unknown. They are not related in any simple way to the synchronized cell division which might be induced by the addition of substrate to cells briefly 'starved' during harvesting, because the shortest generation time recorded for these Euglena, 8 to 12 hr, is much longer than the time scale of the present experiments. It should be noted, however, that damped oscillatory changes in metabolite levels have been found to persist for some time in yeast cells after sudden changes in 'metabolic state', and that these oscillations have been related to feedback mechanisms regulating metabolic rates (Betz & Chance, 1965). It is possible that the much-slower fluctuations observed in Euglena result from some analogous mechanism.

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REFERENCES

BETZ, A. & CHANCE, B. (1965). Phase relationship of glycolytic intermediates in yeast cells with oscillatory metabolic control. *Archs Biochem. Biophys.* **109**, 585.

DANFORTH, W. F. (1953). Oxidative metabolism of Euglena. *Archs Biochem. Biophys.* **46**, 164.

DANFORTH, W. F. (1961). Oxidative assimilation of acetate by Euglena. Carbon balance and effects of ethanol. *J. Protozool.* **8**, 152.

DANFORTH, W. F. (1967). In *Research in Protozoology*. Ed. by T.-T. Chen, vol. I, p. 215. Oxford: Pergamon Press.

DANFORTH, W. F. & WILSON, B. W. (1957). Adaptive changes in the acetate metabolism of Euglena. *J. Protozool.* **4**, 52.

DANFORTH, W. F. & WILSON, B. W. (1961). The endogenous metabolism of *Euglena gracilis*. *J. gen. Microbiol.* **24**, 95.

ELLIOTT, K. A. C. (1965). γ-Aminobutyric acid and other inhibitory substances. *Br. med. Bull.* **21**, 70.

GOLDEMBERG, S. H., MARECHAL, L. R. & DE SOUZA, B. C. (1966). β-1,3-Oligoglucan: orthophosphate glucosyltransferase from *Euglena gracilis*. *J. biol. Chem.* **241**, 45.

HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of the phosphoric esters on filter paper chromatograms. *Nature, Lond.* **164**, 1107.

MARECHAL, L. R. & GOLDEMBERG, S. H. (1964). Uridine diphosphate glucose-β-1, 3-glucan-β-3-glucosyltransferase from *Euglena gracilis*. *J. biol. Chem.* **239**, 3163.

MARZULLO, G. & DANFORTH, W. F. (1964*a*). Kinetic studies on the oxidative assimilation of acetate by a non-photosynthetic strain of *Euglena gracilis*. *J. gen. Microbiol.* **34**, 9.

MARZULLO, G. & DANFORTH, W. F. (1964*b*). Composition of the ethanol-insoluble assimilatory products of oxidative assimilation of acetate by *Euglena gracilis*. *J. gen. Microbiol.* **34**, 21.

MCCALLA, D. R. (1963). Accumulation of extracellular amino acids by *Euglena gracilis*. *J. Protozool.* **10**, 491.

WARBURG, O. (1958). Photosynthesis. *Science, N.Y.* **128**, 68.

WILSON, B. W. & DANFORTH, W. F. (1958). The extent of acetate and ethanol oxidation by *Euglena gracilis*. *J. gen. Microbiol.* **18**, 535.

J. gen. Microbiol. (1969), 55, 267–274
Printed in Great Britain

Biochemistry of Germination in *Peronospora tabacina* (Adam) Conidia: Evidence for the Existence of Stable Messenger RNA

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SUMMARY

In an effort to understand the mechanisms controlling germ-tube differentiation in *Peronospora tabacina* the biochemical events occurring early in germination have been investigated Actidione [2×10^{-6} M] inhibited germination, indicating that differentiation required the synthesis of new protein. However, not all the protein synthesized prior to emergence was essential; only the protein synthesized within 30 min. of the start of germination appeared to be necessary. Inhibitors of RNA synthesis prevented the incorporation of [^{3}H]uridine into RNA, yet had no effect on germination, indicating that differentiation did not require the synthesis of RNA. It is concluded that the protein required for differentiation is synthesized on a stable template of messenger RNA present within the dormant conidium.

INTRODUCTION

Although a number of treatments are known to break the dormancy of fungal spores (for review see Sussman & Halvorson, 1966), at present the biochemical events responsible for the initiation of fungal spore germination are poorly understood. A knowledge of these events would provide information on the control of differentiation, and might also be of use in research aimed at controlling fungal plant pathogens.

In saprophytic species, one of the earliest events occurring in germinating spores is the synthesis of protein (Ohmori & Gottlieb, 1965; Staples, Syamananda, Kao & Block, 1962) but attempts to relate this synthesis to the appearance of certain enzymes have so far yielded only tentative results. In germinating spores of obligate parasites [^{14}C]-labelled amino acids are incorporated into protein indicating that some protein synthesis does occur, but the rates of incorporation are always low and an increase in total protein does not occur. Nucleic acids are also synthesized prior to germ-tube emergence in several fungi (Staples *et al.* 1962) and it is likely, as in *Aspergillus niger*, that this is primarily RNA and not DNA. Gottlieb (1966) suggested that this might be messenger RNA needed for the synthesis of early protein. However, the data of Ono, Kimura & Yanagita (1966) for *A. oryzae* indicated that RNA synthesized early in germination was ribosomal and transfer RNA and that messenger RNA was not formed until germination was well advanced. The fact that many purine and pyrimidine analogues failed to inhibit the germination of *Peronospora tabacina* conidia (Shepherd, 1962) cast doubt on the direct involvement of RNA synthesis in germination. In addition, Shaw (1967) has observed that actinomycin D (20 μg./ml.) failed to inhibit the germination of *Puccinia helianthi* uredospores, although it inhibited the incorporation of [^{3}H]cytidine into RNA.

I have attempted to resolve the problem of whether the germination of *Peronospora tabacina* conidia requires the synthesis of both protein and/or RNA. The obligate parasite *P. tabacina* was chosen because conidia were readily available and, in addition, much was already known about the effects of environmental factors on germination (Shepherd 1962). The present experiments provide evidence that protein synthesis is an important early event in germination whereas RNA synthesis, as measured by the incorporation of [^{3}H]uridine, is not essential before germ-tube emergence. It is concluded that dormant spores contain stable messenger RNA.

METHODS

Preparation of conidia. Infected 8-week-old tobacco plants (*Nicotiana tabacum* cv. *Virginia Gold*) were induced to sporulate by placing them overnight in a chamber at 20° and 100% humidity. Conidia were washed from the fourth youngest leaves with ice-cold water. In these conidia dormancy is maintained by a germination inhibitor which, when washed from the conidia, allows germination to commence (Shepherd & Mandryk, 1962). To remove traces of this inhibitor conidia were washed three times with ice-cold water, centrifuging after each washing (3000 *g* for 5 min.). Conidia were then suspended in a small volume of magnesium + phosphate buffer (1·0 g. $MgSO_4 . 7H_2O$; 1·52 g. KH_2PO_4; 4·608 g. $Na_2HPO_4 . 12H_2O$; 1000 ml. distilled water, pH 7·0) and held in ice while the concentration was determined with a haemocytometer slide. Conidia were germinated in the same buffer in 5·0 cm. diameter Petri dishes at 15°. The final volume in each Petri dish was 2·5 ml. A concentration of $5{\cdot}0 \times 10^5$ conidia per ml. was used unless otherwise stated.

Assessment of germination. A high inoculum concentration ($> 3{\cdot}0 \times 10^6$) and a low temperature kept conidia dormant. The germination process was initiated by raising the temperature to 15° and by lowering the conidial concentration, and was considered complete when the first sign of a germ tube was noted. The first germ tubes appeared 60 min. after the start of incubation, but poor synchronization meant that some conidia only germinated after 4 hr. Therefore, in experiments where percentage germination was recorded, conidia were killed with formalin after 4 hr and an assessment made on a sample of 300 conidia.

Amino acid and nucleotide pool determinations. Both the amino acid and nucleotide pools were measured at intervals during the germination process. $1{\cdot}25 \times 10^6$ conidia were extracted twice with hot ethanol, followed by two further extractions with cold 70% ethanol. Extracts were combined, evaporated to dryness, and purified by elution from Dowex 50 (H^+) with 1·5 N-ammonia. After removing the ammonia, amino acids were estimated by the method of Cocking & Yemm (1954). For nucleotide estimations, $7{\cdot}5 \times 10^6$ conidia were extracted twice with cold 10% perchloric acid. These extracts were combined and the nucleotides adsorbed on to 10 mg. of acid washed charcoal by shaking at room temperature for 30 min. The charcoal was separated by centrifugation and the nucleotides eluted at 37° using 50% (v/v) ethanol + 1% (v/v) ammonia. The $E^{260}_{1\,cm.}$ of this eluate was taken as a measure of the nucleotides.

Radioisotopes. Reconstituted [^{14}C]yeast protein hydrolysate was purchased from Schwarz Biochemicals Inc., Orangeburg, N.Y., U.S.A. [^{3}H]Uridine (13 c/mmole) and [^{3}H]cytidine (30·2 c/mmole) were purchased from the Radiochemical Centre, Amersham, U.K.

RESULTS

Protein synthesis and germination

Figure 1 shows the rate of incorporation of [^{14}C]-labelled amino acids into protein during the first 90 min. of germination. The initial rate of incorporation declined after the first 15 min. period and the lower rate was maintained during the rest of the germination process. This decline was not due to changes in the amino acid pool, as this remained constant throughout germination. Thus, protein synthesis occurred before germ-tube emergence, although no significant increase in total

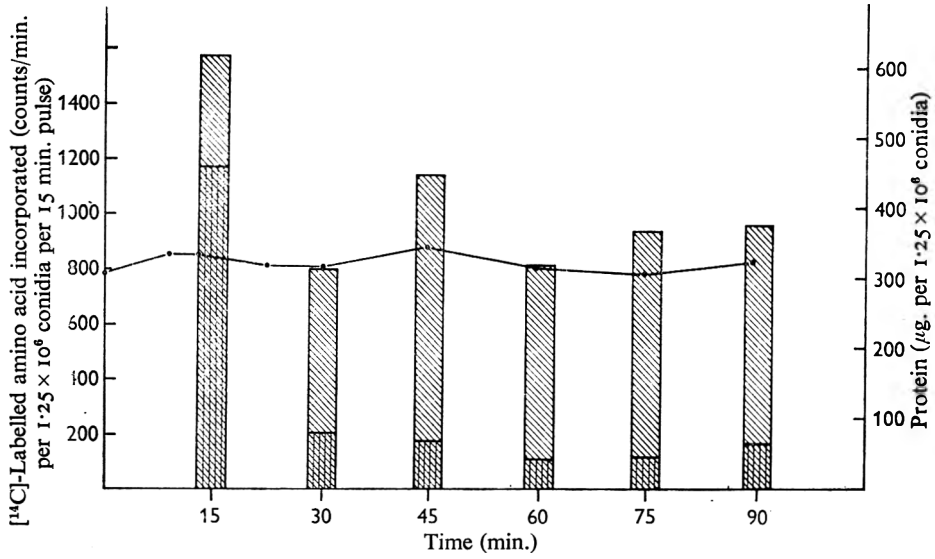

Fig. 1. Effect of actidione on the rate of incorporation of [^{14}C]-labelled amino acids into protein during the early stages of germination in *Peronospora tabacina*. Conidia were labelled with 15 min. pulses of [^{14}C]-labelled amino acids (0·4 μc/ml.) given at 15 min. intervals after the start of germination. Incorporation was stopped by the addition of 0·5 ml. cold 30% (w/v) trichloroacetic acid [TCA] and conidia were transferred to centrifuge tubes washing with cold 5% (w/v) TCA + 0·1% casamino acids. After centrifugation [3000 *g* for 5 min.] the supernatant was discarded and the conidia resuspended in 5% TCA solution, heated to 90° for 10 min., collected on membrane filters [Oxoid], and dried beneath an infrared lamp. Radioactivity was counted in a micromil thin window counter [Nuclear Chicago C. 210]. Each histogram represents the radioactivity incorporated during the preceding 15 min. time interval. 1·25 × 10^6 conidia were extracted with either hot 70% (v/v) ethanol or cold 10% (v/v) perchloric acid, and the residues heated in 2 N-NaOH for 10 min. at 100°. Protein was estimated by the modified Folin–Ciocalteu method suggested by Layne (1957). ▨, Control; ⁘, actidione (2 × 10^{-6} M); ●—●, protein.

protein could be detected (Fig. 1). Evidence obtained by using the specific protein-synthesis inhibitor, actidione (cycloheximide), showed that this protein synthesis was required for germ-tube formation. Actidione (2 × 10^{-6} M) inhibited both [^{14}C]-labelled amino acid incorporation into protein (Fig. 1) and germination. Table 1 shows that actidione only inhibited germination when added within 30 min. of the start of germination, indicating that the protein synthesis required for germ-tube formation occurred early in the germination process. However, in additional experiments germination was not inhibited by puromycin (160 μg./ml.) or amino acid

analogues (*p*-fluorophenylalanine, canavanine, and ethionine, all 200 μg./ml.), even when added at the start of germination perhaps because they failed to penetrate the conidia.

Table 1. *The effect of actidione on the germination of Peronospora tabacina conidia*

Actidione [$2{\cdot}0 \times 10^{-6}$ M] was added to conidia [$2{\cdot}0 \times 10^{5}$/ml.] at intervals after the start of germination. Percentage germination was assessed both at the time of adding actidione and after 4 hr, thus giving a measure of the number of conidia which germinate in the presence of actidione.

Time (min.) of adding actidione after the start of incubation	% germination at time of adding actidione	% germination after 4 hr	% conidia which germinate in the presence of actidione
0	0	1·0	1·0
10	0	3·0	3·0
20	0	11·0	11·0
30	0·5	50·5	50·0
40	0·5	90·0	89·5
50	0·5	94·0	93·5
60	3·25	100·0	97·75

RNA synthesis and germination

When *Peronospora tabacina* conidia were incubated in the presence of [^{3}H]uridine radioactivity was incorporated into RNA (Fig. 2). The rate of incorporation was highest during the first 15 min. period and then declined to a negligible rate after 30 min. This reduction was unlikely to result from a change in pool size because, although the uridine pool was not determined separately, only a small increase in the total nucleotide pool was detected prior to germ-tube emergence.

Actinomycin D (80 μg./ml.) failed to inhibit both germination and the incorporation of [^{3}H]uridine into RNA suggesting that conidia were impermeable to this inhibitor. No conclusions concerning the involvement of RNA synthesis in germination could, therefore, be drawn from this experiment.

Proflavine and ethidium bromide form complexes with DNA by intercalation between adjacent base pairs of the DNA double helix thus preventing the transcription of DNA. At low concentrations both inhibitors had no effect on germination (Fig. 3), although both did inhibit the incorporation of [^{3}H]uridine into RNA (Fig. 2). Thus germ-tube emergence would seem to be independent of RNA synthesis. At higher concentrations proflavine and ethidium bromide inhibited germination but, as Waring (1966) has pointed out, at these concentrations protein synthesis is also inhibited.

Heidelberger (1965) suggested that 5-fluorouracil mainly inhibits DNA synthesis, although a few examples are known where 5-fluorouracil, especially at high levels, directly inhibits RNA synthesis (Cohen *et al.* 1958). No incorporation of [^{3}H]cytidine into alkali-resistant, acid-insoluble material occurred during germination, indicating that [^{3}H]cytidine was not incorporated into DNA. In the absence of any such incorporation, it was possible to show that 5-fluorouracil had a substantial effect on the incorporation of [^{3}H]cytidine into RNA (Table 2) although, at concentrations below 240 μg./ml., it had no effect on germination. This finding demonstrated further that much of the RNA synthesized before germ-tube emergence was inessential for germ-tube differentiation.

The type of RNA being synthesized during germination was examined by extracting RNA from conidia germinated for 60 min. in the presence of [^{3}H]uridine and fractionating it on a sucrose density gradient. The radioactive profile shown in

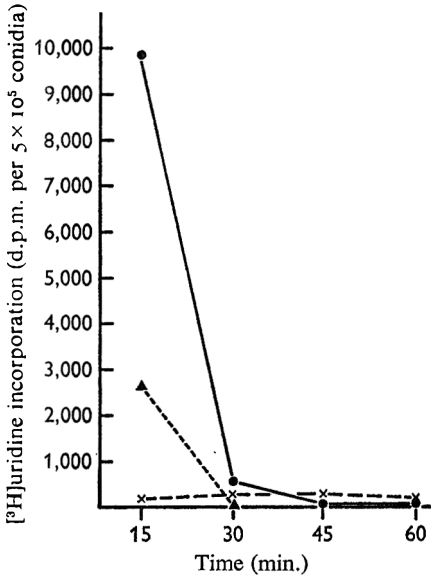

Fig. 2

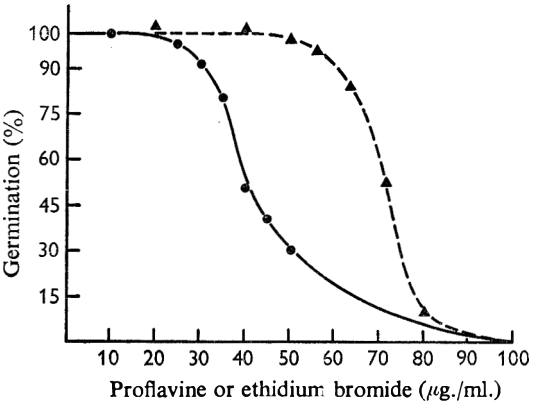

Fig. 3

Fig. 2. Effect of proflavine (25 μg./ml.) and ethidium bromide (50 μg./ml.) on the incorporation of [^{3}H]uridine into RNA during the first 60 min. of germination. Conidia were labelled for 15 min. pulses with 2·0 μc/ml. [^{3}H]uridine. Incorporation was stopped by the addition of cold 30% (w/v) TCA and conidia were transferred to membrane filters as described in Fig. 1, except that the hot TCA wash was omitted. (When heated at 90° for 10 min. all radioactivity was lost from the conidia.) Filters were dried by successive washes with 95% (v/v) ethanol, and ether before the radioactivity was counted in a Packard Tri-carb Liquid Scintillation Spectrometer using 2 to 5 diphenoxazole (6·0 g./l. toluene) as scintillant. ×---×, Proflavine; ▲----▲, ethidium bromide. ●—●, control.

Fig. 3. Effect of proflavine and ethidium bromide on the germination of *Peronospora tabacina*. Proflavine and ethidium bromide were added at the start of germination and the percentage germination was assessed after 4 hr at 15°. ●—●, Proflavine; ▲—▲, ethidium bromide.

Table 2. *The effect of 5-fluorouracil on* [^{3}*H*]*cytidine incorporation into RNA*

Conidia (2·0 × 10^5/ml.) were incubated for 60 min. in the presence of [^{3}H]cytidine (2·0 μc/ml.). Conidia were collected and prepared for scintillation counting as described in Fig. 2. The radioactivity lost on heating conidia for 10 min at 100° in 5% (w/v) TCA was taken as a measure of the incorporation into RNA. After 4 hr incubation in the presence of 5-fluorouracil (240 μg./ml.) the percentage germination was 97%.

Concentration of 5-fluoro-uracil (μg./ml.)	[^{3}H]Cytidine incorporated into RNA (d.p.m.)	% inhibition
0	24,922	0
80	10,720	57
120	10,198	59
160	7,715	69
200	7,474	70
240	5,479	78

Fig. 4 shows two peaks which are characteristic for ribosomal RNA. Some incorporation into soluble RNA was observed, but no class of RNA corresponding to messenger RNA was synthesized. This ribosomal RNA was not required for germ-tube differentia-

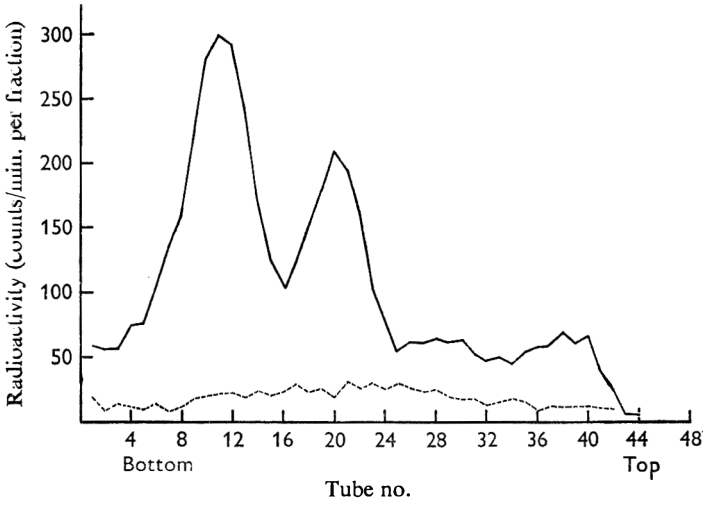

Fig. 4. Sucrose density gradient fractionation of RNA from germinating *Peronospora tabacina* conidia. $1{\cdot}25 \times 10^7$ conidia were incubated for 60 min. with a total of 50 μc [^{3}H]uridine. Uptake was stopped by rapidly cooling to 0°. Conidia were collected by centrifugation and augmented with $2{\cdot}5 \times 10^7$ dormant conidia to provide sufficient RNA for manipulation during the isolation procedure. Conidia were suspended in 3 ml. 0·05 M-tris/HCl, pH 8·0, containing $5{\cdot}0 \times 10^{-3}$ M-$MgCl_2$ and 1 % (w/v) sodium dodecyl sulphate (SDS) and sonicated (Raytheon D.F. 101. setting 50) for 20 min. at 4°. Glass sinter was added to assist in breaking the conidia, with the result that breakage always exceeded 80 %. The sonicate was extracted three times with tris-saturated phenol in the presence of 0·05 M-EDTA and 0·5 % (w/v) SDS. All operations were carried out at 4° and, after adjusting the combined aqueous phases to pH 5·8 with 2·0 M-sodium acetate, RNA was precipitated by the addition of two volumes of cold ethanol. The RNA was purified by precipitation with 1 % (w/v) cetyl trimethylammonium bromide (CTAB). This CTAB + RNA precipitate was washed with 70 % (v/v) ethanol in 0·1 M-sodium acetate and the resultant Na-RNA was dissolved in 0·2 ml. glass distilled water and stored at − 15°. The RNA (250 μg.) was fractionated on a 5 to 20 % (w/v) sucrose density gradient (0·025 M-tris HCl pH 7·0 containing 0·1 M-NaCl) by centrifugation for 4·5 hr at 37,000 rev./min. (Spinco S.W. 39 rotor). Samples were collected dropwise from the bottom after puncturing the tube, and the fractions assayed directly for radioactivity by means of liquid scintillation spectrometry. ——, control; - - - - -, proflavine.

Table 3. *The effect of ethidium bromide, 5-fluorouracil, and proflavine, on the incorporation of* [^{14}C]*-labelled amino acids into protein*

Conidia ($2{\cdot}0 \times 10^5$/ml.) were incubated for 60 min. in the presence of [^{14}C]-labelled amino acids (0·2 μc/ml.). Conidia were collected and prepared for counting as described in Fig. 1. The inhibitors were added at the start of germination and the percentage germination was assessed after 4 hr at 15°.

Inhibitor	% germination	[^{14}C]-labelled amino acids incorporated into protein (c.p.m.)	% inhibition
None	100	2322	0
Ethidium bromide (50 μg./ml.)	100	2116	9
5-Fluorouracil (240 μg./ml.)	96	2252	3
Proflavine (25 μg./ml.)	98	1215	48

tion because, when conidia were labelled for 60 min. in the presence of proflavine (25 μg./ml.), radioactivity was not incorporated into either ribosomal peak.

At concentrations which inhibited RNA synthesis, yet had no effect on germination, ethidium bromide, and 5-fluorouracil had little effect on the incorporation of [^{14}C] amino acids into protein (Table 3), whereas proflavine inhibited this incorporation by 50%. This experiment demonstrated not only that considerable protein synthesis occurred in the absence of RNA synthesis but also showed that not all the protein synthesized was essential for germ tube emergence.

DISCUSSION

These experiments demonstrate that protein synthesis is an essential requirement for germ-tube differentiation, but that RNA synthesis is not. Although [^{3}H]uridine is incorporated into RNA prior to germ-tube emergence, this RNA synthesis is not essential and can be abolished by inhibitors which have no effect on germination. The protein which is involved in germ-tube formation appears, therefore, to be synthesized on a template of stable messenger RNA already present in dormant conidia. Alteration of this RNA in such a way that it is able to act as a template for protein synthesis may be the first step in the germination process.

Fungal spore germination involves changes in both metabolism and morphology, and as such provides a model system for studying the biochemical events controlling differentiation. Any developmental process involves the orderly and sequential expression of genetic information. Information of this nature could readily be controlled at the translation level and would require the presence of already transcribed messenger RNA within the dormant spore. Indeed, examples of translational control of differentiation are known in higher organisms. Fertilized sea-urchin eggs develop rapidly to the blastula stage in the absence of RNA synthesis, indicating the presence of stable messenger RNA within the unfertilized egg (Spirin, 1966). In the alga *Acetabularia mediterranea*, RNA synthesis is not required for the development of spore caps which are formed in the absence of a nucleus (Werz, 1965). Shaw (1967) reported that uredospores of *Puccinia helianthi* germinated when RNA synthesis was inhibited with actinomycin D, suggesting that control of germination in this fungus might also be at the translation level.

During germination, the rate of incorporation of [^{14}C]-labelled amino acids into protein in *Peronospora tabacina* was exceedingly low when compared with similar rates determined for germinating conidia of saprophytic fungi (Staples *et al.* 1962). Low incorporation rates are found also in germinating rust uredospores and may be a property common to obligate parasites. Shaw (1967) attributed these low rates in rust uredospores to an absence of nucleoli, which appear to be the site of ribosomal synthesis (Perry, 1967). In *P. tabacina* ribosomal RNA is synthesized prior to germ-tube emergence, as it is in *Aspergillus oryzae* (Ono *et al.* 1966). Thus it would seem that the low rates of incorporation into protein found in *P. tabacina*, are not caused by a lack of ribosomal RNA synthesis.

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REFERENCES

COCKING, E. C. & YEMM, E. W. (1954). Estimation of amino acids by ninhydrin. *Biochem. J.* **58**, xii.

COHEN, S. S., FLACKS, J. G., BARNER, H. D., LOEB, M. R. & LICHTENSTEIN, J. (1958). The mode of action of 5-fluorouracil and its derivatives. *Proc. natn. Acad. Sci., U.S.A.* **44**, 1004.

GOTTLIEB, D. (1966). In *The Fungus Spore*. Ed. by M. F. MADELIN. London: Butterworths.

HEIDELBERGER, C. (1965). Fluorinated pyrimidines. *Progress in Nucleic Acid Research and Molecular Biology* **4**, 1.

LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Meth. Enzymol.* **3**, 447.

OHMORI, K. & GOTTLIEB, D. (1965). Development of respiratory enzyme activities during spore germination. *Phytopathology* **55**, 1328.

ONO, T., KIMURA, K. & YANAGITA, T. (1966). Sequential synthesis of various molecular species of ribonucleic acid in the early phase of conidia germination in *Aspergillus oryzae*. *J. gen. appl. Microbiol., Tokyo* **12**, 13.

PERRY, R. P. (1967). The nucleolus and the synthesis of ribosomes. *Progress in Nucleic Acid Research and Molecular Biology* **6**, 219.

SHAW, M. (1967). Cell biological aspects of host-parasite relations of obligate fungal parasites. *Can. J. Bot.* **45**, 1205.

SHEPHERD, C. J. (1962). Germination of conidia of *Peronospora tabacina* ADAM. I, Germination *in vitro*. *Aust. J. biol. Sci.* **15**, 483.

SHEPHERD, C. J. & MANDRYK, M. (1962). Auto-inhibitors of germination and sporulation in *Peronospora tabacina* Adam. *Trans. Br. mycol. Soc.* **45**, 233.

SPIRIN, A. S. (1966). On 'masked' forms of messenger RNA in early embryogenesis and in other differentiating systems. *Current Topics in Developmental Biology* **1**, 1.

STAPLES, R. C., SYAMANANDA, R., KAO, V. & BLOCK, R. J. (1962). Comparative biochemistry of obligately parasitic and saprophytic fungi. II. Assimilation of [^{14}C]labelled substrates by germinating spores. *Contr. Boyce Thompson Inst.* **21**, 345.

SUSSMAN, A. S & HALVORSON, H. O. (1966). *Spores: Their Dormancy and Germination*. New York: Harper and Row.

WARING, M. J. (1966). Cross-linking and intercalation in nucleic acids. *Symp. Soc. gen. Microbiol.* **16**, 235.

WERZ, G. (1965). Determination and realization of morphogenesis in Acetabularia. In *Genetic Control of Differentiation. Brookhaven Symp. Biol.* **18**, 185.

J. gen. Microbiol. (1969), **55**, 275–283
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Specificity of Phenotypic Adaptation of *Bacillus cereus* to Tetracycline

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SUMMARY

Bacillus cereus strain 569H became resistant to tetracyclines, arsenite and cyanide. The degree of resistance acquired was independent of the inducing concentration up to 2×10^{-5} M. Recovery from tetracycline and arsenite inhibition involved a change in the bacteria themselves; cyanide resistance involved also the destruction of this inhibitory agent. Although there were many similarities between the tetracycline and arsenite recoveries, two distinct mechanisms were involved. Adaptation to tetracycline was not observed with *B. megaterium*, *Pseudomonas aeruginosa* or *Escherichia coli*. *Bacillus megaterium* and *E. coli* did adapt to cyanide. Although polymyxin B was inactive by itself against *B. cereus*, lytic activity due to the antibiotic was seen when a tetracycline was also present in the medium. The lytic activity ceased when the bacilli recovered from tetracycline. *Bacillus cereus* did not adapt to other inhibitors of protein synthesis, RNA synthesis or oxidative phosphorylation.

INTRODUCTION

A non-genetic adaptation to two different bacteriostatic substances, tetracycline (Connamacher, Mandel & Hahn, 1966, 1967) and arsenite (Mandel, Mayersak & Riis, 1965), occurs with *Bacillus cereus* 569H. In each case, recovery has been due to a decreased intracellular concentration of the inhibitory agent with very little chemical change of the drug. And yet each agent is believed to act by a different mechanism: tetracycline by binding to the 30S ribosomes at the point reserved for tRNA (Connamacher & Mandel, 1968) and arsenite by competition with phosphate in energy-producing mechanisms (Mandel *et al.* 1965). Because of the similarity in the way the bacteria adapt to the two very different substances, it seemed possible that we were working with a general adaptive mechanism to any toxic agent. Therefore, the present work was done to determine: (*a*) the spectrum of toxic agents to which the bacteria could adapt; (*b*) whether there was a relationship between the adaptation due to arsenite and to tetracycline; (*c*) whether any relationship between arsenite and tetracycline could be seen in a genetically resistant *Escherichia coli* K-12. During our work a third agent, cyanide, was found to exhibit the same over-all pattern as did arsenite and tetracycline, and a potentiation was discovered in *B. cereus* between tetracycline and the usually ineffective polymyxin B.

METHODS

Materials. Tetracycline-HCl was provided by the Lederle and Pfizer Laboratories. Chlortetracycline-HCl and demethylchlortetracycline-HCl were supplied by Lederle

Laboratories, oxytetracycline-HCl by Pfizer Laboratories. The author is grateful to both companies for their assistance.

Bacillus cereus strain 569H was provided by Dr H. G. Mandel (Department of Pharmacology, The George Washington University School of Medicine). *Escherichia coli* K-12 strains, one requiring methionine but sensitive to tetracycline and the other requiring thiamine + threonine + leucine and resistant to tetracycline, chloramphenicol, streptomycin and sulphonamides, were kindly furnished by Dr Kazuo Izaki (Department of Agricultural Chemistry, University of Tokyo, now of the Faculty of Agriculture, Tohoku University, Sendai, Japan).

Casein hydrolysate was bought from Difco Laboratories and was the only brand which dissolved completely in the medium of Matthews & Smith (1956). Other inhibitors and materials came from commercial sources.

Growth of bacteria. Bacillus cereus 569H and *B. megaterium* were grown in a casein hydrolysate + salts medium (Matthews & Smith, 1956). *Escherichia coli* K-12 was grown in a nutrient broth + glucose medium supplemented with 10^{-5} M-thiamine. Antimicrobial agents were added in aqueous solution or suspension in volumes less than 2% of the total. All bacteria were grown aerobically in an Eberbach water-bath shaker at 37°. Growth was measured turbidimetrically in a Bausch and Lomb Spectronic 20 or a Gilford Model 240 spectrophotometer.

Inter-relationships between inhibitory agents. Many studies consisted of measuring the growth of bacteria in the presence of two compounds added simultaneously or sequentially In one series of experiments, *Bacillus cereus* was grown in the presence of either 6×10^{-6} M-tetracycline or $1{\cdot}5 \times 10^{-4}$ M-arsenite until recovery. Each sample was then divided into three parts: one received additional 6×10^{-6} M-tetracycline, one received $1{\cdot}5 \times 10^{-4}$ M-arsenite and one was kept as control. The growth of each sample was followed as extinction at 540 mμ (E_{540}). In other experiments, after adaptation, the medium was removed from the culture by centrifugation at room temperature and the deposited bacteria washed once with new medium, resuspended in new medium, and the experiment continued as described above. This type of experiment was also done to determine the interrelationships of adaptation between cyanide and tetracycline, and between cyanide and arsenite.

RESULTS

Adaptation and cross-resistance within the tetracycline family

Bacillus cereus 569H can become phenotypically resistant to tetracycline, oxytetracycline, chlortetracycline (Fig. 1) and demethylchlortetracycline (Fig. 2). The effect of oxytetracycline was the lowest, followed in increasing order by tetracycline, demethylchlortetracycline, chlortetracycline. After bacteria adapted to 6×10^{-6} M-tetracycline, they were completely refractory to media containing that amount of drug plus additional 6×10^{-6} M-tetracycline, demethylchlortetracycline or oxytetracycline. Additional chlortetracycline (6×10^{-6} M) was somewhat inhibitory in tetracycline-adapted bacilli, although the same dose without antibiotic pretreatment caused a complete and irreversible arrest of bacterial growth.

Cross-resistance between antibiotics

Streptomycin ($5{\cdot}2 \times 10^{-5}$ M), chloramphenicol (3×10^{-5} M), erythromycin ($1{\cdot}3 \times 10^{-6}$ M), cyclohexamide ($1{\cdot}1 \times 10^{-4}$ M), puromycin ($8{\cdot}5 \times 10^{-5}$ M) and actinomycin D (10^{-7} M) were added separately to bacterial cultures. All but actinomycin D act by direct inhibition of protein synthesis (Pestka, Marshall & Nirenberg, 1965; Coutsogeorgopoulos, 1966; Wolfe & Hahn, 1964; Lin, Mosteller & Hardesty, 1966; Nathans

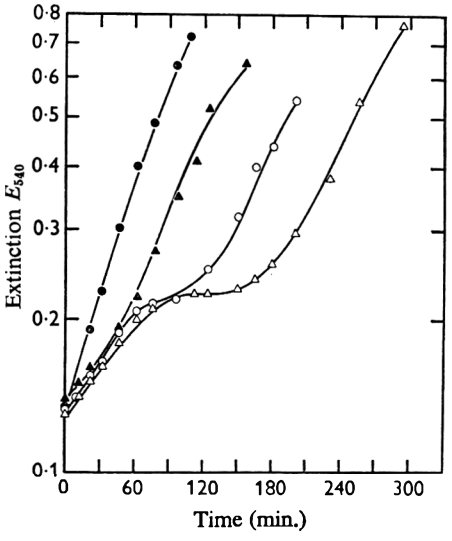

Fig. 1

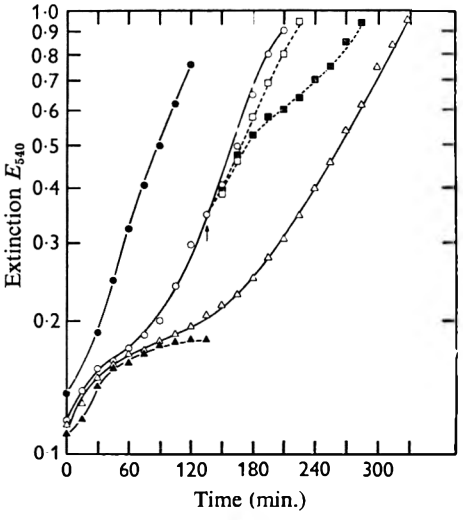

Fig. 2

Fig. 1. Adaptation of *Bacillus cereus* 569H to different tetracyclines. *Bacillus cereus* was grown in casein hydrolysate + salts medium; growth was measured in a Bausch and Lomb Spectronic 20 spectrophotometer. The extinction at 540 mμ was plotted against time. ●, Control; ▲, oxytetracycline (6×10^{-6} M); ○, tetracycline (6×10^{-6} M); △, chlortetracycline (2×10^{-6} M).

Fig. 2. Effect of several tetracyclines before and after adaptation of *Bacillus cereus* to 6×10^{-6} M-tetracycline. Antibiotics were added either at the origin or at arrow (↑). Other details are the same as Fig. 1. ●, Control: ○, tetracycline (6×10^{-6} M); △, demethylchlortetracycline (6×10^{-6} M); ▲, chlortetracycline (6×10^{-6} M). Added at arrow (↑); ■, chlortetracycline (6×10^{-6} M); □, demethylchlortetracycline (6×10^{-6} M); oxytetracycline (6×10^{-6} M), not shown, was ineffective after bacterial adaptation of tetracycline.

& Lipmann, 1961; Reich *et al.* 1962). Although all of the above antibiotics except cyclohexamide were inhibitory to *Bacillus cereus* adaptation to these agents did not occur. Neither did adaptation of *B. cereus* to tetracycline affect the pattern of inhibition shown by these antibiotics (Fig. 3).

Polymyxin B ($1{\cdot}6 \times 10^{-5}$ M) by itself was not inhibitory to *Bacillus cereus*. However, when bacilli were grown also in the presence of 6×10^{-6} M-tetracycline, a steady decrease in extinction was seen (Fig. 4). The lysis continued as long as the bacilli remained sensitive to tetracycline. On recovery from tetracycline inhibition, *B. cereus* lost its sensitivity to polymyxin B.

Cross-resistance between tetracycline and other inhibitors

Because both tetracycline (Jones & Morrison, 1962) and arsenite (Mandel *et al.* 1965) have been shown to interrupt the energy production of the cell, other energy inhibitors and active transport inhibitors were tested for reversible bacteriostatic action. Ouabain (10^{-4} M) did not inhibit *Bacillus cereus*, nor was bacterial inhibition observed with up to 10^{-3} M-phloridzin, 2,4-dinitrophenol, ethionine, potassium fluoride, amobarbital, or malonic acid. These agents also did not affect the ability of

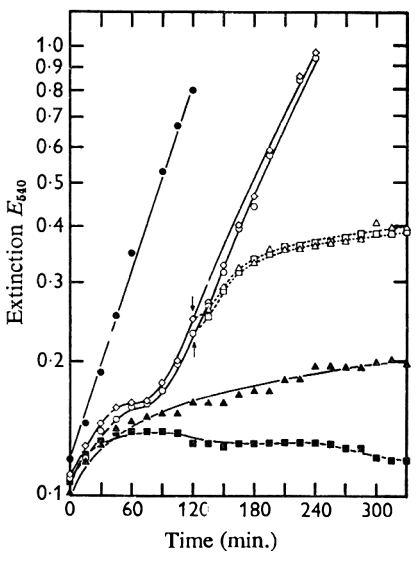

Fig. 3

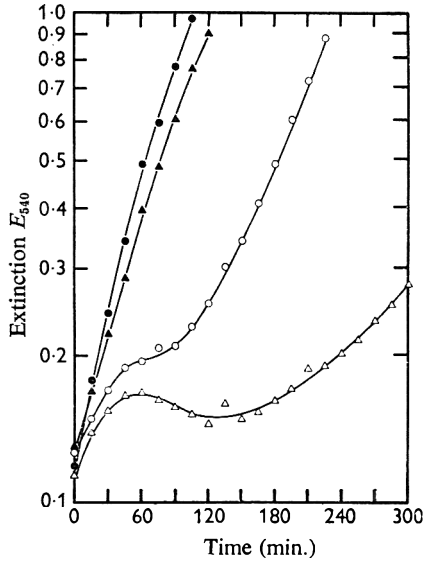

Fig. 4

Fig. 3. Effect of tetracyline adaptation on the action of other antibiotics. Antibiotics erythromycin and puromycin were added to cultures at time O at arrow (↑). ●, Control; ○; ◇, tetracycline (6×10^{-6} M); ▲, △, erythromycin ($1{\cdot}3 \times 10^{-6}$ M); ■, □, puromycin ($8{\cdot}5 \times 10^{-6}$ M), closed symbols if added at time O, open symbols if added at arrow. Dashed line signifies a second drug addition.

Fig. 4. Effect of polymyxin B ($1{\cdot}6 \times 10^{-5}$) M on *Bacillus cereus* 569H alone or with tetracycline (6×10^{-6} M). Experimental details as in Fig. 1. ●, Control; ▲, polymyxin B ($1{\cdot}6 \times 10^{-5}$ M); ○, tetracycline (6×10^{-6} M); △, tetracycline (6×10^{-6} M) + polymyxin B ($1{\cdot}6 \times 10^{-5}$ M).

B. cereus to adapt to tetracycline. Sodium azide, formaldehyde and iodoacetic acid were inhibitory at concentrations between 10^{-4} M and 10^{-5} M, but no recovery was observed. Adaptation of bacteria to tetracycline did not change the inhibitory pattern. Potassium cyanide (10^{-3} M) caused a period of growth inhibition followed by recovery.

Relationship between tetracycline and arsenite resistance in Bacillus cereus

Experiments were made to discover whether or not the adaptive mechanism for tetracycline was involved in arsenite adaptation or vice versa. *Bacillus cereus* was grown in 6×10^{-6} M-tetracycline until resumption of full exponential growth. The culture was then divided, $1{\cdot}5 \times 10^{-4}$ M-arsenite or an additional 6×10^{-6} M-tetracycline was added to the tetracycline already present in the medium, and the growth was measured (see Methods). The same experiment was done with bacilli made resistant to

arsenite. There was a decrease in both cases (Fig. 5*a*). To examine the possibility that the attenuation of the bacteriostatic effect was due to a chelation between tetracycline and arsenite in the medium, detoxifying both agents, adapted bacteria were centrifuged at 3000 *g* for 10 min., washed once with fresh medium, and resuspended in drug-free medium before the second inhibitory agent was added. These tetracycline-adapted bacilli, when treated in this manner, exhibited an increased sensitivity to arsenite over that seen in non-treated cultures (Fig. 5*b*), although adaptation to tetracycline remained. The effect of tetracycline and arsenite after arsenite adaptation remained shortened (Fig. 5*c*). This experiment was repeated three times with consistent results.

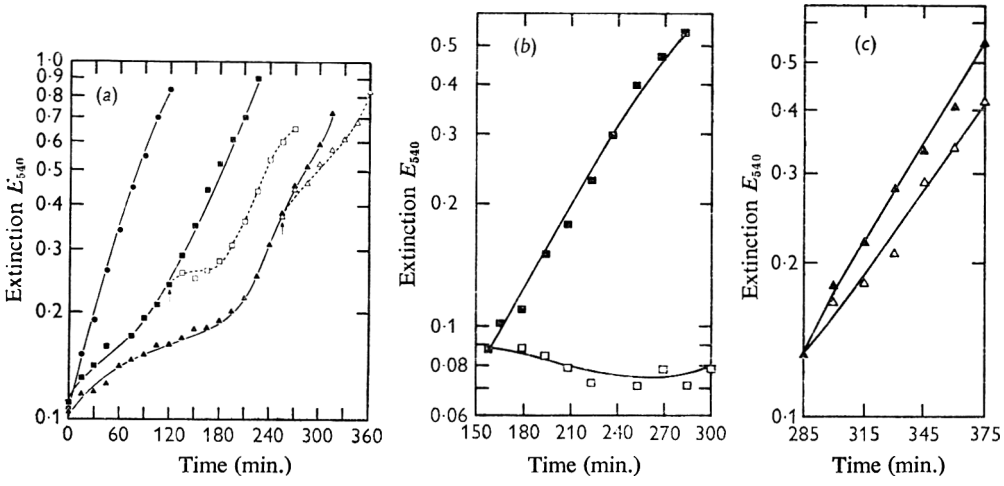

Fig. 5. Interrelationship between tetracycline and arsenite resistances. Bacilli were made resistant to tetracycline or arsenite. (*a*) Second inhibitory agent added at arrow (↑). (*b*) Tetracycline removed by centrifuging culture and resuspending bacilli before arsenite addition. (*c*) Arsenite removed by centrifuging culture and resuspending bacilli before tetracycline addition. Other details are the same as Fig. 1. ●, Control; ■, tetracycline (6×10^{-6} M); □, arsenite ($1{\cdot}5 \times 10^{-4}$ M) added after tetracycline recovery; ▲, arsenite ($1{\cdot}5 \times 10^{-4}$ M); △, tetracycline (6×10^{-6} M) added after arsenite recovery; dashed lines signify the presence of both drugs in the medium.

Relationship between cyanide and arsenite or tetracycline resistances in Bacillus cereus

Experiments were done to determine the relationship between the cyanide and the arsenite or tetracycline adaptations of *Bacillus cereus*. The procedure was that used in the previous section. Cyanide recovery did not change the sensitivity to arsenite or tetracycline, nor could it protect the bacilli from a second treatment with cyanide (Fig. 6). Neither tetracycline nor arsenite adaptation changed the sensitivity of *B. cereus* to cyanide. To see whether the bacteria became resistant to cyanide by destroying it, *B. cereus* was grown in the presence or absence of cyanide until adaptation occurred. Both cultures were centrifuged and half of each resuspended in each medium. Cyanide-resistant and untreated bacteria were also resuspended in medium containing cyanide that had been incubated in cell-free medium (Fig. 7). The untreated bacteria were not inhibited by medium from cultures of bacilli which had recovered from cyanide inhibition. They were as inhibited by the second introduction of cyanide as they were by the original dose.

Adaptation of other bacteria to inhibitory agents

In three other bacterial species, *Escherichia coli* K-12, *Bacillus megaterium* and *Pseudomonas aeruginosa*, recovery from the effects of 6×10^{-6} M tetracycline did not occur within 2·5 hr. Recovery from arsenite and cyanide was observed, however, with *B. megaterium*. *Escherichia coli* K-12 (sensitive) was more sensitive to tetracycline

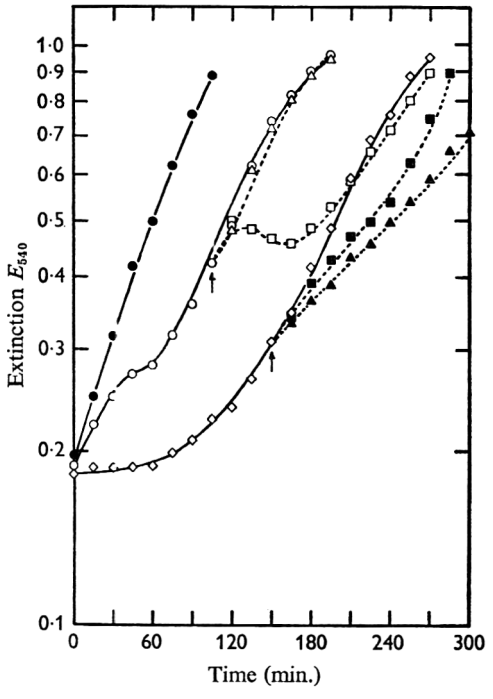

Fig. 6

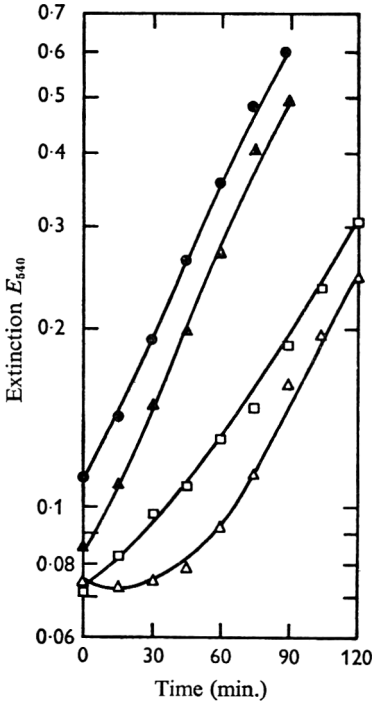

Fig. 7

Fig. 6. Interrelationship between tetracycline and cyanide resistance. Experiment was done in a manner similar to that shown in Fig. 5*a*, except that cyanide (2×10^{-5} M) replaced arsenite. Secondary drug additions were made at arrow (↑), and growth curve followed (dashed lines). ●, Control; ○, tetracycline (6×10^{-6} M); △, +additional tetracycline to (12×10^{-6} M); □, +KCN (2×10^{-5} M); ◇, KCN (2×10^{-5} M); ■ +tetracycline (6×10^{-6}M); ▲, +additional KCN (2×10^{-5} M).

Fig. 7. Destruction of KCN by *Bacillus cereus*. *B. cereus* grown until resistant to KCN (2×10^{-5} M), and medium and bacilli separated. Control bacilli were incubated in that medium, and a sample of medium was incubated with cyanide but without bacilli. KCN-adapted bacilli were grown in similar media.

Symbol	Medium	Bacteria
●	Control	Control
▲	KCN-recovered	Control
□	KCN-bacteria free	KCN-recovered
△	KCN-bacteria	Control

than was *B. cereus*, being inhibited by concentrations as low as 2×10^{-7} M. Unlike *B. cereus* (Connamacher *et al.* 1967), the rate of growth was proportional to the concentration of tetracycline (Fig. 8). The resistant strain did not respond to 10^{-5} M-tetracycline. Neither strain of *E. coli* was sensitive to arsenite; both showed a sensitivity to cyanide and an ability to adapt to that drug.

Relationship between the level of antibiotic resistance and the inducing concentration

The non-genetic resistance to tetracycline exhibited by *Bacillus cereus* was not observed with drug concentrations above 5×10^{-5} M (Connamacher *et al.* 1967). Experiments were done to determine whether the degree of resistance achieved was related to the original 'inducing' concentration of tetracycline. Bacilli were made resistant to concentrations of tetracycline from 2×10^{-6} M to 2×10^{-5} M. The degree of resistance achieved was measured by growing the bacilli in additional 2×10^{-5} M to 10^{-4} M-tetracycline. The degree of resistance achieved was independent of the 'inducing' concentration (Fig. 9).

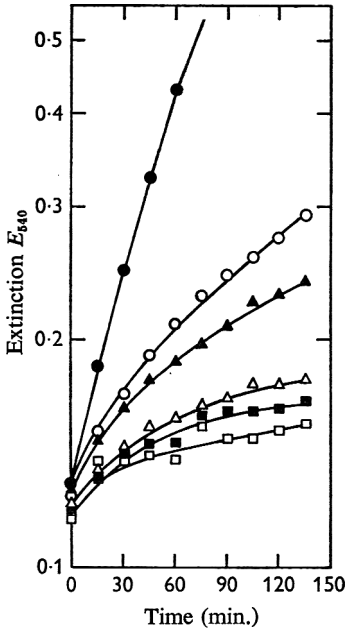

Fig. 8

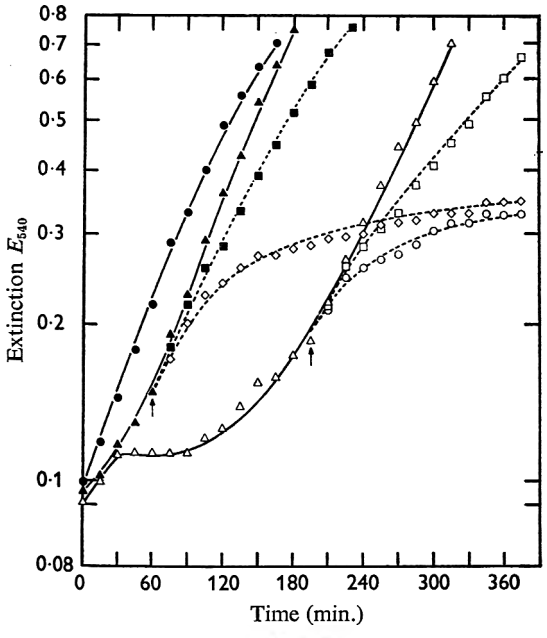

Fig. 9

Fig. 8. Growth of *Escherichia coli* K-12 (sensitive) in the presence of different concentrations of tetracycline. *E. coli* were grown aerobically in nutrient broth + glucose medium, and turbidity at 540 mμ was plotted against time. ●, Control; tetracycline (M): ○, 4×10^{-7}; ▲, 8×10^{-7}; △, 2×10^{-6}; ■, 4×10^{-6}; □, 10^{-5}.

Fig. 9. Action of higher concentrations of tetracycline on *Bacillus cereus* adapted to 6×10^{-6} M or $1{\cdot}8 \times 10^{-5}$ M tetracycline. Additional drug added at arrow (↑), and growth curves followed (dashed lines). ●, Control; ▲, tetracycline (6×10^{-6} M); ■, +additional tetracycline (10^{-5} M); △, +additional tetracycline (5×10^{-5} M); △, tetracycline ($1{\cdot}8 \times 10^{-5}$ M) □, +additional tetracycline (10^{-5} M); ○, +additional tetracycline (5×10^{-5} M).

DISCUSSION

Two aspects of non-genetic resistance to tetracycline are at present under investigation. They are: (*a*) what are the biochemical events that occur during the adaptation of *Bacillus cereus* to tetracycline; (*b*) do they occur in bacilli made resistant through genetic mutation? Because protein, and therefore enzyme, synthesis is totally inhibited

by 6×10^{-6} M-tetracycline (Connamacher & Mandel, 1964; Hash, Wishnick & Miller, 1964), and because energy is required for the adaptation to take place (Connamacher *et al.* 1966), active mechanisms other than protein synthesis must be responsible. The specificity of the adaptation might give a clue as to the biochemical mechanisms involved.

Interrelationships between the mechanisms of resistance and the inhibitory agent

The similarity between the adaptations to tetracycline and to arsenite suggested the possibility that both use the same mechanism of adaptation. In both cases, the resistant bacteria contained less drug than did the sensitive ones (Connamacher *et al.* 1967; Mandel *et al.* 1965). In the author's experience, the recovery from both compounds must occur within 3 hr or not at all. And both tetracycline- and arsenite-adapted bacilli remained susceptible to higher concentrations of the inhibitory agents. Further evidence for the interrelationship between tetracycline and arsenite was seen in the decreased sensitivity to arsenite after tetracycline adaptation in the absence of washing. It was difficult to evaluate the apparent decrease in tetracycline sensitivity after bacilli became resistant to arsenite since it was possible that tetracycline chelated with arsenite in the medium, inactivating both (the arsenite concentration was 25 times that of tetracycline, so that even washing might not remove enough of the trivalent anion). Despite this apparent relationship, it now appears that each resistance has its own mechanism of onset. When the medium from tetracycline-adapted bacilli was exchanged for drug-free medium before addition of arsenite (Fig. 5), the bacilli remained resistant to tetracycline. However, they became more sensitive to arsenite than they were before adaptation to tetracycline. Secondly, the related organism, *Bacillus megaterium*, remained sensitive to tetracycline even when it had adapted to arsenite. Lastly, if this adaptation was a general mechanism, it would be expected to be seen in many instances. Actually, phenotypic resistance could only be demonstrated with tetracycline, arsenite and cyanide. The recovery of bacilli from cyanide inhibition appeared to use a mechanism different from either of the two described above. Firstly, the recovery occurred in all bacilli studies, whether or not tetracycline or arsenite adaptation also took place. Secondly, bacilli destroyed the cyanide in the medium, which was not the case with the other two drugs.

Independence of adaptive tetracycline concentration and degree of resistance achieved

With most non-genetic adaptations there is a relationship between the amount of a drug used to promote resistance and the degree of resistance achieved. With tetracycline this relationship was not observed. Whatever the concentration of tetracycline used, from the slightly inhibitory 2×10^{-6} M to 2×10^{-5} M, above which the bacilli did not recover, the degree of resistance remained the same. This would suggest that tetracycline was acting as a derepressor, except that protein synthesis was completely inhibited. A more likely possibility suggested by Connamacher *et al.* (1967) is that two mechanisms are involved in tetracycline entry, one active and one passive. All drug concentrations used completely inhibited the active phase. However, high extracellular concentrations of tetracycline still caused sufficient entry of the antibiotic by passive diffusion to inhibit the bacterial growth.

Interrelationship between tetracycline and polymyxin B

Polymyxin B is normally effective against only those Gram-positive bacteria which are highly sensitive to lysozyme (Newton, 1956). *Bacillus cereus* is not such an organism, and it is not normally susceptible to polymyxin B. In the presence of tetracycline, however, a bactericidal effect of polymyxin B was seen. Once tetracycline adaptation occurred, polymyxin action ceased. Tetracycline thus appeared to alter the bacilli in such a way as to allow polymyxin B access to the cell membrane of Gram-positive bacteria. On recovery of the bacilli from the effects of tetracycline, this access was lost. Work on this aspect is continuing.

This work was supported by Grant no. AI-08192-01 from the United States National Institute of Allergy and Infectious Diseases. The author thanks Miss Marlease Williams for careful technical assistance and Dr W. S. Vincent for aid in the preparation of this manuscript. He also thanks the Lederle Laboratories and Pfizer Laboratories for gifts of the tetracyclines used in this paper.

REFERENCES

CONNAMACHER, R. H. & MANDEL, H. H. (1964). Studies on the mechanism of action of tetracycline on *B. cereus*. *Fedn. Proc.* **23**, 388.

CONNAMACHER, R. H. & MANDEL, H. G. (1968). Intracellular localization of tetracycline in bacteria. *Biochim. biophys. Acta* **166**, 475.

CONNAMACHER, R. H., MANDEL, H. G. & HAHN, F. E. (1966). Non-genetic resistance by *Bacillus cereus* to tetracycline. *Fedn. Proc. Fedn. Am. Socs exp. Biol.* **25**, 344.

CONNAMACHER, R. H., MANDEL, H. G. & HAHN, F. E. (1967). Adaptation of populations of *Bacillus cereus* to tetracycline. *Molec. Pharmac.* **3**, 586.

COUTSOGEORGOPOULOS, C. (1966). On the mechanism of action of chloramphenicol in protein synsynthesis. *Biochim. biophys. Acta* **129**, 214.

HASH, J. H., WISHNICK, M. & MILLER, P. A. (1964). On the mechanism of action of the tetracycline antibiotics in *Staphylococcus aureus*. *J. biol. Chem.* **239**, 2070.

JONES, J. G. & MORRISON, G. A. (1962). The bacteriostatic actions of tetracycline and oxytetracycline. *J. Pharm. Pharmac.* **14**, 808.

LIN, S. Y., MOSTELLER, R. D., & HARDESTY, B. (1966). The mechanism of sodium fluoride and cyclohexamide inhibition of haemoglobin biosynthesis in the cell-free reticulocyte system. *J. molec. Biol.* **21**, 51.

MANDEL, H. G., MAYERSAK, J. S. & RIIS, M. (1965). The action of arsenic on *Bacillus cereus*. *J. Pharm. Pharmac.* **17**, 794.

MATTHEWS, R. E. F. & SMITH, J. D. (1956). Distribution of 8-Azaguanine in the nucleic acids of *Bacillus cereus*. *Nature, Lond.* **177**, 271.

NATHANS, D. L & LIPMANN, F. (1961). Amino acid transfer from amino acylribonucleic acids to protein on ribosomes of *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.*, **47**, 497.

NEWTON, B. A. (1956). The properties and mode of action of polymyxin. *Bact. Rev.* **20**, 14.

PESTKA, S., MARSHALL, R. & NIRENBERG, M. (1965). RNA codewords and protein synthesis. V. Effect of streptomycin on the formation of ribosome-sRNA complexes. *Proc. natn. Acad. Sci., U.S.A.* **53**, 639.

REICH, E., FRANKLIN, R. M., SHATKIN, A. J. & TATUM, E. L. (1962). Action of actinomycin D on animal cells and viruses. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1238.

WOLFE, A. D. & HAHN, F. E. (1964). Erythromycin: mode of action. *Science, N.Y.* **143**, 1445.

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The Utilization of Thiocyanate by a Heterotrophic Bacterium

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SUMMARY

A heterotrophic bacterium has been isolated which can use thiocyanate as its sole source of cellular nitrogen and also sulphur; ammonium ions inhibit the utilization of thiocyanate. It can utilize both phenol and thiocyanate simultaneously; it is a pseudomonad and is most similar to *Pseudomonas stutzeri.*

INTRODUCTION

The biodegradation of the thiocyanate ion, especially in biological effluent-treatment systems, is usually considered to be due to the action of autotrophs as, for example, the Thiobacillus described by Happold, Johnstone, Rogers & Youatt (1954). It occurred to us that there might well be organisms growing heterotrophically which were capable of using thiocyanate as their source of cellular nitrogen and also, perhaps, sulphur. This paper reports the isolation of such an organism.

METHODS

Chemicals. All chemicals were either of AnalaR or the purest commercial grade obtainable from British Drug Houses Ltd., Poole, Dorset.

Media. The mineral-salts medium contained (g./l. distilled water): KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0·2; $CaCl_2$, 0·02; and 0·1 ml. 60% (w/v) $FeCl_3.6H_2O$. 'Sulphate-free' media contained 0·2 g. $MgCl_2$ instead of $MgSO_4$. The solution was filtered and the filtrate adjusted to between pH 7·0 and 7·4 with NaOH; it was then sterilized at approx. 10^5 N/m^2 for 15 min. Disodium succinate (final concn. 25 mM) or phenol (final concn. 5 mM) were added as the carbon and energy source. For the source of nitrogen either KSCN (final concn. 4 mM) or $(NH_4)_2SO_4$ (final concn. 2 mM) was used. All supplements were added aseptically after sterilization as above.

Thiobacillus-enrichment medium was medium no. 2 described by Parker & Prisk (1953).

Media were solidified when necessary by the addition of 3% (w/v) washed Ionagar no. 2 (Oxoid).

Isolation and maintenance of the organism. The organism was isolated by plating out, on to solid Thiobacillus-enrichment medium, a sample taken from an activated-sludge tank treating primarily NH_4SCN. After overnight incubation at 30° eight different colonial types were observed, which were individually transferred on succinate + thiocyanate + mineral-salts agar. Only one of these eight organisms had grown

well after overnight incubation at 30°. An individual colony from this plate was used to inoculate a liquid succinate + thiocyanate + mineral-salts medium and the culture was shaken at 30° overnight. Good growth resulted which was coupled with the disappearance of thiocyanate; the culture obtained was morphologically homogeneous.

The organism was maintained by subculturing on to both nutrient-agar and succinate + thiocyanate + mineral-salts agar. Growth studies were always performed in 500 ml. Erlenmeyer flasks containing 100 ml. medium; these were shaken at 150 rev./min. in an orbital incubator at 30°.

Analytical methods. All turbidimetric or spectrophotometric assays were done with a Unicam SP. 600 with cuvettes with a 1 cm. light-path.

The extinction, at 450 mμ, of undiluted samples of culture medium was used as a measurement of growth.

Thiocyanate was estimated by adding to a 0·5 ml. sample, 0·5 ml. 5 N-nitric acid, 9·5 ml. distilled water and 0·5 ml. 10% (w/v) iron (III) nitrate; in the corresponding control the iron(III) nitrate was replaced by an equal volume of water. The extinction of the final colour was read at 410 mμ.

Phenol was estimated by the 4-aminophenazone method of Shaw (1951).

Sulphate was determined by the barium chloranilate method of Bertolacini & Barney (1957).

Nessler reagent was used to assay ammonia.

Diagnostic tests. The tests used to identify the organism were the same as those described by Stanier, Palleroni & Doudoroff (1966), except that the number of flagella was determined from electron-micrographs of the whole organism.

RESULTS

Characters of the organism

The isolated organism was a Gram-negative unicellular straight rod (measuring 1·0 $\mu \times 0{\cdot}8\ \mu$), motile by means of one to eight flagella at one pole of the organism. It gave a very rapid positive result in the oxidase test and utilized denitrification as an anaerobic respiratory mechanism. Besides succinate and phenol (catechol is not cleaved by the meta-mechanism) it grew with either glucose, maltose or glycollate as the sole source of carbon and energy; starch was hydrolysed, gelatin was not. No growth took place on nutrient agar at 4° over a period of ten days; growth was good at 41°.

Pigment was not produced under any conditions of growth tried, even when cultured on both of the complex media (A and B) designed by King, Ward & Raney (1954) to detect the two commonest types of pigments produced by pseudomonads. On medium B it exhibited the unusual colony structure which has been used to distinguish *Pseudomonas stutzeri* (van Niel & Allen, 1952): both the rough, wrinkled, dry and coherent form together with the smooth colonial variants were readily observed, from either a single rough or a single smooth parent colony.

Growth studies with thiocyanate

In either a phenol or succinate + mineral-salts medium the organism used thiocyanate or ammonium ion as sole source of cellular nitrogen. Growth of the organism and disappearance during growth of both phenol and thiocyanate is shown in Fig. 1;

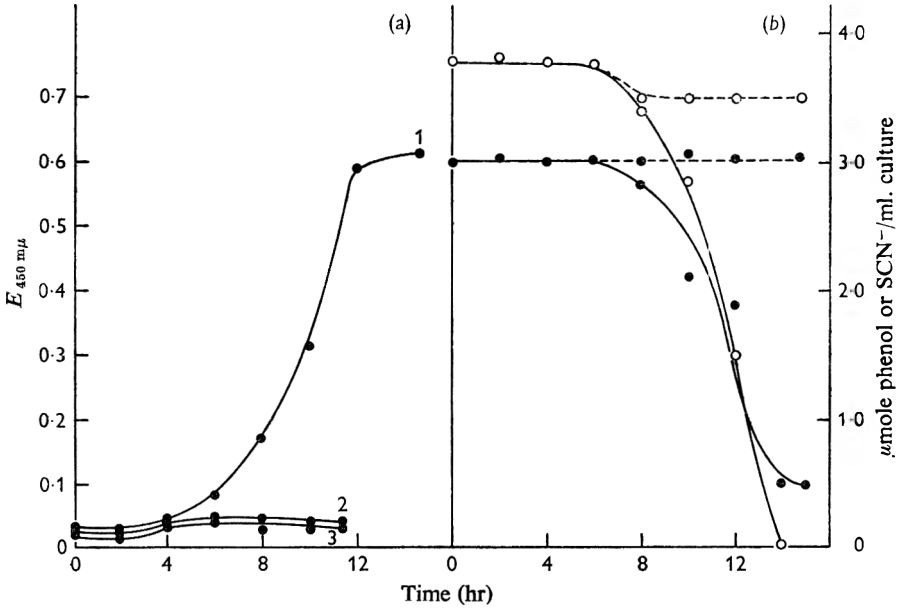

Fig. 1. (*a*) Growth of the organism at 30° in a shaken ammonium ion-free mineral-salts medium supplemented with (1) phenol as the sole source of carbon and energy, and thiocyanate as the sole source of nitrogen; (2) phenol only; (3) thiocyanate only. (*b*) Disappearance of phenol and thiocyanate during growth. —○—, phenol concentration in medium 1 (*a*); – –○– –, phenol concentration in medium 2, (*a*); —●—, thiocyanate concentration in medium 1 (*a*); – –●– –, thiocyanate concentration in medium 3 (*a*).

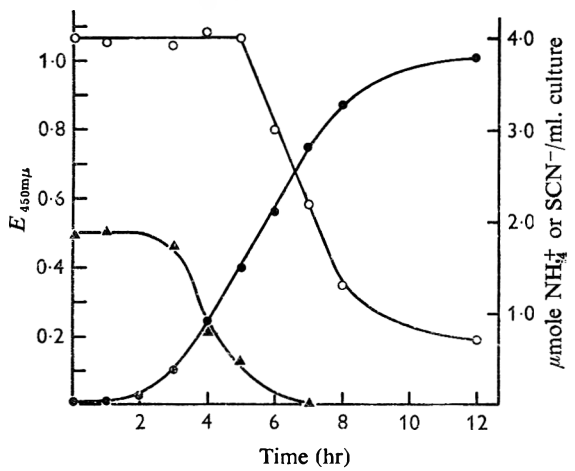

Fig. 2

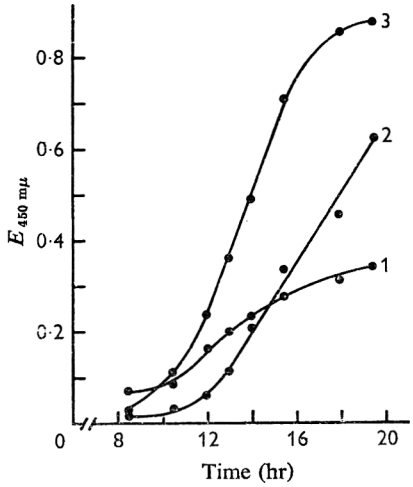

Fig. 3

Fig. 2. Disappearance of NH_4^+ and SCN^- during growth of the organism at 30° in a shaken succinate/thiocyanate/ammonium-salt/mineral-salts medium. ●, growth; ○, thiocyanate concentration; ▲ ammonium ion concentration. For experimental details see text.

Fig. 3. Growth of the organism at 30° in a shaken 'sulphate-free' medium with succinate as the sole source of carbon and energy, supplemented with (1) NH_4^+ and SCN^- as the only sources of added nitrogen and sulphur, (2) SCN^- as the single source of nitrogen and sulphur, (3) NH_4^+, SCN^-, and SO_4'' as the sources of nitrogen and sulphur.

ammonium ions were detected in the culture filtrate towards the end of the growth cycle. At the usual concentration of the carbon and energy sources used, 3 mM-thiocyanate was sufficient for maximal growth. Whenever the carbon and energy source was exhausted in the presence of excess thiocyanate growth stopped abruptly and the concentration of thiocyanate ceased to decrease.

When thiocyanate and ammonium ions were present together in the growth medium thiocyanate degradation did not occur until the ammonium ions were nearly used up (Fig. 2). Addition of ammonium ions to a culture growing with thiocyanate inhibited the further utilization of this compound though this effect did not appear to be instantaneous.

No intra- or extra-cellular sulphur was produced, nor was sulphide detected, during growth with thiocyanate as the sole source of nitrogen. The sulphate concentration of the medium increased during growth with thiocyanate acting as the sole source of added sulphur as well as of nitrogen; when ammonium ions were added to such a medium growth was markedly inhibited (Fig. 3).

DISCUSSION

The organism described in this paper fits the definition of a pseudomonad given by Stanier *et al.* (1966) (its DNA base content has not been measured). Especially characteristic is the organism's rough and smooth colonial variation. This property has traditionally been used to identify such a pseudomonad as *Pseudomonas stutzeri* (for example, van Niel & Allen, 1952), but our organism differs from the Stanier *et al.* (1966) description of *Pseudomonas stutzeri* in that it is not monotrichous and it contains a constitutive 'arginine dihydrolase'.

Another feature is the ability of our organism to metabolize thiocyanate, for thiocyanate degradation is usually considered to be due entirely to the activities of certain thiobacilli. One of the 'Thiobacillus' strains (no. 5C) isolated by Hutchinson, Johnstone & White (1965) was stated to be very similar to *Pseudomonas stutzeri* though no data on the similarity were given. Our organism, though growing readily in media suitable for non-exacting heterotrophs, has never been cultured autotrophically and, in our hands, has never utilized thiocyanate as a sole source of energy.

Thiocyanate was readily shown to be a sole source of nitrogen; it was not so straightforward to demonstrate that it could be the sole source of sulphur. Sulphate-free media are not easy to prepare because traces of sulphate were present in most of the chemicals used. Furthermore, since sulphate is produced during thiocyanate utilization some sulphate was always carried over with the inocula used in the growth experiments. The organism did grow, though after a much longer lag, as well in the 'sulphate-free' media as in media containing added sulphate.

The marked inhibition of growth in 'sulphate-free' media caused by the addition of ammonium ions could be due to their inhibition of thiocyanate utilization which would effectively cut the supply of sulphur. That ammonium ions do repress thiocyanate utilization suggests that they lie on the pathway of nitrogen assimilation from thiocyanate.

The ability of the organism to metabolize phenol and thiocyanate simultaneously makes it potentially very useful in the biological treatment of effluents containing such compounds. The removal of both these compounds in a one-stage process is not

usually efficiently achieved, yet this organism grew in a medium containing 8 mM-phenol + 4 mM-thiocyanate, both of which disappeared during growth. A prior removal of ammonia from liquors to be treated by such organisms would, however, be necessary.

We are indebted to Mr W. Hodgkiss (Torry Research Station, Aberdeen) for taking electron micrographs of the organism and determining the number of flagella; also to Dr G. Hobbs (Torry Research Station) for helpful advice. We are grateful to the Science Research Council for a training grant to one of us (D.A.S.) and to the National Coal Board for additional financial assistance. The valuable technical assistance of Mr J. Rees is greatly appreciated.

REFERENCES

BERTOLACINI, R. J. & BARNEY, J. E. (1957). Colourimetric determination of sulphate with barium chloranilate. *Analyt. Chem.* **29**, 281.

HAPPOLD, F. C., JOHNSTONE, K. I., ROGERS, H. J. & YOUATT, J. B. (1954). The isolation and characteristics of an organism oxidising thiocyanate. *J. gen. Microbiol.* **10**, 261.

HUTCHINSON, M., JOHNSTONE, K. I. & WHITE, D. (1965). The taxonomy of certain thiobacilli. *J. gen. Microbiol.* **41**, 357.

KING, E. O., WARD, M. K. & RANEY, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. clin. Med.* **44**, 301.

PARKER, C. D. & PRISK, J. (1953). The oxidation of inorganic compounds of sulphur by various sulphur bacteria. *J. gen. Microbiol.* **8**, 344.

SHAW, J. A. (1951). Determination of phenols in aqueous wastes from coke plants. *Analyt. Chem.* **23**, 1788.

STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *J. gen. Microbiol.* **43**, 159.

VAN NIEL, C. B. & ALLEN, M. B. (1952). A note on *Pseudomonas stutzeri*. *J. Bact.* **64**, 413.

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Cell Volume per Nucleus in Haploid and Diploid Strains of *Aspergillus nidulans*

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(*Accepted for publication* 9 *October* 1968)

SUMMARY

Diploid conidiospores of *Aspergillus nidulans* are uninucleate and have twice the volume of haploid conidiospores. On the other hand, the hyphae are coenocytic, and it has been found that the dimensions of the hyphal cell are unaffected by ploidy but that diploid cells have half as many nuclei as haploid ones. It is concluded from this that both cessation of growth in cells of fixed size and the timing of mitoses in coencytes are determined by a critical volume of cytoplasm per genome, rather than per nucleus.

In the conidial apparatus, the dimensions of the coenocytic conidiophore, like the hyphae, are unaffected by ploidy, while the uninucleate sterigmata are larger in the diploid. In the case of the primary sterigmata, however, the volume in the diploid is less than twice that in the haploid, suggesting that the mechanism controlling cytoplasmic volume is not fully operative at this stage.

INTRODUCTION

Cell volume is frequently proportional to the DNA content of the cell nucleus: this can be seen in polyploid, multinucleate or polytene tissues (List, 1963), in comparisons of members of a polyploid series (Noggle, 1946), or in inter-species comparisons (Commoner, 1964; Yčas, Sugita & Bensam, 1965).

A relationship between cell size and the initiation of mitosis is also well known, particularly from the experiments of Hartmann (1928) and Prescott (1956) on Amoeba, and the significance of this has been discussed in a number of reviews (Swann, 1957; Mazia, 1961; Yčas *et al.* 1965).

The Ascomycete, *Aspergillus nidulans*, contains both coenocytic hyphae, and, in the conidial apparatus, uninucleate cells. Comparison of haploids and diploids in this system shows a close connexion between the above two sets of relationships, since in the coenocytes, cell volume is independent of ploidy, but the number of nuclei per unit volume is not. The special feature of this system is that in a constant growing coenocyte the only feature that can regulate the number of nuclei per unit volume is the frequency of mitosis. In the uninucleate conidia, on the other hand, this regulation depends on the timing of the cessation of growth, and here again, the cell volume is proportional to ploidy and DNA content (Heagy & Roper, 1952; Pontecorvo, Roper, Hemmons, Macdonald & Bufton, 1953).

The transition between the coenocytic and uninucleate situations occurs in the sterigmata, and examination of these suggests that differences in ploidy are expressed as differences in cell volume only at certain stages of the cell cycle.

METHODS

Strains. Strains were all derived from the stocks of *Aspergillus nidulans* in the Department of Genetics, Glasgow University (Pontecorvo *et al.* 1953; Käfer, 1958). Hyphal measurements were made on the haploid: *y;w3;pyro4* and the diploid:

$$\frac{+ \quad + \quad bi1 \quad w3 \quad pyro4}{paba1 \quad y \quad + \quad + \quad pyro4}.$$

Measurements of conidial apparatus were made on the haploid: *bi1*, and the diploid:

$$\frac{paba1 \quad y \quad bi1 \quad + \quad +}{+ \quad + \quad bi1 \quad w2 \quad orn2}.$$

(*w* = white conidia, *y* = yellow conidia; *bi*, *pyro*, *paba*, *orn* = requirements for biotin, pyridoxine, *p*-aminobenzoic acid, and ornithine respectively).

The haploid and diploid strains to be compared thus had the same nutritional requirements (pyridoxine in the first case, biotin in the second), and the strains for conidial apparatus measurements were both green, since all the markers used are recessive. The diploids were obtained by the method of Roper (1952), however, so they are necessarily heterozygous for nutritional markers not present in the haploids.

Media. The minimal medium of Pontecorvo *et al.* (1953) was used, supplemented with 550 μg./l. pyridoxine or 22 μg./l. biotin as appropriate. Cultures were incubated at 37°.

Microscopy. Nuclei were examined by means of acridine fluorescence (Anderson, Armstrong & Niven, 1959). Adaptation of this technique to Aspergillus was suggested by photographs of other acridine stained fungi (Anderson, unpublished work). Details of the present slide culture and staining technique have been given before (Clutterbuck & Roper, 1966).

Measurements of hyphae (Table 1) were taken from camera-lucida drawings of hyphae at the colony growth front. Hyphal diameter was measured on a scale that read directly in terms of cross-sectional area, on the assumption that the hyphae are cylindrical. Material for measurements of the conidial apparatus (Tables 2 and 4) was grown on ordinary agar plates. Measurements of conidiophore length and conidial column diameter were made on material wetted with xylene; for other measurements alcohol was used. The length of primary sterigmata was measured on heads bearing primary and secondary sterigmata only, while the combined length of primary and secondary sterigmata was measured on heads bearing the first conidia. Measurements were made with a micrometer eyepiece and only one measurement was made on any head.

The diameter of the vesicle and the spacing of the primary sterigmata were measured on camera-lucida drawings of conidiophores from old cultures which had been shaken with 0·1 % (v/v) Tween 80 (Koch–Light) detergent solution to wash off conidia and sterigmata. The drawings included the holes in the vesicle marking the former positions of the sterigmata, and the area of a triangle between the three holes most directly facing the microscope objective was measured on each drawing. This triangle is referred to as the 'sterigmata triangle' (see Fig. 1, *f*).

For fluorescence microscopy of sterigmata, pieces of a colony on agar, taken from the colony edge and therefore bearing developing conidial heads, were placed, face

downwards, on microscope slides kept at −20°. As soon as the surface of the colony had frozen on to the slide, the rest of the agar was removed and the slide was freeze-dried. The preparation was fixed and stained as for hyphal fluorescence.

RESULTS AND DISCUSSION

Hyphae. Studies were confined to the terminal cell (i.e. from the hyphal tip to the first septum) of each hypha since this cell is largely independent, at least as far as growth is concerned, of the older parts of the colony (Clutterbuck & Roper, 1966). Side branches of the cell, where these occurred, were ignored. The results, given in Table 1, show that the dimensions of the terminal cell are very similar in haploids and

Table 1. *The dimensions and numbers of nuclei of the terminal hyphal cells of Aspergillus nidulans*

Figures are means with standard errors.

	Number of cells	Length (μ)	Cross-sectional area (μ^2)	Volume (μ^3)	Number of nuclei	Volume per nucleus (μ^3)
Haploid	50	451 ± 13	12·9 ± 0·4	5715 ± 296	66·6 ± 2·4	88·6 ± 3·2
Diploid	52	433 ± 12	11·8 ± 0·4	5169 ± 236	31·7 ± 1·3	166·3 ± 6·1
Ratio: diploid/haploid		0·96	0·91	0·90	2·10	1·88
't' test: diploid = haploid		t = 1·02 Non-sig.	t = 1·90 Non-sig.	t = 1·44 Non-sig.	t = 12·7 P < 0·001	t = 11·4 P < 0·001
't' test: diploid = 2 × haploid		—	—	—	t = 1·06 Non-sig.	t = 1·45 Non-sig.

diploids. The numbers of nuclei in the two types of cell, however, are very different, with the result that the volume of cytoplasm per nucleus in the diploid is approximately twice that in the haploid. This means that the hyphae of *Aspergillus nidulans* are similar to the multinucleate conidia of *A. sojae* (Ishitani, Uchida & Ikeda, 1956) in that constancy of cytoplasm per genome in haploids and diploids is not obtained by alteration of cell size as in uninucleate cells, but by alteration of number of nuclei in cells of the same mean volume. Haploid and diploid hyphae of *A. nidulans* elongate at a similar rate (*c.* 210 μ/hr under the conditions used), so that establishment of a double volume of cytoplasm per nucleus in the diploid requires a reduced frequency of mitosis. It has long been known that in any continuously growing organism a critical volume of cytoplasm, or some particular feature of it, triggers mitosis (see Swann, 1957, and Mazia, 1961, for reviews). The present work stresses that it is a critical volume of cytoplasm *per genome* that is the trigger, just as in uninucleate cells of determinate growth (of which the conidia of *A. nidulans* are examples) a critical volume of cytoplasm per genome signals the cessation of growth. Another way of expressing these results is to say that in haploid and diploid hyphae the dimensions of the cell and the volume of cytoplasm per genome are all constant, it is only the packaging of genomes into nuclei that differs. It should be noted that once the characteristic volume of cytoplasm per nucleus is established in each strain, haploid or diploid, the rate of mitosis will also be the same, since in both types of strain, doubling of the nuclei must correspond with the doubling time of the cytoplasm. It is only in adjustment of the volume of cytoplasm per nucleus that the rates of mitosis will differ.

Conidial apparatus. The conidia of *Aspergillus nidulans*, in contrast to the hyphae just described, are uninucleate, and diploid conidia have double the volume of haploid ones (Pontecorvo *et al.* 1953). It is of interest, therefore, to examine the structures lying between the multinucleate hyphae and the uninucleate conidia, i.e. the conidiophore which is coenocytic, and primary and secondary sterigmata which are uninucleate.

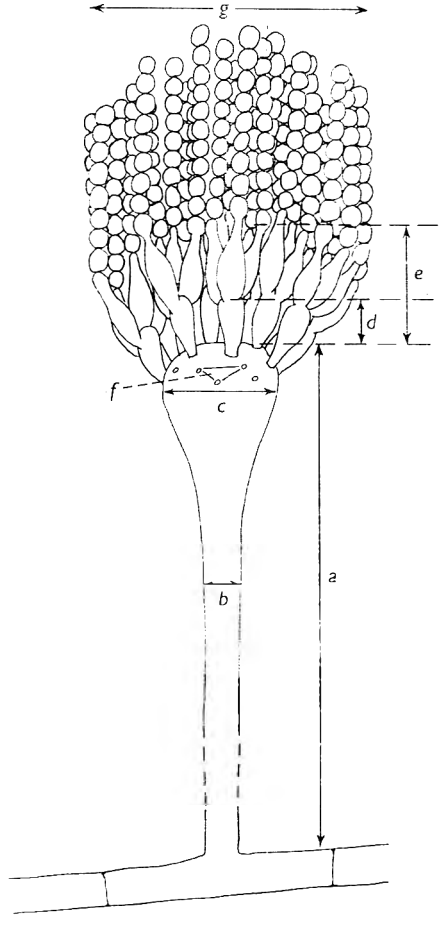

Fig. 1. Diagram of a typical conidial head of *Aspergillus nidulans*. The lettering refers to parameters measured in Tables 2 and 4.

A conidial head is shown diagrammatically in Fig. 1, along with the parameters whose dimensions are given in Tables 2 and 4. It can be seen from Table 2 that the multinucleate conidiophore resembles the hyphae in that its dimensions are independent of ploidy, while haploid and diploid sterigmata lengths are significantly different from one another. The sterigmata have also been tested to see whether their lengths differ by a factor of $\times \sqrt[3]{2}$; this would be the expected difference if the diploid sterigmata had double the volume of haploid ones and all their dimensions were increased proportionately to achieve this. Only the figures for the secondary sterigmata (obtained by subtraction) are in agreement with this hypothesis, the primary sterigmata differing by a considerably smaller factor.

Following this result a study was made with the fluorescence microscope so that the nuclear state of the sterigmata could be related to size. Sterigmata form as buds on the vesicle. After repeated mitosis of the nuclei in the vesicle one nucleus migrates into each sterigma bud. Similarly, the secondary sterigmata form as buds on the primary sterigmata and are later provided with nuclei by division of the primary sterigma nuclei. Conidia then bud from the secondary sterigmata. To follow size changes during this process, total sterigma length was measured on 80 randomly chosen heads of haploid and diploid strains at each of the following stages: (1) primary sterigmata before entry of nuclei from the vesicle, (2) nucleated primary sterigmata, (3) primary plus secondary sterigmata before division of the nucleus, (4) primary plus secondary sterigmata where both are nucleated, (5) primary plus secondary sterigmata where these bear conidial buds.

Table 2. *The dimensions of the conidial aparatus of Aspergillus nidulans*

Dimensions in microns are given as means with standard errors. Letters (*a*)–(*e*) refer to Fig. 1.

	Conidiophore		Vesicle diameter (*c*)	Primary sterigma length (*d*)	Total sterigma length (*e*)	Secondary sterigma length (by subtraction)
	Length (*a*)	Diameter (*b*)				
Haploid	98·8 ± 4·0	4·4 ± 0·1	10·2 ± 0·2	5·18 ± 0·08	11·52 ± 0·10	6·34 ± 0·12
Diploid	94·9 ± 4·4	4·6 ± 0·2	9·8 ± 0·2	5·54 ± 0·08	13·69 ± 0·19	8·15 ± 0·20
Number of observations	50	20	50	40	40	40
Ratio: diploid/haploid*	0·96	1·05	0·96	1·07	1·19	1·29
'*t*' test: diploid = haploid	*t* = 0·65 Non-sig.	*t* = 0·60 Non-sig.	*t* = 1·35 Non-sig.	*t* = 3·53 *P* < 0·001	*t* = 10·4 *P* < 0·001	*t* = 7·70 *P* < 0·001
'*t*' test: diploid = $\sqrt[3]{2}$ × haploid	—	—	—	*t* = 8·15 *P* < 0·001	*t* = 3·68 *P* < 0·001	*t* = 0·65 Non-sig.

* Where a diploid structure has double the volume of a haploid, its diameter or length is expected to differ by a factor of $\sqrt[3]{2} = 1{\cdot}26$.

The results are combined in a histogram in Fig. 2. Only the mean of stage 5 is given since this is taken as the finishing point of sterigmata development. The differences between haploid and diploid may be obtained by estimating the position of boundaries between stages: an attempt to do this is shown by the arrows in Fig. 2. Alternatively, the variance may be taken as a measure of the spread of values in a stage, and hence of its duration (Table 3). Using the first method, the apparent elongation of stage 1 may well be an artifact of the sampling method, reflecting only the wider distribution of values of stage 2. All stages other than 1, however, appear genuinely protracted. Both methods agree that the greatest increase occurs in stage 3, although none of the variances differ significantly between the two strains. On the other hand, the means for all stages, again with the exception of the first, do differ significantly between haploid and diploid. A difference between haploid and diploid means for a stage, however, cannot be interpreted exactly since it may be due to prolongation of that stage, or any of the previous ones.

In all, therefore, it appears that although diploid primary sterigmata are slightly larger than haploid ones, the main difference in volume does not arise until the stage when the secondary sterigmata are formed, but the nucleus has not yet divided.

Another character of the conidiophore has also been studied. This is the spacing of the primary sterigmata on the vesicle and is measured by the area of a 'sterigmata

triangle' (Fig 1, *f*). It can be seen from Table 4 that this also is independent of ploidy. Taking it that the sterigmata cover the upper hemisphere of the vesicle, it can be calculated from the sterigmata triangle area and the diameter of the vesicle that the average conidial head, of either ploidy, bears 31 primary sterigmata. Since haploid and diploid

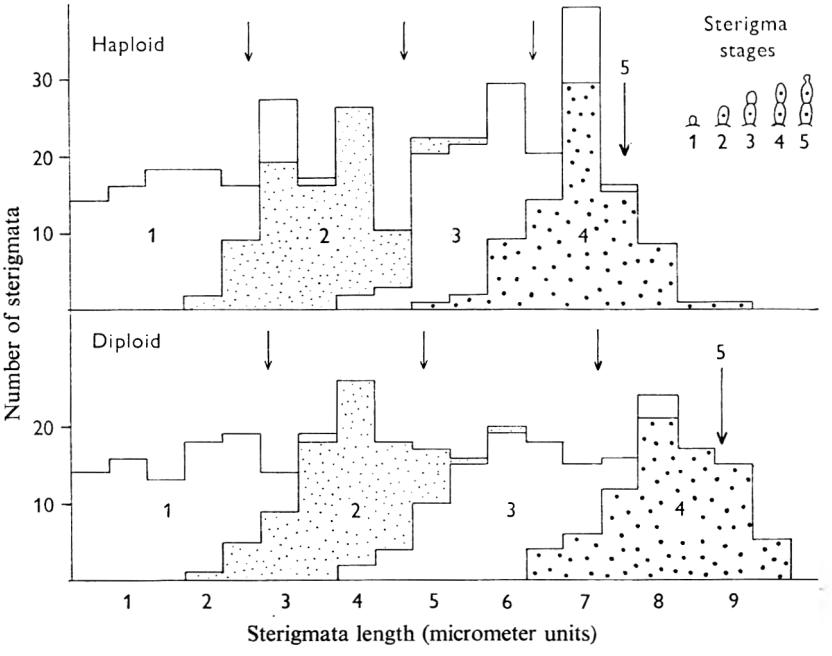

Fig. 2. Total sterigmata length in *Aspergillus nidulans* at various stages of development. The numbering of the histograms refers to the stages illustrated inset. 1 micrometer unit = 1·57 μ.

Table 3. *Aspergillus nidulans sterigmata length: tests of significance of the difference between haploid and diploid means and variances at the different stages of development shown in Fig. 2*

	Stage 1. Anucleate primary sterigmata	Stage 2. Nucleated primary sterigmata	Stage 3. Primary sterigmata plus anucleate secondary sterigmata	Stage 4. Primary plus secondary sterigmata both nucleated
'*t*' test for difference of means	t = 0·33 Non-sig.	t = 3·04 $P < 0·01$	t = 2·27 $P < 0·05$	t = 10·17 $P < 0·001$
Variance ratio: diploid/haploid*	1·00	1·06	1·18	1·09

* To reach the 5% level of probability the variance ratio with 80/80 degrees of freedom must exceed 1·46 (Fisher & Yates, 1963).

conidial heads bear the same numbers of primary sterigmata, and diploid conidia have twice the volume of haploid ones, it would be expected that the diameter of the tightly packed column of conidia would differ in the two ploidies by a factor of $\sqrt[3]{2}$ = 1·26

The diameters differ but not to this extent (see Table 4). This is almost certainly related to the observation that in the diploid, but not in the haploid, some of the primary sterigmata fail to receive nuclei and therefore do not develop further. This would be expected to occur if the vesicle initially contained the same number of nuclei per unit volume as the hyphae: there would then be approximately 6 nuclei in the haploid vesicle and 3 in the diploid (taking the vesicle as a sphere). At least three rounds of division would then be required to provide nuclei for the sterigmata in the haploid, but this would be insufficient in the diploid. The actual numbers of nuclei in the vesicle cannot readily be determined due to the difficulty of identifying the stage before divisions begin, and thereafter, because of the tendency of a mass of nuclei stained by fluorescence to obscure one another.

Table 4. *The sterigmata triangle area and conidial column diameter in the conidial head of Aspergillus nidulans*

Dimensions are means with standard errors. Letters (*f*) and (*g*) refer to Fig. 1.

	Sterigmata triangle area (μ^2) (*f*)	Conidial column diameter (μ) (*g*)
Haploid	$2{\cdot}54 \pm 0{\cdot}12$	$46{\cdot}7 \pm 0{\cdot}9$
Diploid	$2{\cdot}60 \pm 0{\cdot}10$	$50{\cdot}0 \pm 1{\cdot}0$
Number of observations	50	50
'*t*' test: diploid = haploid	$t = 0{\cdot}48$	$t = 2{\cdot}47$
	Non-sig.	$P < 0{\cdot}02$
'*t*' test:		$t = 6{\cdot}03$
Diploid = $\sqrt[3]{2} \times$ haploid	—	$P < 0{\cdot}001$
Ratio: diploid/haploid*	1·02	1·07

* $\sqrt[3]{2} = 1{\cdot}26$.

If the same number of cycles of division occurs in the vesicle in both ploidies, as suggested by this argument, then entry of nuclei into the primary sterigmata should occur at the same time. Since the primary sterigmata have been growing as extensions of the vesicle up to this point, and vesicle size is independent of ploidy, this may explain why the primary sterigmata do not differ before the nuclei enter.

The lack of difference is not made up once the nuclei have entered. This may mean that the interval of growth before budding is already determined at the time of entry of the nuclei into the primary sterigmata. This idea is supported by observations (Clutterbuck, unpublished) on a series of mutants designated '*aps*' ('*a*nucleate *p*rimary *s*terigmata') in which the nuclei fail to enter the primary sterigmata at all. In these strains, the anucleate sterigmata grow only slightly beyond the normal size at nuclear entry (achieving a final length of $4{\cdot}96 \pm 0{\cdot}08$ μ), and then produce secondary buds that do not develop further. It is clear that in this case budding, but not growth, is possible in the absence of the nucleus.

Two hypotheses may be put forward to explain the dependence of cell volume on ploidy. The simpler postulates that there is a symbiotic type of relationship between nucleus and cytoplasm such that if either component is in excess its own growth is depressed while that of the other is stimulated. In such a system, a diploid nucleus would be able to support the same volume of cytoplasm as two haploid ones. Such a simple system would fit any of the data presented here.

Bacterial work (Donachie & Masters, 1968) suggests a more positive control system involving the triggering of DNA synthesis and cell division by critical ratios of cytoplasmic and nuclear components. A mechanism of this sort has also been proposed by Yčas *et al.* (1965). Mazia (1961) suggested that cell growth is dependent on the 'growth potential' that is released from the nucleus at certain stages of the cell cycle. In yeast this stage has been shown to be soon after budding (Mitchison, 1958), possibly as a feature of the stepwise increase in enzyme levels that has now been shown in yeast (Tauro, Halvorson & Epstein, 1968) as well as in bacteria (Masters & Pardee, 1965). The similarity of the sterigmata of Aspergillus to the cells of budding yeasts suggests that a similar release of growth potential, of an amount proportional to ploidy, may occur some time after the entry of the nuclei into the primary sterigmata, and only at this time will the difference between diploids and haploids be fully expressed. This would provide an alternative explanation for the lack of difference between haploids and diploids at the primary sterigma stage.

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REFERENCES

ANDERSON, E. S., ARMSTRONG, J. A. & NIVEN, J. S. F. (1959). Fluorescence microscopy: observations of virus growth with aminoacridines. *Symp. Soc. gen. Microbiol.* **9**, 224.

CLUTTERBUCK, A. J. & ROPER, J. A. (1966). A direct determination of nuclear distribution in heterokaryons of *Aspergillus nidulans*. *Genet. Res. Camb.* **7**, 185.

COMMONER, B. (1964). Roles of deoxyribonucleic acid in inheritance. *Nature, Lond*, **202**, 960.

DONACHIE, W. D. & MASTERS, M. (1968). In *Cell Cycle. Gene Enzyme Interactions*. Ed. I. L. Cameron G. M. Padilla and G. L. Whitson. New York and London: Academic Press (In the Press.)

FISHER, R. A. & YATES, F. (1963). *Statistical Tables*, 6th Ed. Edinburgh and London: Oliver and Boyd Ltd.

HARTMANN, M. (1928). Über experimentelle Unsterblichkeit von Protozoen-Individuen. *Zool. Jb.* **45**, 973.

HEAGY, F. C. & ROPER, J. A. (1952). Deoxyribonucleic acid content of haploid and diploid Aspergillus conidia. *Nature, Lond.* **170**, 713.

ISHITANI, G., UCHIDA, K. & IKEDA, K. (1956). The relation of DNA content to cell size in Aspergillus. *Expl Cell Res.* **10**, 737.

KÄFER, E. (1958). An 8-chromosome map of *Aspergillus nidulans*. *Adv. Genet.* **9**, 105.

LIST, A. (1963). Some observations on DNA content and cell and nuclear volume growth in the developing xylem cells of certain higher plants. *Am. J. Bot.* **50**, 320.

MASTERS, M. & PARDEE, A. B. (1965). Sequence of enzyme synthesis and gene replication during the cell cycle of *Bacillus subtilis*. *Proc. natn. Acad. Sci. U.S.A.* **54**, 64.

MAZIA, D. (1961). Mitosis and the physiology of cell division. In *The Cell*. Ed. by J. Brachet and A. Mirsky, vol. 3. New York and London: Academic Press.

MITCHISON, J. M. (1958). The growth of single cells. II. *Saccharomyces cerevisiae*. *Expl Cell Res.* **15**, 214.

NOGGLE, G. R. (1946). The physiology of polyploidy in plants. I. Review of the literature. *Lloydia* **9**, 153.

PONTECORVO, G., ROPER, J. A., HEMMONS, L. M., MACDONALD, K. D. & BUFTON, A. W. J. (1953). The genetics of *Aspergillus midulans*. *Adv. Genet.* **5**, 141.

PRESCOTT, D. M. (1956). Relation between cell growth and cell division. II. The effect of cell size on cell growth rate and generation time in *Amoeba proteus*. *Expl Cell Res.* **11**, 86.

ROPER, J. A. (1952). Production of heterozygous diploids in filamentous fungi. *Experentia* **8**, 14.

SWANN, M. M. (1957). The control of cell division: a review. I. General mechanisms. *Cancer Res.* **17**, 727.

TAURO, P., HALVORSON, H. O. & EPSTEIN, R. L. (1968). Time of gene expression in relation to centromere distance during the cell cycle of *Saccharomyces cerevisiae*. *Proc. natn. Acad. Sci. U.S.A.* **59**, 277.

YČAS, M., SUGITA, M. & BENSAM, A. (1965). A model of cell size regulation. *J. theor. Biol.* **9**, 444.

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Mutants of the Arginine-proline Pathway in *Aspergillus nidulans*

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SUMMARY

Proline suppressor mutant *su-19* was analysed for arginase and ornithine δ-transaminase (OTA) activity. Both enzymes are constitutively produced by this mutant. Five other suppressor loci not linked with *su-19* or to each other are involved in regulation of the two enzymes.

A mutant showing no OTA activity was isolated. Properties of this mutant and its effect in various strains of the *pro su* type were studied.

INTRODUCTION

Mutants of one (*su-19*) out of three proline suppressor loci studied previously in *Aspergillus nidulans* showed the constitutive synthesis of ornithine δ-transaminase (OTA), the enzyme involved in the conversion of ornithine to glutamic γ-semialdehyde (GSA) (Weglenski, 1966, 1967). It was suggested that due to high level of the OTA in *su-19* strain, ornithine is diverted from the arginine pathway and utilised for GSA and proline synthesis. Thus, mutations in the *su-19* locus were considered responsible for opening the alternative route of the proline synthesis which by-passes the metabolic block (between glutamate and GSA) in the major proline pathway. Assays for arginase showed that the activity of this enzyme is also higher than in the wild strain. However, the arginase results were not reliable as the assay procedure applied does not work well in *A. nidulans* (cf. Weglenski, 1967); moreover, all assays were carried out with strains of the *pro su* type and not of the *pro*$^+$ *su* type. As previously shown (Weglenski, 1967), the presence of the *pro* mutation strongly influences the activity of the enzymes metabolizing arginine. The relations between the pathways of arginine and proline synthesis are shown in Fig. 1.

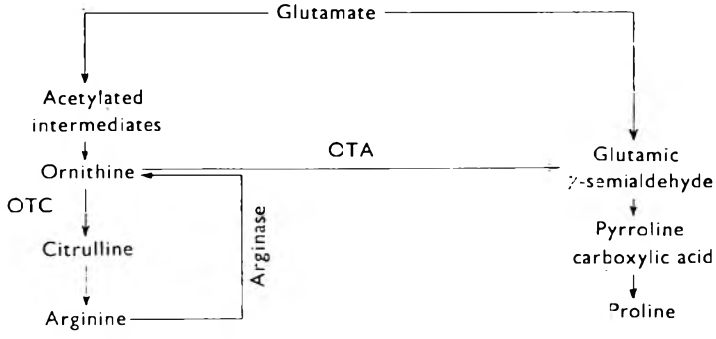

Fig. 1. *Aspergillus nidulans*: the pathway of proline and arginine synthesis. OTC, ornithine transcarbamylase; OTA, ornithine δ-transaminase.

The aim of the experiments presented in this paper was: (1) to obtain the *pro*$^+$ *su-19* strain and to test if the *su-19* mutation affects only OTA or both OTA and arginase synthesis, (2) to test if other loci are involved in regulation of arginase and/or OTA synthesis, (3) to select OTA deficient mutants and to construct a strain blocked both in the major and in the alternative pathway of proline synthesis, which would make it possible to test decisively whether the opening of the alternative pathway is responsible for the suppression phenomenon in the case of *su-19* and other suppressor loci.

METHODS

Media and standard techniques used in this work were as in Pontecorvo *et al.* (1953).

Material. All proline suppressor mutants except *su-1* were obtained as spontaneous mutants from the *pro-6 pab-9 bio-1* or the *pro-6 ade-9 y phe-2* strains (Weglenski, 1966). Strain *pro-1 su-1* was obtained from the Department of Genetics, Glasgow University.

Isolation of mutants. Conidia of the *pro-6 su-19 pab-9 bio-1* strain were treated for 1 hr with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (500 μg./ml.). After removing mutagen conidia were resuspended in water and plated on the medium containing all required supplements. Colonies obtained were tested by needle inoculations for growth on a prolineless medium.

Isolation of the pro$^+$ *su-19 strain.* Locus *su-19* maps on chromosome I between *pro-1* (an allele of *pro-6*) and *ade-9* showing with these two loci about 6 and 4% recombination respectively. To obtain the *pro*$^+$ *su-19* strain the following cross was made:

$$\frac{pro\text{-}6\ su\text{-}19 \quad + \quad pab\text{-}9 \quad + \quad bio\text{-}1}{+ \quad + \quad ade\text{-}9 \quad + \quad y \quad +}.$$

From the progeny of this cross colonies of the *pab bio* phenotype were selected and tested in heterokaryons against the *pro-6 ade-9 y phe-2* strain. By this method *pro-6* can be distinguished from the *pro*$^+$ colonies since those belonging to the former class will not form heterokaryons which can grow on the prolineless medium. To the latter class belong colonies of the *pro*$^+$ *su*$^+$ *pab-9 bio-1* and *pro*$^+$ *su-19 pab-9 bio-1* genotypes, which can be distinguished after crossing them with the *pro-6 su-19* strain. If a colony used in the cross was *pro*$^+$ *su-19*, no proline requiring progeny will be obtained.

Methods of culture and harvesting mycelium and of enzyme extraction and assay for OTA were as previously described (Weglenski, 1967).

Assay of arginase. 0·2 ml. of enzyme extract (equiv. 0·3 mg. protein) in 0·1 M-potassium phosphate buffer (pH 7·0) was preincubated at 37° for 1 hr with 0·1 μmole of $MnCl_2$. After preincubation 50 μmole of arginine-HCl, pH 10·0, were added and the reaction mixture was incubated for 20 min. at 37°; total volume of reaction mixture 1·0 ml. The reaction was stopped by adding 0·2 ml. 0·2 M-HCl. Urea formed in the reaction was split to ammonium by adding an excess of urease. Ammonium was estimated by the Conway microdiffusion method (Conway, 1962).

Determination of protein. Protein estimations were made according to the Lowry, Rosebrough, Farr & Randall (1951) method, with crystalline bovine serum albumin as a standard.

Abbreviations used: pro = proline; *ade* = adenine; *pab* = p-aminobenzoic acid; *phe* = phenylalanine; *bio* = biotin; *y* = yellow conidia; *su* = suppressor.

RESULTS

su-19 and other mutants constitutive for arginase and OTA synthesis

Activities of arginase and OTA were compared in *pro-6 su-19*, *pro*$^+$ *su-19* and wild strains. The high level of synthesis of both enzymes results from the suppressor mutation and is not influenced by the presence or absence of the *pro-6* mutation (Table 1). Several other suppressor mutants were tested for arginase and OTA activities. Five suppressor mutants showed constitutive levels of both enzymes (Table 1). All these mutants are recessive and they complement with *su-19* and with each other. Crosses were made of all possible pairs of these suppressors. No linkage between suppressor loci was observed. One suppressor mutant which we studied, *su-1*, was isolated and mapped by Forbes (1956) in linkage group III. Two other mutants, *su-11* and *su-22* were assigned to linkage groups V and III respectively by means of mitotic haploidisation (Sawicka, personal communication).

Table 1. *Aspergillus nidulans: specific activities of arginase and ornithine δ-transaminase (OTA) in wild, ota-1 and various suppressor strains*

Strain	Arginase activity (μg. N-NH_3/mg protein/min.)	OTA activity (mμmole ornithine/mg. protein/min.)
Wild	4·0	12
Wild, induced by arginine	39·1	About 200
pro-6 su-19	28·5	144
pro$^+$ *su-19*	27·8	145
pro-6 su-11	26·7	163
pro-6 su-22	25·8	134
pro-6 su-1	23·8	128
pro-6 su-30	24·2	150
pro-6 su-35	26·5	171
pro-6 su-19 ota-1	30·2	About 1
pro$^+$ *su*$^+$ *ota-1*	3·8	About 1

Table 2. *Aspergillus nidulans: crosses involving the ota-1 mutant*

Cross	Number of colonies tested	Number of *pro* colonies	% *pro*
$\frac{\textit{pro-6 su-19 ade-9} \quad + \quad \textit{ota-1}}{+ \quad + \quad + \quad \textit{pab-9} \quad +}$	251	42	17
$\frac{\textit{pro-6} \quad + \quad \textit{pab-9} \quad + \quad \textit{ota-1}}{\textit{pro-6 ade-9} \quad + \quad \textit{su-6} \quad +}$	225	168	75

Isolation and characterisation of the ota-1 mutant

The mutant isolation procedure applied to the *pro-6 su-19* strain provided several mutants unable to grow on the prolineless medium. They could represent: (1) back mutations in the *su-19* locus, (2) mutations in the major pathway of proline synthesis resulting in metabolic blocks between GSA and proline, (3) mutations in the alternative pathway of proline synthesis. Mutants of the first class can be easily distinguished as their growth responses are the same as of the *pro-6* strain which grows on proline or arginine. Only one mutant which does not respond to arginine, but still grows on

proline, was found; it will be henceforth referred to as *ota-1*. In order to distinguish whether this mutant belongs to the second or to the third class, it was crossed to the wild strain. The *pro-6* and *su-19* loci are closely linked, thus if the *ota-1* mutation is concerned only with the supplementary proline pathway, 25% of proline requiring colonies should result from this cross rather than the 50% which would be expected if *ota-1* blocks the major proline pathway. The result (Table 2) is close to the former figure. To test the third possibility, i.e. that the *ota-1* mutation causes a metabolic block in the alternative pathway of proline synthesis, arginase and OTA activities were assayed. Strains carrying the *ota-1* mutation show no OTA activity, while arginase activity remains unaffected (Table 1). The *pro-6 su-19 ota-1* strain was tested in a heterokaryon against the *pro-6 su-19 ota*$^+$ strain; *ota-1* appeared to be recessive to the *ota-1* allele.

Effects of the ota-1 mutation in the pro-6 su-6 strain

Mutations in the *su-6* locus result in partial deficiency of ornithine transcarbamylase, the enzyme which converts ornithine to citrulline (Weglenski, 1967). It was suggested that in suppressors strains of this type ornithine accumulates and becomes available for transamination to GSA. If this hypothesis is correct, the *ota-1* mutation introduced into the *pro-6 su-6* strain should cancel the effects of the suppressor on the proline mutation. The *pro-6*, *su-6* and *ota-1* loci are not linked; thus when *ota-1* cancels the *su-6* effect, one can expect 75% of proline requiring colonies among progeny of the cross between *pro-6 su-6* and *pro-6 ota-1* strains. The results of this cross (Table 2) are in full agreement with this supposition.

DISCUSSION

The results presented in this paper show that the synthesis of arginase and OTA is controlled by the same regulatory system. Similar observations were made with *Bacillus subtilis* by De Hauwer, Lavalle & Wiame (1964). These authors demonstrated the co-ordinate induction of arginase and OTA together with arginine permease and isolated mutants constitutive for all three enzymes. In terms of Jacob & Monod (1961) operon model, *su-19* should be considered as a regulatory gene, while *ota-1* as the structural gene for OTA. However, the presence of five suppressor mutations mapping in different loci but resulting in the same effects as the mutation in the *su-19* locus suggests that the genetic regulation of arginine break-down enzymes is complicated and can not be explained by the previously proposed models.

From properties of the *ota-1* mutant it can be concluded that the arginine–ornithine–GSA pathway is the only one by which arginine (and ornithine) can be utilised for proline synthesis. The results clearly show that the proline suppressors observed in this study cause the opening of this particular pathway. However, the proline suppressor mutants not linked to the *pro-6* locus were found, which are active in the strain *pro-6 ota-1* being blocked both in major and alternative pathway of proline synthesis. The presence of suppressors of this type suggests a possibility for existence of a third pathway of proline synthesis in *Aspergillus nidulans*. Genetical and enzymological analysis of these mutants is now in progress.

REFERENCES

CONWAY, E. J. (1962). *Microdiffusion Analysis and Volumetric Error*. London: Grosby Lockwood.

DE HAUWER, G., LAVALLE, R. & WIAME, J. M. (1964). Étude de la pyrroline déshydrogénase et de la regulation du catabolisme de l'arginine chez *Bacillus subtilis*. *Biochim. biophys. Acta* **81**, 257.

FORBES, E. C. (1956). Recombination in the *pro* region in *Aspergillus nidulans*. *Microb. Genet. Bull.* **13**, 9.

JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. molec. Biol.* **3**, 318.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**, 265.

PONTECORVO, G., ROPER, J. A., HEMMONS, L. M., MACDONALD, R. D. & BUFTON, A. W. J. (1953) The genetics of *Aspergillus nidulans*. *Advanc. Genet.* **5**, 141.

WEGLENSKI, P. (1966). Genetical analysis of proline mutants and their suppressors in *Aspergillus nidulans*. *Genet. Res.* **8**, 311.

WEGLENSKI, P. (1967). The mechanism of action of proline suppressors in *Aspergillus nidulans*. *J. gen. Microbiol.* **47**, 77.

J. gen. Microbiol. (1969), **55**, 307–317
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Transformation in *Micrococcus lysodeikticus*

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SUMMARY

Phage N6 lysates of *Micrococcus lysodeikticus* and *Sarcina lutea* were very viscous and were found to mediate genetic transfer of an adenine marker (ade^+) to adenine-dependent strains of *M. lysodeikticus*. The transfer activity of these lysates was found to reside in a high molecular weight DNA fraction rather than in the phage particles themselves. DNA that was isolated and purified from phage lysates and lysozyme-treated cells was shown to possess transforming activity. Recipient competence was maximal during late logarithmic growth (14–18 hr). The cell density of the transfer suspension was optimum at about 10^7 colony forming units/ml. Transformants were recovered after 1 hr exposure to DNA and continued to increase in number up to 6 hr. Transformants appeared in higher frequencies on Difco agars that were less purified. Under optimal conditions, transformation frequencies up to several percent per colony forming unit were obtained. All strains of *M. lysodeikticus* tested (ATCC 4698, ATCC 15801, CCM 1335, PU, UM, WRU, ISU) could serve as donors of the ade^+ marker. All strains, except ATCC 4698, were competent recipients of the adenine marker.

INTRODUCTION

The 'species' *Micrococcus lysodeikticus* originated from a single culture isolated by Fleming (1922). He discovered that the organism was very susceptible to the lytic action of lysozyme and, since this early study, it has been widely used as a source of cell-wall substrate for lysozyme assay. Numerous studies have been conducted on its cell wall and membrane structure and various enzyme systems.

Cultures maintained by different laboratories have shown some variation over a period of time (Feiner, Meyer & Steinberg, 1946; Grula, 1962), presumably as a result of subculture and other selective processes. More specific variation has been obtained by the isolation of certain mutants to study various features of the organism (Feiner, 1967; Fleming & Allison, 1927; Naylor & Burgi, 1956; Okubo, Nakayama, Sekiguchi & Takagi, 1967; Stechschulte, 1961). Only recently, however, has this organism been subjected to genetic analysis. The first evidence of genetic exchange was reported in certain crosses using phage N6 lysates capable of transmitting an adenine marker (Kloos, 1968).

This report will provide evidence of the mechanism of genetic transfer mediated by phage N6 lysates. The nature of the transfer agent (e.g. free DNA or phage particles)

was first elucidated by treating phage lysates with deoxyribonuclease (DNase), heat, and phage-specific antiserum. Since these studies indicated that free DNA and not phage particles themselves were involved in transfer, we continued to pursue the isolation of transforming DNA. Preliminary studies are included which determine some of the optimum conditions for transformation and survey donor and recipient competence of a number of different strains.

Table 1. *Strains and mutants of Micrococcus lysodeikticus*

Strains	Genetic characters	Source
ATCC 4698	*ade pig-y*	J. B. Evans, North Carolina State Univ., Raleigh, N.C., U.S.A.
ATCC 1580	*ade pig-p*	American Type Culture Collection, Rockville, Maryland, U.S.A.
ATCC 1580	*ade*$^+$*-4 pig-p*	—
CCM 1335	*ade*$^+$ *pig-y*	M. Kocur, Czechoslovak Collection of Microorganisms, J. E. Purkyne Univ., Brno, CSSR
PU	*ade pig-y*	E. A. Grula, Oklahoma State Univ., Stillwater, Okla., U.S.A.
PU	*ade*$^+$*-8 pig-y*	—
UM	*ade pig-y*	E. A. Grula
UM	*ade*$^+$*-1 pig-y*	—
WRU	*ade pig-y*	E. A. Grula
WRU	*ade*$^+$*-1pig-y*	—
ISU	*ade pig-y*	E. A. Grula
ISU	*ade pig-o*	—
ISU	*ade*$^+$*-1 pig-y*	—

METHODS

Bacterial strains. Strains and mutants of *Micrococcus lysodeikticus* used in this study are listed in Table 1. The strain of *Sarcina lutea* used in crosses with *M. lysodeikticus* was ATCC 272 obtained from M. Kocur.

The phenotypic markers used to test genetic exchange included: (1) the ability of a strain to grow in the absence of adenine and (2) colonial pigment. These characters differed among several strains and could be easily scored. Most of the laboratory strains required adenine for growth. With the adenine-dependent strains used in this study, adenosine, inosine, or hypoxanthine could replace adenine and even produce a more rapid growth response (Grula, 1962). Strain CCM 1335 was adenine-independent and was used as a donor. Spontaneous adenine-independent mutants were readily obtained from all strains, except ATCC 4698, and were also tested as donors. The two spontaneous pigment mutants used in this study were isolated from wild-type, yellow strains. The marker system used to test genetic exchange between *Sarcina lutea* and *Micrococcus lysodeikticus* utilized the natural adenine-independent *S. lutea* as donor with adenine-dependent strains of *M. lysodeikticus*.

The designation of markers was made according to the scheme of Demerec, Adelberg, Clark & Hartman (1966) whenever possible. The adenine-dependent strains were designated *ade*. Adenine-independent mutants were designated *ade*$^+$ followed by an arabic numeral to indicate the order of isolation; e.g. *ade*$^+$-1. Colonial pigment was denoted by the term *pig* with the addition of a hyphenated symbol to indicate the particular colour. The following terms were used: *pig-y*, yellow; *pig-p*, pale pink or beige; *pig-o*, orange or apricot.

Media and conditions of growth. The complex broth (P broth) and agar (P agar) used throughout this study were described by Naylor & Burgi (1956). The defined agar used was a modification of the defined medium of Wolin & Naylor (1957) supplemented with certain amino acids required for rapid growth on agar (Rose & Kloos, unpublished data). The composition of the synthetic agar is as follows: K_2HPO_4, 2 g.; NH_4Cl, 1 g.; $MgSO_4.7H_2O$, 0·1 g.; $FeSO_4.7H_2O$, 4 mg.; $MnCl_2.4H_2O$, 2 mg.; glucose, 7 g.; biotin, 10 μg.; monosodium glutamate, 10 g.; L-arginine, 656 mg.; L-phenylalanine, 400 mg.; L-tyrosine, 500 mg.; L-proline, 900 mg.; L-isoleucine, 500 mg.; L-cysteine, 480 mg.; L-methionine, 270 mg.; Special Agar-Noble (Difco), 15 gm.; de-ionized water, 1000 ml. Glucose was added aseptically after sterilization of the medium. The pH of the medium was adjusted to 7·2 before autoclaving.

Bacteria were maintained at 4° on P agar slants in cotton-plugged tubes. The inocula for all experiments were obtained from subcultures of stocks which were prepared on P agar slants incubated for 18 hr and then stored. Cultures were shaken with a Burrell Wrist-Action Shaker (Burrell Corporation, Pittsburgh, Pennsylvania) at a setting of 4 with arms mounted over water baths at 32°.

Bacteriophage. The micrococcal phage N6, propagated on *Micrococcus lysodeikticus* ATCC 15801, was used to prepare lysates of *M. lysodeikticus* strains. This phage was described by Naylor & Burgi (1956) and was obtained from M. Kocur. A host-range mutant of phage N6 was isolated and used to prepare lysates of *Sarcina lutea.* Phages were stored in P broth in screw-capped tubes at 4°.

Preparation of phage lysates. Phages were propagated on the appropriate donor strain by a modification of the agar-layer technique of Swanstrom & Adams (1951). Propagation was for 18 to 24 hr. The soft P agar layer was suspended in 8 ml. of P broth and homogenized by repeated passage through a 10 ml. pipette. After centrifugation, lysates were bacteriologically sterilized by filtration through an 02 Selas Filter (Selas Flotronics, Spring House, Pennsylvania, U.S.A.) and then stored at 4°. They usually had titres from 10^{10} to 10^{11} plaque forming units (p.f.u.)/ml.

Preparation of lysozyme lysates. Lysozyme lysates were obtained by treating 18 hr broth cultures ($2–4 \times 10^9$ colony-forming units/ml. c.f.u./ml.) of the appropriate donor strain with lysozyme (Difco) (20 μg./ml.) for 1 hr. The suspension was then centrifuged to remove cell debris and bacteriologically sterilized by filtration through an 20 Selas Filter. The filtrate was heated at 75° for 30 min. to remove lysozyme activity.

Procedure for crosses with lysates. An 18 hr P agar slant culture ($5–10 \times 10^9$ colony forming units) of the recipient strain was suspended in 1 ml. P broth. A 0·1 ml. sample of this suspension was then added to 1 ml. of phage or lysozyme lysate and the mixture (transfer suspension) shaken for 30 min. The shaken suspension was washed once by centrifugation and resuspended in 1 ml. saline. Samples of 0·1 ml. were spread on the appropriate medium and scored after incubation for 6 days.

DNase treatment. DNase (1 × crystallized, Worthington Biochemical Corporation, Freehold, New Jersey) was added to phage lysates at concentrations of 10^{-4} μg./ml. to 100 μg./ml. Magnesium sulphate was added to a final concentration of 0·005 M. The mixture was incubated at 37° for 30 min. and then used to prepare the transfer suspension.

Heat treatment. Phage lysates were heated at different temperatures from 32° to 100° for 1 hr and then cooled rapidly in an ice-bath for 15 min. The cooled lysate was then warmed at 32° and used to prepare the transfer suspension.

Preparation of serum. The preparation of phage N6 antigen was according to the method of Burgi (1955). Phage specific antiserum was prepared by injecting rabbits intravenously with 1 ml. of antigen on days 1, 3, 7, 15, and 23. Antiserum was collected 7 days after the last injection. Normal serum and antiserum were diluted 1/10 in 0·01 M-phosphate buffer and heated at 75° for 45 min. to remove complement and nuclease activity. Sera were incubated with cells of the recipient strain for 4 hr to remove most of the cell antibodies. Further purification by ammonium sulphate fractionation and dialysis was required to remove material in both normal serum and antiserum that reduced the yield of recombinants in tests.

Serum treatment. Phage lysates were treated with normal serum or antiserum by adding 1 ml. of serum to 1 ml. of lysate and incubating the mixture for 2 hr at 32°. Transfer suspensions were prepared by using the total volume (2 ml.) of treated lysates.

Procedure for DNA isolation and purification. The procedure used to obtain purified DNA was that of Marmur (1961) with the modification of Saito & Miura (1963) using T1 ribonuclease (RNase) (Sankyo Company, Ltd., Tokyo, Japan) as well as bovine pancreatic RNase (Calbiochem, Los Angeles, California). DNA was isolated from *Micrococcus lysodeikticus* cells by treatment with lysozyme (Pentex Incorp., Kankakee, Illinois) and from phage N6 lysates without prior lysozyme treatment. All preparations were de-proteinized 4 times both before and after RNase treatment. After the final ethanol precipitation, DNA was dispersed into a 1/10 dilution of standard saline-citrate buffer (SSC: 0·15 M-NaCl + 0·015 M-sodium citrate, pH 7·0) which was later adjusted to SSC. Chloroform was added to purified DNA preparations to maintain bacterial sterility.

DNA concentration was determined by the diphenylamine reaction of Dische (1955). RNA was estimated by the orcinol reaction (Brown, 1946; Mejbaum, 1939) and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). DNA base ratios were determined by the thermal denaturation method of Marmur & Doty (1962).

Procedure for crosses using purified DNA. An 18 hr P agar slant culture of the recipient strain was suspended in 1 ml. P broth. A 10^{-2} dilution of this suspension in broth was prepared and a 0·1 ml. sample added to 1 ml. of purified DNA preparation. The transfer suspension was shaken for 2 hr, washed once by centrifugation, and resuspended in 1 ml. saline. Samples of 0·1 ml. from the concentrated suspension and a 10^{-1} dilution in saline were spread on duplicate synthetic agar plates and scored after incubation for 6 days.

RESULTS

Demonstration of genetic transfer. The ability of phage N6 lysates to transfer genetic information from donor to recipient cells of *Micrococcus lysodeikticus* was demonstrated in two separate crosses using strains that differed in genetic characters (Table 2). Several hundred recombinant clones from each cross were isolated and tested for genetic stability. All maintained the recombinant phenotype on repeated agar transfer. Donor contamination was eliminated as a source of clones by the following observations: (*a*) samples from phage lysates that were filtered did not contain donor cells; (*b*) donor cells which were added to phage lysates appeared on synthetic agar at least 24 hr earlier than Ade^+ recombinants and possessed characteristics (colonial pigment, ability to support phage N6 plaque formation, growth) of the donor strain; (*c*) recombinants possessed other characteristics of the recipient strain.

Ade$^+$ recombinants began to appear on defined agar in 40–48 hr when strain PU (*ade*$^+$-*8*) was used as the donor or 56–72 hr when ATCC 15801 (*ade*$^+$-*4*) was donor. It should be noted that the PU mutant grows more rapidly than the ATCC 15801 mutant on synthetic agar (Rose & Kloos, unpublished data). Recombinants continued to appear up until about 6 days of incubation and varied from 0·2 to 3·5 mm. diameter depending on the time of emergence. Pigment recombinants were scored among Ade$^+$ clones and Ade clones appearing on synthetic agar supplemented with inosine (60 mg./l.). The number of cells giving rise to a clone (colony forming unit) was usually two or four. Microscopic examination of wet mounts of broth and agar slant cell suspensions, that were well mixed, indicated that less than 5% of total cell clusters were composed of more than four cells.

Table 2. *Genetic exchange in Micrococcus lysodeikticus*

Cross: donor × recipient	Recombinant phenotype	Clones/ml. suspension*	Recombinant frequency/c.f.u.
ATCC 15801 (*ade*$^+$-*4 pig-p*) × ISU (*ade pig-y*)	Ade$^+$	10,880	$4{\cdot}2 \times 10^{-5}$
	Pig-p	2,340	$8{\cdot}9 \times 10^{-6}$
	Ade$^+$ Pig-p	4	$1{\cdot}5 \times 10^{-8}$
ATCC 15801 (*ade pig-p*) × ISU (*ade pig-y*)	Ade$^+$	1,020	$4{\cdot}0 \times 10^{-6}$
ISU (*ade pig-y*) cells	Ade$^+$	40	$1{\cdot}5 \times 10^{-7}$
	Pig-p	0	0
PU (*ade*$^+$-*8 pig-y*) × ISU (*ade pig-o*)	Ade$^+$	30,630	$1{\cdot}1 \times 10^{-4}$
	Pig-y	7,040	$2{\cdot}6 \times 10^{-5}$
	Ade$^+$ Pig-y	10	$3{\cdot}6 \times 10^{-8}$
PU (*ade pig-y*) × ISU (*ade pig-o*)	Ade$^+$	1,220	$4{\cdot}4 \times 10^{-6}$
ISU (*ade pig-o*) cells	Ade$^+$	45	$1{\cdot}6 \times 10^{-7}$
	Pig-o	0	0

* Transfer suspensions contained $2{\cdot}6$–$2{\cdot}7 \times 10^8$ recipient c.f.u./ml.

Identification of the transfer agent in phage lysates. It was quite natural to expect that genetic exchange in the above experiment was a consequence of transduction. However, in addition to possessing high phage titres, phage lysates appeared to contain high levels of polymerized DNA. Lysates were unusually viscous. Treatment with DNase resulted in a rapid loss of viscosity and, at all levels tested, completely destroyed the ability of lysates to produce Ade$^+$ recombinants. DNase treatment did not effect the phage titre, phage adsorption, or the viability of the recipient strain.

When the temperature of the phage lysate was raised to 70°, the phage N6 was inactivated and unable to form plaques on an indicator strain. In addition, several other effects of phage adsorption on the recipient strain did not appear. On the other hand, the yield of Ade$^+$ recombinants remained high at all temperatures up to the melting-point of *Micrococcus lysodeikticus* DNA (Rosypalova, Bohacek & Rosypal, 1966) (Table 3).

Phage specific antiserum reduced the concentration of active phages in lysates to very low levels without reducing the yield of Ade$^+$ recombinants (Table 4). Centrifugation of phage lysates, to remove large quantities of phages, produced supernatants that possessed high transfer ability. Treatment of the supernatant with antiserum decreased

the number of active phages to 7 p.f.u./ml. without reducing the yield of recombinants. On the other hand, phages which were washed by centrifugation failed to produce recombinants. Each of the above experiments suggests that free DNA and not the phage N6 was responsible for genetic transfer of the *ade*$^+$ marker. This would imply that genetic exchange was a result of transformation.

Table 3. *Effect of heat treatment of phage lysates on the yield of Ade*$^+$ *recombinants in the cross ATCC 15801 (ade*$^+$*-4) × ISU (ade)*

Temperature of lysate	Phage N6 plaque-forming ability (p.f.u./ml.)*	Ade$^+$ clones/ml. transfer suspension†
32°	$7{\cdot}3 \times 10^{10}$	8,720
70°	0	10,430
80°	0	10,015
90°	0	6,760
100°	0	35
ISU cells		50

* After cooling and re-warming to 32°, lysates were assayed on the donor strain.

† Transfer suspensions contained $5{\cdot}2 \times 10^8$ recipient c.f.u./ml. The number of clones is the average from duplicate plates.

Table 4. *Effect of the addition of specific antiserum to phage lysates and lysate supernatants on the yield of Ade*$^+$ *recombinants in the cross ATCC 15801 (ade*$^+$*-4) × ISU (ade)*

Treatment	Phage N6 plaque-forming ability (p.f.u./ml.)*	Ade$^+$ clones/ml. transfer suspension†
Phage lysate:		
No treatment	$3{\cdot}4 \times 10^{10}$	6640
Normal serum	$2{\cdot}5 \times 10^{10}$	4980
Antiserum	$8{\cdot}7 \times 10^5$	5030
Lysate supernatant:		
No treatment	$9{\cdot}8 \times 10^7$	6180
Normal serum	$9{\cdot}4 \times 10^7$	5310
Antiserum	$7{\cdot}0 \times 10^0$	8420
ISU cells	—	40

* After treatment, lysates were assayed on the donor strain.

† Transfer suspensions contained $7{\cdot}8 \times 10^8$ recipient c.f.u./ml. The number of clones is the average from duplicate plates.

Transformation by purified DNA. Reasonably high levels of DNA were isolated from both phage N6 lysates (52–56 μg./ml.) and lysozyme-treated cells (1·29–1·35 mg./g. wet packed cells). The ratio RNA : DNA varied from less than 0·01 to 0·1 in different preparations. DNAs contained 2–20% protein. The percent G-C of DNA isolated from phage lysates and lysozyme-treated cells was 67·9 and 69·8, respectively.

Purified DNA isolated from phage lysates and cells of *Micrococcus lysodeikticus* showed transforming activity for the *ade*$^+$ marker (Fig. 1). The specific transforming activity of DNA isolated from lysozyme-treated cells was about 350 Ade$^+$ clones/μg. DNA. The activity of DNA isolated from phage lysates was about 42 Ade$^+$ clones/μg. DNA. These results confirm the implied transformation demonstrated above with crude lysates.

Optimum conditions for transformation. Several factors which affected the yield of transformants were examined to determine conditions that would support maximum transformation frequencies. These included the age of the recipient culture, the cell density of the transfer suspension, the time of exposure to DNA, and the type of agar used in the plating medium. All experiments were conducted with the cross ATCC 15801 (*ade*$^+$-4) × ISU (*ade*). Transfer suspensions were prepared with either phage lysates (heated 1 hr at 70° to inactivate phage N6) or purified DNA (36 μg./ml.). Both sources of transforming DNA indicated similar optimal conditions for all factors tested.

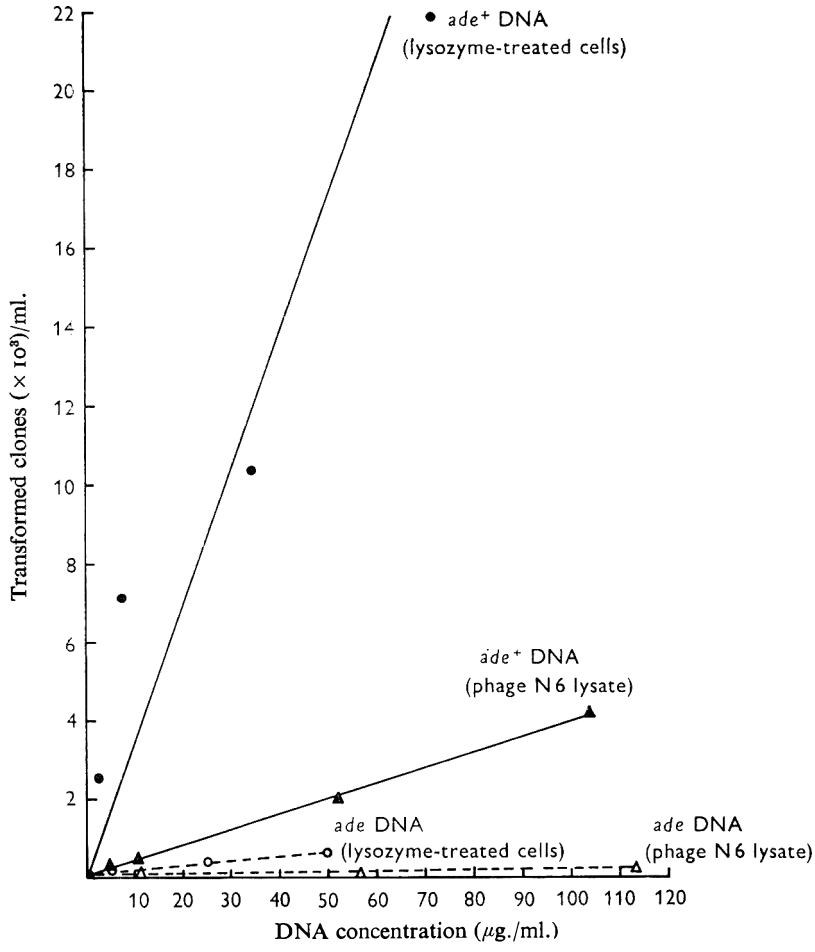

Fig. 1. Effect of DNA concentration on the yield of Ade$^+$ transformants. ● DNA isolated from lysozyme-treated ATCC 15801 (*ade*$^+$-4) cells. ▲ DNA isolated from phage N6 lysate of *ade*$^+$-4. ○ DNA isolated from lysozyme-treated ATCC 15801 (*ade*) cells. △ DNA isolated from phage N6 lysate of ATCC 15801 (*ade*). The recipient strain was ISU (*ade*).

Competence of broth and agar slant recipient cultures was maximal at 14–18 hr of incubation, corresponding to late logarithmic growth. Cultures continued to show significant competence over extended periods of incubation. For example, 72 hr cultures produced 1/10 the number of transformants produced by cultures with maximum competence.

The concentration of the transfer suspension was optimum at about 10^7 colony forming units (c.f.u.)/ml. At this concentration, the transformation frequency/c.f.u. was $2{\cdot}1 \times 10^{-3}$. At concentrations, the total number of transformants was not increased significantly. By reducing the concentration to 10 ^{6}c.f.u./ml., the number of transformants was reduced to 40% and the transformation frequency/c.f.u. was increased to $1{\cdot}3 \times 10^{-2}$.

The time course of the reaction between DNA and recipient organisms was followed over an 8 hr period. After the appropriate exposure time, DNase (5 μg./ml.) and $MgSO_4$ (0·005 M) were added to the transfer suspension to prevent any further reaction by transient DNA. Transformants began to appear after 1 hr exposure to DNA and continued to increase in frequency up to 6 hr. Transfer suspensions that were not treated with DNase produced essentially the same number of transformants over the total exposure period. The concentration of the transfer suspension did not increase during the first 2 hr of incubation. However, after this lag, the concentration increased 5-fold by 6 hr.

Table 5. *Survey of crosses of strains of Micrococcus lysodeikticus and Sarcina lutea*

	Recipient: Ade$^+$ clones/ml. transfer suspension*					
Donor	ATCC 4698 (*ade*)	ATCC 15801 (*ade*)	PU (*ade*)	UM (*ade*)	WRU (*ade*)	ISU (*ade*)
None	0	5	5	15	25	10
ATCC 4698 (*ade*)	0	—	—	—	—	940
ATCC 15801 (*ade*$^+$-*4*)	0	620 (770)	0	—	—	4,010 (2,260)
ATCC 15801 (*ade*)	—	5 (5)	—	—	—	420 (370)
CCM 1335 (*ade*$^+$)	—	—	—	—	—	7,940
PU (*ade*$^+$-*8*)	0	100	15	25	150	17,590
PU (*ade*)	—	—	5	—	—	780
UM (*ade*$^+$-*1*)	—	—	—	55	—	3,420
UM (*ade*)	—	—	—	10	—	470
WRU (*ade*$^+$-*1*)	—	—	—	—	(85)	—
WRU (*ade*)	—	—	—	—	(25)	—
ISU (*ade*$^+$-*1*)	—	—	—	—	—	(2,480)
ISU (*ade*)	—	(40)	—	—	—	(15)
Sarcina lutea						
ATCC 272 (*ade*$^+$)	0	105	25	20	210	16,470

* Transfer suspensions were prepared with heat-inactivated phage N6 lysates (without brackets) or with lysozyme lysates (brackets). Suspensions contained $1{\cdot}2$–$1{\cdot}7 \times 10^7$ recipient c.f.u./ml. Crosses that were not performed are indicated by symbol —. The number of clones is the average from duplicate plates.

Different Difco agars used in the preparation of the defined medium had a pronounced effect on the recovery of transformants. The three agars tested and their degrees of purity are as follows: Purified Agar > Special Agar-Noble > Bacto-Agar. The number of transformants was found to vary inversely with the degree of purity of the agar. For example, the transformation frequency/c.f.u. on Purified Agar was $4{\cdot}5 \times 10^{-6}$, on Special Agar-Noble, $2{\cdot}5 \times 10^{-3}$, and on Bacto-Agar, $6{\cdot}7 \times 10^{-2}$.

Survey of donor and recipient competence of strains. The ability of different strains of *Micrococcus lysodeikticus* to act as donors and recipients of an adenine marker was tested by a number of intra- and inter-strain crosses (Table 5). In addition, the related *Sarcina lutea* was tested as a donor. Crosses were initially made with phage-inactivated lysates, except when strains ISU and WRU were donors. Both of these strains were unable to propagate phage N6 to high titres and strain ISU was not lysed by this phage. In crosses with these donors, lysozyme lysates were substituted to prepare transfer suspensions. Control crosses using DNase-treated lysates failed to produce Ade$^+$ recombinants. All strains were competent donors of the adenine marker. The relative donor competence of strains must be taken with some caution as the amount of DNA in lysates was not determined. Significant recipient competence was demonstrated in strains ISU, ATCC 15801, and WRU; the competence of strains PU and UM was questionable in this experiment, with Special Agar-Noble in the plating medium. It was interesting to find that on Bacto-Agar all strains, except ATCC 4698, showed good recipient competence. A comparison of recipient competence on the two different agars is shown in Table 6. As was expected, the relative competence of strains was similar in crosses prepared with either lysates or purified DNA.

Table 6. *Recipient competence of Micrococcus lysodeikticus strains on different plating agars*

Recipient strain (*ade*)	Ade$^+$ clones/ml. transfer suspension*	
	Bacto-Agar	Special Agar-Noble
ATCC 4698	0	0
ATCC 15801	14,120	440
PU	1,740	10
UM	7,380	30
WRU	9,880	80
ISU	216,780	8,170

* Transfer suspensions were prepared with purified DNA (36 μg. in 0·5 ml. SSC + 0·5 ml. P broth) isolated from lysozyme-treated ATCC 15801 (*ade*$^+$-*4*) cells. Suspensions contained 3·0–3·4 × 10^6 recipient c.f.u./ml. The number of clones is the average from duplicate plates.

DISCUSSION

Transformation in *Micrococcus lysodeikticus* was not initially observed by the usual method of testing purified DNA, but was found unexpectedly with micrococcal phage lysates which were being screened for transducing activity. The role of free DNA in genetic transfer was implied by the absence of recombinants in crosses with phage N6 lysates treated with DNase. The phage N6 did not appear to be necessary for genetic transfer as inactivation by heat or specific antiserum failed to decrease the yield of recombinants. Conclusive evidence of transformation was obtained by the isolation of transforming DNA. The specific transforming activity of DNA isolated from lysozyme-treated cells was higher than with DNA isolated from phage lysates. However, it would be expected that the rather vigorous treatment used in the preparation of phage lysates could result in shearing of the highly polymerized DNA. Also it is uncertain to what extent propagation of phage may have influenced DNA activity.

Transformation mediated by a phage lysate is unusual, particularly with the high frequencies observed in this study. One explanation may be that *Micrococcus lysodeikticus* releases transforming DNA into the surrounding medium during phage-induced lysis without significant degradation. If the organism possesses active DNase(s), it probably is not active on the homologous DNA. Studies by Campbell, Evans, Perry & Niven (1961) have indicated that the extracellular DNase, produced by the related *Micrococcus sodonensis*, was inactive against the homologous DNA. Further studies would be required to correlate the observations in the two species.

Recombination between various adenine-dependent donor strains and the ISU adenine-dependent recipient suggests that the auxotrophs may carry mutations at different sites. That these mutations may be point mutations is implied from the ease with which adenine-independent mutants can be obtained from auxotrophic strains. By comparison, strain ATCC 4698 appears to have a deletion or several mutant sites, as adenine-independent mutants were not recovered in numerous attempts, and it could not be transformed with an adenine marker. It would be of interest to know the adenine phenotype of Fleming's original isolate before it was subjected to selective processes in different laboratories.

Genetic exchange between *Sarcina lutea* and *Micrococcus lysodeikticus* provides additional evidence of the close relationship of these organisms. Kocur & Martinec (1962) have suggested that both be classified as *Micrococcus luteus*. The transformation system described in this paper may be useful in testing the genetic compatability of different micrococci to further clarify taxonomic relationships.

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REFERENCES

BROWN, A. H. (1946). Determination of pentose in the presence of large quantities of glucose. *Archs. Biochem.* **11**, 269.

BURGI, E. (1955). *A study of abortive infection in a mutant strain of Micrococcus lysodeikticus infected with a bacteriophage*. Ph.D. thesis, Cornell University, New York.

CAMPBELL, J. N., EVANS, J. B., PERRY, J. J. & NIVEN, C. F., JUN. (1961). An extracellular material elaborated by *Micrococcus sodonensis*. *J. Bact.* **82**, 828.

DEMEREC, M. E., ADELBERG, E. A., CLARK, A. J. & HARTMAN, P. E. (1966). A proposal for a uniform nomenclature in bacterial genetics. *Genetics* **54**, 61.

DISCHE, Z. (1955). Color reactions of nucleic acid components. In *The Nucleic Acids*. Ed. by E. Chargaff and J. N. Davidson, vol. 1, p. 285. New York and London: Academic Press.

FEINER, R. R. (1967). Ultraviolet radiation-sensitive mutants of *Micrococcus lysodeikticus*. *J. Bact.* **94**, 1270.

FEINER, R. R., MEYER, K. & STEINBERG, A. (1946). Bacterial lysis by lysozyme. *J. Bact.* **52**, 375.

FLEMING, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc.* B **93**, 306.

FLEMING, A. & ALLISON, V. D. (1927). On the development of strains of bacteria resistant to lysozyme action and the relation of lysozyme action to intracellular digestion. *Br. J. exp. Path.* **8**, 214.

GRULA, E. A. (1962). A comparative study of six cultures of *Micrococcus lysodeikticus*. *Can. J. Microbiol.* **8**, 855.

KLOOS, W. E. (1968). Evidence of genetic exchange in *Micrococcus lysodeikticus*. *Bact. Proc.*, p. 55.

KOCUR, M. & MARTINEC, R. (1962). A taxonomic study of the genus *Micrococcus*. *Folia Fac. Sci. Natur. Univ. Purkynianae Brunensis* **3**, 1.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. molec. Biol.* **3**, 208.

MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. molec. Biol.* **5**, 109.

MEJBAUM, W. (1939). Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Z. physiol. Chem.* **258**, 117.

NAYLOR, H. B. & BURGI, E. (1956). Observations on abortive infection of *Micrococcus lysodeikticus* with bacteriophage. *Virology* **2**, 577.

OKUBO, S., NAKAYAMA, H., SEKIGUCHI, M. & TAKAGI, Y. (1967). A mutant of *Micrococcus lysodeikticus* defective in a deoxyribonuclease activity specific for ultraviolet-irradiated DNA. *Biochem. biophys. Res. Commun.* **27**, 224.

ROSYPALOVA, A., BOHACEK, J. & ROSYPAL, S. (1966). Deoxyribonucleic acid base composition of some micrococci and sarcinae. *Antonie van Leeuwenhoek* **32**, 192.

SAITO, H. & MIURA, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochem. biophys. Acta* **72**, 619.

STECHSCHULTE, A. L. (1961). Isolation of biochemical and chromogenic mutants of *Micrococcus lysodeikticus* using lysozyme as the selective agent. *Biol. Ser. Cath. Univ. Am.* No. 68.

SWANSTROM, M. & ADAMS, M. H. (1951). Agar layer method of production of high titre phage stocks. *Proc. Soc. expt. Biol. Med.* **78**, 372.

WOLIN, H. L. & NAYLOR, H. B. (1957). Basic nutritional requirements of *Micrococcus lysodeikticus*. *J. Bact.* **74**, 163.

Note added in proof

It has been brought to our attention that Chakrabarti & Litwack (*Bact. Proc.*, p. 75, 1960) may have had an indication of a transformation process in *M. lysodeikticus* by their apparent transfer of lysozyme resistance to lysozyme sensitive cultures. Recently workers in two other laboratories (Mahler & Grossman; Okubo & Nakayama) have independently conducted preliminary studies of transformation of ultra-violet radiation resistance markers in *M. lysodeikticus*.