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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

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

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

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

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

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

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

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

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

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

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

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

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

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

The following books may be found useful:

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

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

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

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

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

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A Study of the Esterases and Their Function in *Candida lipolytica*, *Aspergillus niger* and a Yeast-like Fungus

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(*Accepted for publication 24 June 1970*)

SUMMARY

Esterases, determined by polyacrylamide gel electrophoresis, were present when *Candida lipolytica* was grown in a liquid, shaken, glucose–mineral salts medium. Intracellular esterase activity increased during growth, but extracellular esterase activity was small and increased only marginally. Esterases were not detected in organisms grown on solid glucose–mineral salts medium, but were present when glycerol tributyrin replaced glucose.

At the onset of asexual sporulation in a yeast-like fungus, three new esterases occurred. Intracellular esterase activity increased, intracellular lipid utilization occurred, and the respiratory quotient decreased. No extracellular esterase activity was detected.

Esterases were only detected in *Aspergillus niger* at late stages of conidiation when intracellular lipid decreased. Esterase activity was not detected in the mitochondria or in the cell-free growth medium.

Esterases of all organisms tested hydrolysed glycerol tributyrin and were arbitrarily classified as lipases; intracellular lipid decreased with increase in esterase activity function. Esterase and profile changes may reflect a role of lipids in sporulation and physiological ageing.

INTRODUCTION

Gel electrophoresis has been shown by several workers to be a suitable technique for the study of esterases, and in some cases the esterase profile has been found to consist of a group of molecular species recognizable by their common enzymic activity on a 'general' ester substrate (Arnason & Pantelouris, 1966; Robinson, 1966). The esterases comprise a family of enzymes exhibiting a very broad substrate specificity, and consequently a classification of esterases based on specificity is of little value. However, it is customary to distinguish lipases as a special class, defined by the International Union of Biochemistry (1961) as a glycerol ester hydrolase, and it is recommended that glycerol ester emulsions should be used as substrates. However, the comments of Lawrence, Fryer & Reiter (1967*a*) on bacterial lipases may apply equally to fungal lipases; that is since nothing is known of their natural substrates or their physiological role the choice of any substrate to detect these lipases must be arbitrary.

A study of the esterase profiles from various stages of the growth cycle of *Bacillus cereus* has revealed a changing pattern of enzymes (Baillie & Norris, 1963). A single esterase band was demonstrated in young cultures and the development of a second band was noted after 11 h. growth.

As the physiological roles of fungal esterases have not been fully elucidated, a study

of these enzymes in three fungi was undertaken. This paper reports and discusses the role of esterases in the vegetative growth of *Candida lipolytica*, and in asexual sporulation in *Aspergillus niger* and an unidentified yeast-like fungus; their ability to hydrolyse glycerol tributyrin was also investigated.

METHODS

Organisms. (1) *Candida lipolytica* was isolated by Ross (1963); (2) an unidentified yeast-like fungus, referred to as isolate 48, was isolated from a marine source (Lloyd, 1970) and produces arthrospores at the end of the growth cycle; and (3) *Aspergillus niger* van Tieghem (IMI 41873). Stock cultures of (1) and (2) were maintained on malt extract–yeast extract–glucose–peptone (MYGP) slopes at 3° and (3) on potato–dextrose agar at 27°.

Media. For *Candida lipolytica* the glucose–mineral medium contained $(NH_4)_2SO_4$, 5·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 0·1 g.; $CaCl_2.2H_2O$, 0·1 g.; glucose, 10·0 g./l. dist. H_2O. To examine the influence of the carbon source on esterase production, the above mineral medium was solidified with 2% (w/v) agar (Oxoid no. 2) and either 1% (w/v) glucose or 1% (v/v) glycerol tributyrin (B.D.H.) was added.

For isolate 48, arthrospore production was induced in MYGP medium; this contained malt extract, 3·0 g.; yeast extract, 3·0 g.; glucose, 10·0 g.; peptone, 5·0 g./l. dist. H_2O. One ml. of a 50% aqueous solution of silicon MS antifoam emulsion RD (Hopkins & Williams Ltd, Freshwater Road, Chadwell Heath, Essex) was added/500 ml. MYGP.

For *Aspergillus niger*, the basal-mineral medium used in the conidiation studies, referred to as 'B' medium, contained NH_4NO_3, 2·5 g.; KH_2PO_4, 1·0 g.; $CaCl_2$, 46·7 mg.; $MgSO_4.7H_2O$, 1·1 mg.; $MnCl_2.4H_2O$, 3·5 mg.; $CuSO_4.2H_2O$, 0·234 mg.; $FeSO_4.7H_2O$, 6·32 mg.; $ZnSO_4.7H_2O$, 1·1 mg./l. dist. H_2O. Conidiating cultures of *A. niger* were obtained on (i) 'B' medium supplemented with (*a*) 20 mM-sodium α-oxoglutarate, or (*b*) 20 mM-fumaric acid, or (*c*) 20 mM-glutamic acid, or (*d*) 20 mM-glycine, or (*e*) 20 mM-leucine, and (ii) $NaNO_3$ medium. The latter was identical to 'B' medium except that 60 mM-$NaNO_3$ replaced NH_4NO_3. Sterile cultures were grown on unsupplemented 'B' medium (Galbraith & Smith, 1969*a*).

The media for *Candida lipolytica* and isolate 48 were adjusted to pH 5·5 and for *Aspergillus niger* to pH 5·0. All media were autoclaved for 15 min. at 121° except for glycerol tributyrin–mineral salts agar; in which case the glycerol tributyrin was sterilized separately by dry heat at 160° for 2 h. and was then emulsified, aseptically, with the autoclaved mineral salts–agar in a blender for 3 min.

Turbidity measurements. All turbidity measurements were referred to water using the logarithmic scale on an EEL nephelometer with a neutral density filter.

Preparation of inocula. Liquid cultures of *Candida lipolytica* were grown in conical 1 l. flasks containing 500 ml. of medium. Surface cultures were grown in squat 500 ml. conical flasks containing 150 ml. of agar medium. Liquid cultures were inoculated with 1 ml., and solid media with 2 ml., of a fungal suspension of turbidity 20 prepared from a 24 h. MYGP slope culture grown at 25°.

Cultures of isolate 48 were grown in 1 l. conical flasks containing 500 ml. of medium; each flask was inoculated with 1 ml. of an arthrospore suspension of turbidity 20 prepared from a 7 day MYGP slope culture grown at 20°.

Cultures of *Aspergillus niger* were grown in 250 ml. conical flasks containing 75 ml. of medium, and each flask was inoculated with approximately 8×10^6 conidia.

Incubation. Liquid cultures of the three organisms were incubated on an orbital shaker (Gallenkamp) at 150 rev./min. at the following temperatures: *Candida lipolytica*, 25°; isolate 48, 20°; *Aspergillus niger*, 27°. Samples were harvested throughout the growth cycle. Agar cultures of *C. lipolytica* were harvested after 5 to 7 days growth at 25°.

Growth measurement. A turbidimetric method was used to estimate the amount of growth of *Candida lipolytica* and isolate 48 in liquid media because it was a simple method showing close linear correlation with dry wt determinations under the condition of growth employed for these studies. Isolate 48 and *Aspergillus niger* were examined microscopically for signs of sporulation.

Preparation of cell-free extracts. Candida lipolytica and isolate 48 were harvested by centrifugation, washed three times and resuspended in distilled water. The concentration of organisms in the suspensions was determined by diluting a sample of suspension to give a turbidity reading of 80 units and then adjusting the concentration of the suspension to equate to 1600 units. The organisms were disintegrated for 5 min. by an M.S.E. ultrasonicator (100 W output), the cups being immersed in solid CO_2+ water. The extracts were centrifuged and the supernatant fluid stored at −20°. Extracts of mycelial pellets of *Aspergillus niger* were prepared by sonication of 2 g. of filter paper dried mycelium which had been ground for a short period in 5 ml. distilled water using a mortar and pestle. Samples of all extracts were freeze dried for 18 h. and reconstituted for electrophoresis in a minimum quantity of 0·05 M-phosphate buffer, pH 8·0.

Electrophoresis. The cell-free extracts were analysed by electrophoresis in polyacrylamide gels by the method of Lund (1965) in a refrigerated room (5 to 10°) at constant voltage (13·75 V/cm.gel). The initial current was 20 mA.

Detection of esterases. After electrophoresis the gels were sliced to four layers 1·0 mm. thick. The middle two slices were stained for esterases by a method adapted from that of Nachlas & Seligman (1949). The slices were incubated for 2 h. in a freshly prepared mixture of 0·1 M-tris-maleate buffer (pH 6·4), 100 ml., + 1% (w/v) α-naphthyl acetate in 50% (v/v) acetone in water, 2 ml., + fast blue B salt (Gurr) *ca.* 0·2 g. Esterase activity was indicated by red bands and the position of the mid-point of each band was measured from the origin.

Quantitative esterase activity determination. Esterase activity was estimated by the method of Hobson & Summers (1966) using: (1) intact *Candida lipolytica* and isolate 48; (2) the following preparations of *Aspergillus niger* grown on 'B' medium + sodium α-oxoglutarate: (*a*) mycelial extracts, and (*b*) mitochondrial suspensions prepared according to the method of Watson & Smith (1967); and (3) cell-free growth medium of all three organisms. The buffered-substrate solution consisted of 10 mg. of α-naphthyl acetate dissolved in 2 ml. acetone and 98 ml. 0·1 M-tris-maleate buffer, pH 5·4. The reaction mixture consisted of 6 ml. buffered-substrate solution and 1 ml. of a suspension of washed organisms of turbidity 80 in distilled water, or 1 ml. of mycelial extract, or 1 ml. of cell-free growth medium. After 20 min. incubation at 20, 25 and 27° for isolate 48, *C. lipolytica* and *A. niger* respectively, 1 ml. of fast blue B solution was added with 1 ml. of 40% (w/v) trichloracetic acid aqueous solution to facilitate extraction of the azo dye from the protein-containing solutions. The complex

formed by the naphthol, released from the hydrolysed substrate, with the dye, was extracted in ethyl acetate. The extinction rates of the extracts were measured in a spectrophotometer at 540 nm. The extinction readings were converted to mg. of α-naphthol liberated using a calibration curve obtained with α-naphthol. The results are expressed as mg. α-naphthol liberated/mg. dry wt organism for *C. lipolytica* and isolate 48, as mg. of α-naphthol liberated/mg. protein for *A. niger* mycelial extracts and mitochondrial suspensions, and as mg. α-naphthol liberated/ml. of cell-free growth medium. The dry wt of cell material used was determined by drying 5 ml. of each suspension to a constant weight in an oven at 100°.

Assay of esterase bands for lipase activity. After electrophoresis, one slice was removed from the top of the gel and the esterases detected. The stained slice was washed with water, replaced in its original position on the top of the gel and the underlying esterase-containing gel excised. These unstained sections were pulverized in about 1 ml. of water and tested for lipolytic activity against glycerol tributyrin using the thin-layer agar diffusion method described by Lawrence, Fryer & Reiter (1967*b*).

Manometric determinations. Gas exchanges by *Candida lipolytica* and isolate 48 were measured using 2 ml. of fresh culture. Oxygen uptake and carbon dioxide evolution were measured by standard Warburg techniques (Umbreit, Burris & Stauffer, 1964).

Lipid determinations. Lipid contents were determined by the method of Pedersen (1962).

RESULTS

Candida lipolytica. Esterase profiles from organisms in early and mid-exponential and early stationary phases of growth in liquid glucose–mineral salts medium all contained the same number of esterase bands (Fig. 1). In the profile of organisms from the early exponential growth phase all the bands stained with approximately the same intensity, whereas in the profiles of organisms from early stationary growth phase bands 44 and 66 showed the greatest size and staining intensity. The esterase activity of intact organisms increased throughout the growth cycle but was negligible in the growth medium over the same period (Fig. 3). The respiratory quotient decreased at the end of the growth cycle to a value indicating lipid utilization (Fig. 3).

No esterases were detected in cell-free extracts of *Candida lipolytica* grown for 5 to 7 days on the solid media when glucose was the sole carbon source; however, when glycerol tributyrin was substituted for glucose an esterase profile was demonstrated in which band 66 stained very heavily (Fig. 1).

Isolate 48. A microscopic examination of this organism in MYGP broth showed that differentiation of vegetative cells into arthrospores occurred at approximately 72 h. and was complete after 145 h. Electrophoretic analysis of esterase preparations from organisms at different stages of the growth cycle revealed a changing esterase profile (Fig. 2). The major esterases present after 24 h. growth, bands 27, 51 and 57, became less prominent as growth proceeded. At the onset of sporulation (72 h.) they were eclipsed by two heavily staining esterases, bands 59 and 67. The increase in esterase activity between 48 and 96 h. (Fig. 4) probably reflects the appearance of these two bands. Esterase activity was not measured beyond 96 h. because the arthrospores were inert towards the ester substrate although extracts contained esterases. At 96 h. approximately 20 to 30% of the cells were arthrospores and, therefore, the reported esterase activity is lower than would have been obtained with a suspension

of only vegetative cells. No extracellular activity was noted. Another change which occurred at the onset of sporulation (72 h.) was a sharp increase in lipid content followed by a decrease during sporulation. A fall in the respiratory quotient from approximately 1·0 at 72 h. to 0·748 at 96 h. was consistent with decrease of the lipid content of the organism.

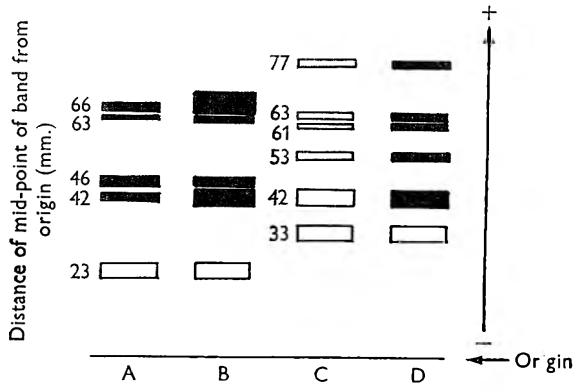

Fig. 1. Diagram of esterase profiles obtained by electrophoresis on polyacrylamide gel of extracts of *Candida lipolytica* and *Aspergillus niger*. (A) *C. lipolytica* from early exponential phase of growth in liquid glucose–mineral salts medium. (B) *C. lipolytica* from late exponential/early stationary phase of growth both in liquid glucose–mineral salts medium and on solid glycerol tributyrin–mineral salts medium. (C) *A. niger* from 48 h. growth, and (D) from 72 h. and 96 h. growth in liquid medium M + α-oxoglutarate. NB. No esterases were detected in *A. niger* after 24 h. growth in liquid medium M + α-oxoglutarate. □, Weak esterase activity; ■, intense esterase activity.

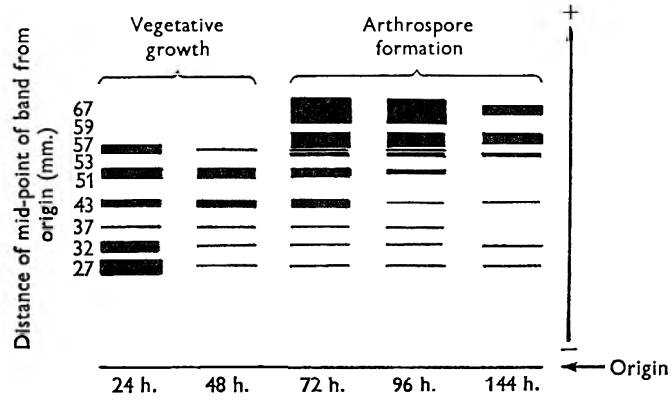

Fig. 2. Diagram of esterase profiles obtained by electrophoresis of extracts of isolate 48, on polyacrylamide gels, from different stages of the growth cycle on MYGP medium.

Aspergillus niger. Esterases were always detected during conidiation but never in vegetative growth in conidiating media nor in sterile cultures. Esterase profiles obtained from different stages of growth of *A. niger* in 'B' medium + α-oxoglutarate show that a weakly staining multimolecular profile appeared at the onset of conidiophore formation and an intensely staining profile appeared during conidia formation (Fig. 1). This change in the staining intensity is reflected in the increase in esterase activity (Fig. 5). Esterase activity was absent both from mitochondrial suspensions and from

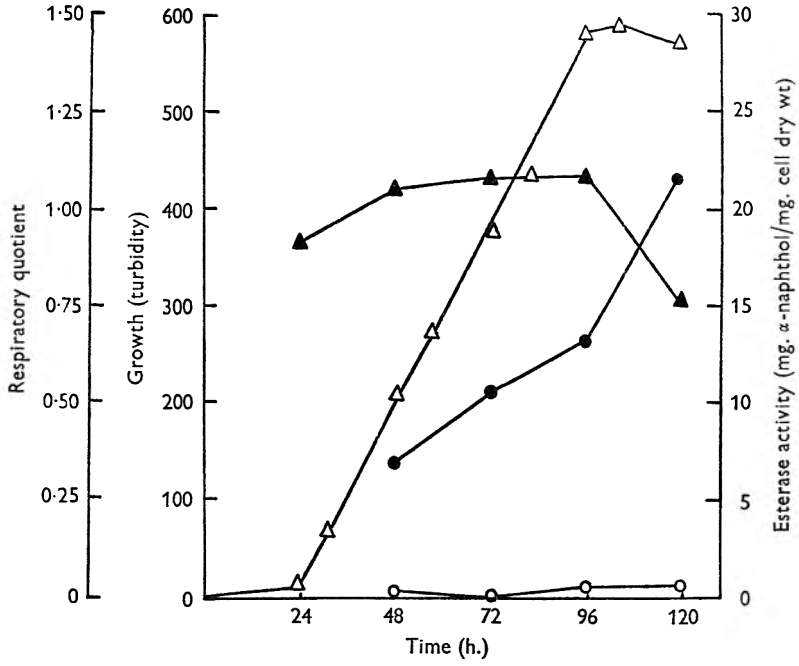

Fig. 3. Comparison of esterase activity, respiratory quotient, and growth of *Candida lipolytica* grown in liquid glucose–mineral salts medium. △——△, Growth measured as turbidity units in EEL nephelometer; ▲——▲, respiratory quotient; ●——●, esterase activity of yeast; ○——○, esterase activity of medium freed of yeast.

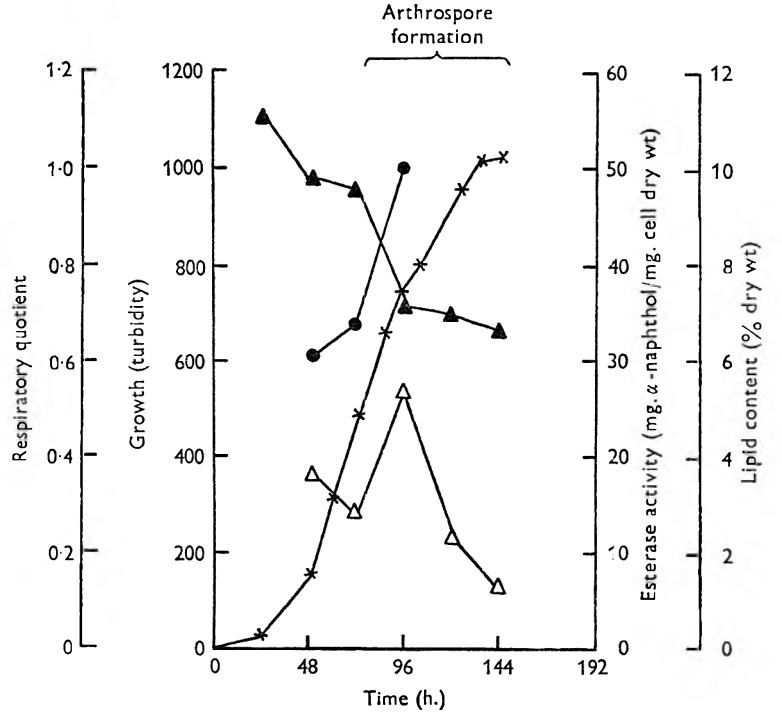

Fig. 4. Comparison of esterase activity, respiratory quotient, growth and lipid content of isolate 48 grown in MYGP medium. ×——×, Growth as measured by turbidity units; ▲——▲, respiratory quotient; ●——●, esterase activity of organism; △——△, lipid content of organism.

the medium throughout the growth period. Esterase profiles from conidiating cultures in the other media were similar to those shown in Fig. 1; the two major bands in all profiles were 42 and 53. All esterases of the three fungi hydrolysed glycerol tributyrin.

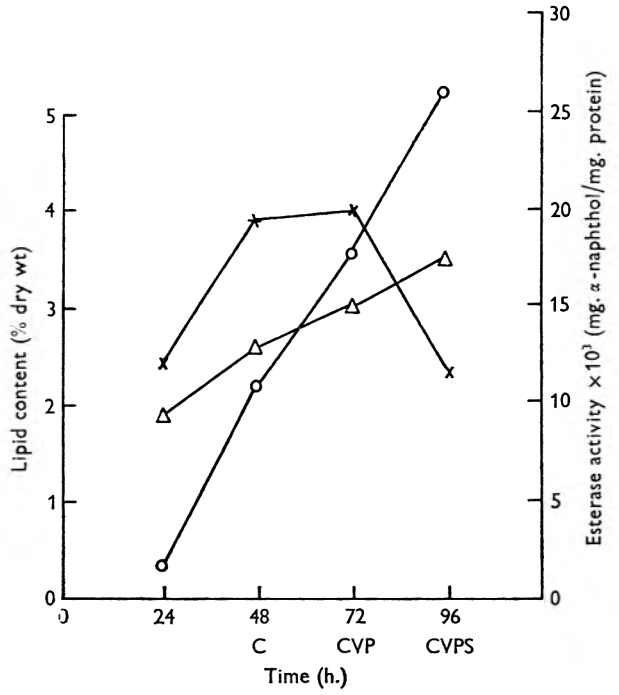

Fig. 5. Comparison of esterase activity and lipid content of *Aspergillus niger* in medium M or medium M+α-oxoglutarate. C = Conidiophores; V = vesicles; P = phiallides; S = spores. O——O, Esterase activity of organism grown in medium M+α-oxoglutarate (no esterase activity from organism grown on medium M); ×——×, lipid content of organism grown in medium M+α-oxoglutarate; △——△, lipid content of organism grown in medium M.

DISCUSSION

Cell-free extracts from *Candida lipolytica* grown in liquid medium and examined by electrophoresis contained five distinct esterase bands. Esterase profiles of the organism from different stages of the growth cycle revealed the same five enzymes. In the early stationary phase profile the relative increase in size and intensity of bands 42 and 66 probably reflects the increase in esterase activity which occurred throughout the growth cycle. The failure to demonstrate esterase profiles in extract of *C. lipolytica* grown on solid glucose medium is unexplained.

Three observations suggest that the esterases of *Candida lipolytica* may be involved in the metabolism of intracellular and extracellularly added lipids. The first is that all the excised esterases of *C. lipolytica* hydrolysed glycerol tributyrin and can be classified as lipases according to the arbitrary definition of Lawrence (1967*a*) that lipases attack triglycerides. The second is that the natural substrate of the esterases may be lipid as they are induced when *C. lipolytica* is grown on solid glycerol tributyrin medium. The third is the finding that the very small increase in extracellular esterase activity did not parallel the large increase in intracellular activity. This indicates that

some, if not all, of the esterases are situated on or in the organism. Support for this is provided by Peters & Nelson (1948), who were unable to detect any extracellular esterase activity in *C. lipolytica*. The validity of the suggestion for the physiological role of the esterases in *C. lipolytica* is supported by the work of Nyns, Chiang & Wiaux (1968). Using similar growth conditions and liquid medium as in this paper, they showed that *C. lipolytica* contained twice as much lipid in the exponential phase as in the stationary phase of growth. The decrease in lipid was attributed to its consumption in the late phases of growth.

Esterase profiles of isolate 48 indicate that some of these enzymes may have an important function at a particular phase in its life cycle because a significant change in the profile occurred at the onset of arthrospore formation with the appearance of bands 55, 59 and 67. The appearance of these esterase bands coincided with the rise in esterase activity. The ability of the excised esterases to hydrolyse tributyrin implies that they may be involved in intracellular lipid utilization, particularly during arthrospore production. The extent of the contribution of intracellular lipid to the metabolism of sporulation can be judged from the large decrease in lipid content which occurs during the process. The rapid accumulation of lipid which occurs at the end of vegetative growth may indicate the point of cessation of the phase of balanced growth due to the exhaustion of some particular nutrient component such as N or P, which, according to Bu'Lock & Powell (1965), may lead to secondary metabolism, an example of which is fat synthesis in yeasts in the absence of assimilable N. Events which terminate balanced growth in fungi, e.g. substrate limitation such as the limitation of assimilable nitrogen (Morton, 1967), also act as morphogenetic stimuli. Thus by extrapolation of the original observation of the change in esterase profile and quantitative esterase activity the following sequence of events leading to differentiation can be visualized. The termination of balanced growth leads to an accumulation of intracellular lipid which is rapidly followed by the induction of differentiation for which process the lipid provides an endogenous carbon and energy source. Although there is no direct evidence that esterase band 27, which is very prominent in the 24 h. profile, is involved in germination of the arthrospores, this possibility cannot be excluded as lipid metabolism has been shown to be predominant in the germination of *Penicillium roqueforti* conidia (Lawrence, 1967*b*) and in stem rust uredospores (Farkas & Ledingham, 1959).

In *Aspergillus niger*, lipolytic esterases are always present during conidiation irrespective of the mode of induction of the latter. Furthermore, esterases have neither been detected in vegetative mycelium from these cultures nor throughout the growth of sterile cultures. From the electrophoresis studies, the subjective impression is that the greatest esterase activity occurred at the time of conidia production and not during conidiophore development. This impression was confirmed by measurements of esterase activity on *A. niger* grown in 'B' medium + α-oxoglutarate. In this particular case the change in the level of lipolytic esterase activity and the intracellular lipid consumption during differentiation appear to be interrelated. If this is so, the implication is that the lipid functions as a source of carbon and energy during conidiation. Such a function for lipid reserves occurs in other organisms, e.g. during the differentiation of amoebae to cysts, the lipid of the vegetative organism is converted via the glyoxylate cycle to cyst-wall cellulose (Tomlinson, 1967). An active glyoxylate cycle has also been shown to occur during differentiation in *A. niger* (Galbraith & Smith, 1969*b*). This cycle may be metabolically co-ordinated with lipid utilization and act

as an anaplerotic pathway or, as suggested by Galbraith & Smith, may provide a source of glyoxylate which could be transaminated to glycine which may act as a purine precursor in the nucleic acid synthesis necessary for transcription of genes active in differentiation.

In postulating a probable correlation between esterase production and conidiation in *Aspergillus niger*, it is necessary to consider the suggestion by Mandelstam (1969), that before any correlation can be regarded as being established it is necessary to show that an event is invariably correlated with a particular morphological stage. The present study has shown that esterase production is always associated with conidiation irrespective of the type of sporulation medium, and initial studies by Craig, Lloyd & Smith (unpublished material) have shown that by using a replacement medium to induce conidiation (J. G. Anderson & J. E. Smith, personal communication), esterases are produced during conidiation irrespective of the previous growth medium. Although a correlation does not establish a causal relationship, such a relationship is implicit in the suggestion that differentiation in isolate 48 and *A. niger* is assisted by or even dependent upon the utilization of stored lipid which can provide an endogenous carbon and energy source. This interpretation is consistent with the hypothesis of Wright (1967, 1970), that the characteristic common to most, if not all, forms of morphogenesis is that they are essentially endogenous, self-sufficient systems which have the ability to use endogenous reserves both extensively and efficiently. Wright (1970) also considers that each differentiating system starts with a fixed amount of endogenous reserve material which is used in an orderly and sequential manner and that the changes in endogenous metabolism alter the composition of small molecules or populations of inducers essential for gene activation.

Experiments in progress, using different techniques to obtain cell-free extracts, suggest that the multimolecularity of the esterase profile in *Aspergillus niger* is not an artefact. The importance of this enzyme heterogeneity may lie in its ability to control lipid metabolism during differentiation. Each esterase could, for example, be subject to regulation by a different end-product or each may be associated with a different lipid pool, each pool being used at one unique stage in differentiation; these control mechanisms may even be the pacemakers of differentiation itself.

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Two Mutations Affecting Utilization of C_4-Dicarboxylic Acids by *Escherichia coli*

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SUMMARY

Studies with a mutant of *Escherichia coli* reported to be deficient in phospho*enol*pyruvate carboxykinase activity (AB257^{suc-}) indicate that it possesses two mutations. One affects growth on succinate (S^-), and the other primarily affects growth on fumarate and malate and affects growth on succinate to a lesser extent (FM^-). Neither of these mutations has a direct effect on carboxykinase activity. Presence of the S^- mutation also prevents growth on acetate. It delays growth on other substrates such as lactate, glycerol and malate and it specifically inhibits the oxidation of succinate by mutant suspensions. The only biochemical lesion which could be detected was a deficiency in succinate dehydrogenase and it is thought that the S^- locus may be situated in the structural gene for this enzyme. Transduction with phage P1 indicates linkages between S^- and *gltA* and *sucA* of 97 and 90%, respectively and the gene order *S*...*sucA*...*gal* was established. The FM^- mutation was also found in the parental strain AB257. It affects the oxidation of succinate, fumarate and malate by mutant suspensions. The uptake of the dicarboxylic acids is also impaired but no other biochemical lesion could be detected. Genetic studies indicate that the FM^- mutation is located near *xyl*.

INTRODUCTION

Several mutations specifically affecting the ability of *Escherichia coli* to utilize C_4-dicarboxylic acids such as succinate, fumarate or malate as sole carbon and energy sources have been described. A mutant lacking succinate dehydrogenase is capable of growth on fumarate but not on succinate (Hirsch, Rasminsky, Davis & Lin, 1963). Several mutants blocked in the biosynthesis of ubiquinone (*ubi*$^-$) are unable to grow on malate or succinate (Cox, Gibson & Pittard, 1968; Cox, Young, McCann & Gibson, 1969). Mutants with a defective C_4-dicarboxylic acid transport system (*dct*$^-$) have been isolated by virtue of their resistance to 3-fluoromalate. These are unable to grow on malate or fumarate and their ability to grow on succinate is markedly impaired (Kay & Kornberg, 1969). Mutants which are virtually devoid of phospho*enol*pyruvate carboxykinase (Kornberg, 1965) or possess abnormally low carboxykinase activity (Hsie & Rickenberg, 1966) are also unable to grow on intermediates of the tricarboxylic acid cycle, presumably due to inadequate provision of phospho*enol*pyruvate for biosynthetic purposes.

Carrillo-Castaneda & Ortega (1967) have reported the isolation of a phospho*enol*pyruvate carboxykinase-less strain of *Salmonella typhimurium*, apparently the result of a deletion which also imposes requirements for nicotinic acid and lysine + methionine.

This indicated that the *pck* and *lys*+*met* loci are close. In view of the high degree of homology between the linkage maps of *Escherichia coli* and *S. typhimurium* (Sanderson, 1967), evidence for the same proximity in *E. coli* was sought during genetic studies with two types of lysine+methionine-requiring mutant: *lip*$^-$, blocked in lipoate biosynthesis, and *suc*$^-$, lacking α-ketoglutarate dehydrogenase (Herbert & Guest, 1968). The phospho*enol*pyruvate carboxykinase-deficient mutant of Hsie & Rickenberg, AB257^{suc-}, was therefore examined. This strain was selected for its ability to utilize glycerol but not succinate after treatment of the parent strain AB257 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. It grew on a variety of hexoses and trioses but not on intermediates of the tricarboxylic acid cycle; however, in its uptake of succinate the mutant was comparable with the parental strain. Our genetic studies indicate that AB257^{suc-} possesses at least two mutations, one affecting succinate utilization (*S*$^-$) and another which primarily affects growth on fumarate and malate (*FM*$^-$). The latter mutation is unstable and it is also present in the parental strain AB257.

METHODS

Organisms. Escherichia coli strain AB257 and its derivative AB257^{suc-}, which is unable to grow on succinate, were kindly provided by Dr H. V. Rickenberg. Both strains were *metB*$^-$ and although Hfr activity could not be detected, a weak polarized donor activity remained. To avoid confusion in the use of *suc*$^-$ to describe the requirement for succinate (e.g. in mutants lacking α-ketoglutarate dehydrogenase) and inability to utilize succinate, and because the parent strain proved to be unable to grow on fumarate and malate, these strains will be designated AB257 *FM*$^-$ and AB257 *FM*$^-$, *S*$^-$. A revertant of the former which grows on fumarate and malate will be called AB257 *FM*$^+$. The characteristics of other strains of *E. coli* K12 used are as follows: AB1325 (*F*$^-$, *proA*$^-$, *lacY*$^-$, *gal*$^-$, *purB*$^-$, *his*$^-$, *str*$^-$, *mtl*$^-$, *xyl*$^-$, *thi*$^-$); XG3 *lip*32 (*F*$^-$, *leu*$^-$, *ara*$^-$, *lac*$^-$, *proC*$^-$, *tsx*$^-$, *purE*$^-$, *lip*$^-$, *trp*$^-$, *str*$^-$, *xyl*$^-$, *thi*$^-$); W620 (*F*$^-$, *gltA*$^-$, *galK*$^-$, *ura*$^-$, *str*$^-$); W1485 (*F*$^+$); HfrH (*Hfr*: *o-thr-pro-gal*. . . *F*); P10 (*Hfr*, *thr*$^-$, *leu*$^-$, *lacY*$^-$, *thi*$^-$, *malA*$^-$, λ^r, $T_{1.5}{}^r$: *o-thi-metA-ilv*. . . *F*) and P13 (*Hfr*, *his*$^-$, *cys*$^-$, *thi*$^-$, *gal*$^-$, $T_{1.3}{}^r$: *o-ilv-met-thi*. . . *F*). The origins of several derivatives of these strains, AB1325 *lip*13 (*lip*$^-$), AB1325 *suc*1 (*sucA*$^-$) and W1485 *suc*1 (*sucA*$^-$) have been described previously (Herbert & Guest, 1968). In addition, W1485 *S*$^-$ was isolated by P1 transduction between AB257 *FM*$^-$, *S*$^-$ and W1485 *gal*$^-$ and selection for *gal*$^+$; the isolation of *FM*$^-$ and *S*$^-$ derivatives of AB1325 is described in the Results section. Phage P1 *kc* was originally obtained from Dr C. Yanofsky and T_6 was kindly provided by Dr B. A. Fry.

Media. The minimal medium used throughout contained (/l.): $NaH_2PO_4.2H_2O$, 6·08 g.; K_2HPO_4, 10·6 g.; $(NH_4)_2SO_4$, 2 g. and 5 ml. of a salts solution which in turn contained (g./l.): $MgSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 1; $FeSO_4.7H_2O$, 0·05; $CaCl_2$, 0·1 and a trace of conc. HCl to clarify. Carbon sources were added to a final concentration of 50 mM for acetate, succinate, DL-malate, fumarate, lactate, glycerol, 0·5 % for galactose, mannitol, xylose and 0·2 % for glucose. Media were supplemented with vitamins and amino acids according to the particular requirements of the bacteria to be grown or selected. The complex medium was L-broth (Lennox, 1955) and bacterial stocks were maintained on plates of L-agar. All incubations were at 37°.

Genetic techniques. Mixtures of log phase cultures containing 2×10^7 donor and 2 to 4×10^8 recipient bacteria/ml. were used in interrupted mating experiments. The

methods of Pittard & Wallace (1966) and Herbert & Guest (1968) were used when exconjugant donor organisms were to be killed with phage T_6 or streptomycin respectively. Transduction with phage P1 *kc* was according to Guest (1969). For estimating linkages, recombinant colonies were picked, diluted and purified by single colony isolation on the medium used for their selection, prior to scoring the inheritance of non-selective markers.

Growth tests. Growth was followed in L-tubes rocked in a water bath at 37° according to Herbert & Guest (1968). Turbidities were measured with an EEL colorimeter fitted with a neutral density filter; an EEL reading of ten was equivalent to 0·3 mg. dry wt/ml. All cultures which grew were tested for reversion.

Oxygen consumption. The oxidation of several substrates was recorded polarographically using a simple oxygen electrode in a reaction vessel of 1 ml. capacity (Estabrook, 1967). Organisms were grown in glucose minimal medium, harvested late in the log phase, washed twice and resuspended at 1 mg. dry wt/ml. in potassium phosphate buffer (0·04 M, pH 7·0). Substrate oxidation is expressed as μl. O_2 consumed/mg. dry wt/h. at 25° after subtracting the endogenous rate.

Enzyme assays. Unless otherwise stated, cell-free extracts were prepared from organisms harvested from minimal medium in late log phase, washed twice and resuspended in potassium phosphate buffer (0·04 M; pH 7·0) at 50 mg. dry wt/ml. These suspensions were disrupted with an ultrasonic cell disintegrator (M.S.E., 100 W) for 4 min. at 0°. Cell debris was removed by centrifuging at 23,000*g* for 10 min. Protein was estimated according to Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard. All spectrophotometric assays were carried out in 1 cm. light-path cuvettes at 25° with a Beckman DB spectrophotometer. The results are expressed as μmoles substrate transformed/mg. protein/h.

Succinate dehydrogenase (EC.1.3.99.1) was measured according to Arrigoni & Singer (1962). The following reactants were contained in 3·0 ml.: potassium phosphate buffer, pH 7·4, 300 μmoles; KCN (freshly neutralized with HCl), 10 μmoles; 2,6-dichlorophenol indophenol (DCPIP), 0·2 mg.; *N*-methylphenazonium methosulphate, 2 mg.; sodium succinate, 20 μmoles and protein up to 1·5 mg. The reaction was started with succinate and DCPIP reduction was followed at 600 nm. using a control which lacked succinate. The oxidation of succinate involves the reduction of two molecules of DCPIP and millimolar extinction coefficient of 21 was used for DCPIP.

Fumarase. (EC.4.2.1.2) was measured by the method of Racker (1950). For this assay extracts were centrifuged at 100,000*g* for 1 h. and the reaction mixture (3 ml.) contained: potassium phosphate buffer pH 7·3, 100 μmoles, and protein 0·5 mg. The reaction was started with sodium L-malate, 50 μmoles and fumarate formation was assessed at 240 nm. using a milliomolar extinction coefficient of 2·44 for fumarate.

Malate dehydrogenase (EC.1.1.1.37) was assayed according to Ochoa (1955). The reaction mixture contained in 3·0 ml.: potassium phosphate buffer, pH 7·4, 75 μmoles; NADH, 0·15 μmoles; protein, 10 μg. and reaction was started with 0·76 μmole of freshly prepared oxalacetate. The decrease in extinction at 340 nm. was compared with that of a control lacking oxalacetate.

Malic enzymes. The NAD- and NADP-linked malic enzymes (EC.1.1.1.38 and EC.1.1.1.40) were assayed in extracts prepared in 0·05 M-tris-HCl buffer, pH 7·9, containing 10 mM-$MgSO_4$ and centrifuged for 20 min. at 24,000*g* and 90 min. at 100,000*g* by the methods of Takeo (1969).

Phosphoenolpyruvate carboxykinase was assayed by a modification of the method of Bridgeland (1968) using organisms grown with succinate or fumarate as substrate. The reaction mixture contained (μmole in 1 ml.): tris-maleate-NaOH buffer (equimolar tris and maleic acid; pH 7·5), 5; phospho*enol*pyruvate, 5; ADP, 5; $MnCl_2$, 5; potassium L-glutamate, 10; $NaH^{14}CO_3$ (0·19 μCi/μmole), 15; glutamate-oxalacetate transaminase, 3·6 units and extract containing 0·5 mg. protein. Incubation was at 30° and at 3 min. intervals, samples (0·1 ml.) were pipetted into 0·9 ml. formic acid (0·1 M) in scintillation vials and left overnight at room temperature to remove dissolved $^{14}CO_2$. Then 12 ml. of dioxan-based liquid scintillator NE220 (Nuclear Enterprises Ltd) was added and the radioactivity measured in a Nuclear-Chicago scintillation counter (Model 6801), at an efficiency of approximately 72 %. Controls lacking phospho*enol*pyruvate or ADP were run with all experiments; CO_2-fixation in the absence of ADP never exceeded 14 % of the total activity and was deducted from activities quoted.

Malate oxidation by particulate fractions. The oxidation of L-malate by subcellular fractions was measured according to Cox, Snoswell & Gibson (1968). The results quoted for the small particulate fraction are expressed in μmole O_2 uptake/mg. protein/h.

Quinone content. The presence of ubiquinone and vitamin K was detected using the methods for extraction and thin-layer chromatography described by Cox, Gibson & Pittard (1968).

Uptake of fumarate and succinate. The uptake of ^{14}C-labelled fumarate and succinate was measured at 37° with log phase bacteria washed and suspended in phosphate buffer (0·1 M, pH 7·4) at 70 μg. dry wt/ml. by the method of Kay & Kornberg (1969). The radioactive acids were added at 0·1 mM (1 Ci/mole), samples (1 ml.) were withdrawn at intervals over 20 min. and the bacteria were collected on Millipore filters (0·45 μm. pore size; presoaked in 0·1 mM-fumarate or succinate), washed three times with 3 ml. of buffer and transferred to vials containing 4 ml. of NE220 scintillation fluid. Radioactivity was assayed by scintillation counting, as above, and the initial rates of C_4-dicarboxylic acid uptake are expressed in nmoles/mg. dry wt organisms/h.

Materials. Phospho*enol*pyruvate and glutamate-oxalacetate transaminase were from C. F. Boehringer & Soehne, Mannheim, Germany. [2,3-^{14}C]-Fumaric and succinic acids were purchased from The Radiochemical Centre, Amersham, and 3-fluoromalate was kindly provided by Dr P. W. Kent.

RESULTS

Genetic Studies with AB257 and AB257^{suc-}

Conjugation

The first indication that AB257^{suc-} contained more than one mutation affecting C_4-dicarboxylic acid metabolism came from preliminary conjugation studies aimed at mapping its *pck*$^-$ lesion. With AB257^{suc-} as donor and AB1325*lip*13 as recipient, a high proportion of *gal*$^+$ and *lip*$^+$ recombinants inherited the donor strain's inability to use succinate as substrate (Table 1). However, despite their inability to use succinate, the majority grew on fumarate and malate. Derivatives lacking the ability to use fumarate and malate were found in significant numbers only amongst the *xyl*$^+$ recombinants. The presence of at least two mutations was confirmed by reversion

studies. Revertants of AB257^{suc-} selected for ability to grow on either fumarate or malate invariably grew on both these substrates, but the majority would not grow on succinate. Likewise, most of the revertants selected on succinate failed to use fumarate and malate although some revertants capable of using all three substrates were again

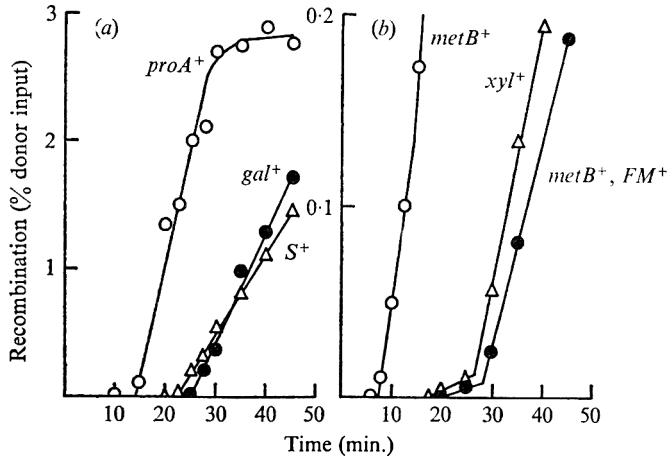

Fig. 1. Kinetics of marker transfer from HfrH to AB1325*S*$^-$, *proA*$^-$, *gal*$^-$, *str*$^-$ (*a*) and from P10 to AB1325*FM*$^-$, *metB*$^-$, *xyl*$^-$, *str*$^-$, *tsx*$^-$ (*b*). In (*a*) counter-selection against the donor was with streptomycin and *S*$^+$ recombinants were selected on media containing succinate as the carbon source. In (*b*) counter-selection against the donor was with phage T_6 and *FM*$^+$, *metB*$^+$ recombinants were selected on fumarate medium to avoid undue interference from reversion of the *FM*$^-$ marker.

Table 1. *Conjugation with AB257^{suc-} and AB257 as donors*

Overnight cultures were diluted into fresh L-broth to 5×10^8 bacteria/ml. for each strain and incubated at 37° for 90 min. Recombinants were selected on appropriate media containing methionine and streptomycin; 50 or 100 were purified by single colony isolation and scored for their inability to grow on succinate (*S*$^-$) or on fumarate and malate (*FM*$^-$).

	AB257^{suc-} × AB1325 *lip* 13		AB257 × AB1325	
Selected marker	Recombinants/ml.	*S*$^-$ (%)	Recombinants/ml.	*FM*$^-$ (%)
pro$^+$	76,000	< 1	186,000	< 1
mtl$^+$	—	—	7,300	33
xyl$^+$	3,400	< 2	9,500	57
his$^+$	—	—	1,500	< 1
gal$^+$	260	62	430	< 2
lip$^+$	150	65	—	—

recovered. The parental strain, AB257, grew on succinate but, in contrast to published results (Hsie & Rickenberg, 1966), it failed to grow on fumarate and malate. A high frequency of reversion occurred on fumarate and particularly on malate. All the revertants selected on fumarate grew on malate and most of the revertants selected on malate grew on fumarate, but some grew less well than others on this substrate. On succinate the revertants grew better than AB257 itself. The simplest interpretation of these results is that AB 257 and AB257^{suc-} possess a mutation (*FM*$^-$) which primarily

affects growth on fumarate and malate and to a lesser extent on succinate, and that, in addition, AB257^{suc-} possesses a mutation which primarily affects growth on succinate. The strains may therefore be designated AB257 *FM*$^-$ and AB257 *FM*$^-$, *S*$^-$. The revertants of AB257 *FM*$^-$, *S*$^-$ which grow on all three carbon sources are thought to arise by double events which would be favoured by the instability of the mutations and the fact that each mutation seems to impair growth on any of the three selective media. The presence of a mutation affecting fumarate and malate utilization in AB257 was confirmed by transfer to AB1325 during conjugation (Table 1). Some 57 % of *xyl*$^+$ recombinants simultaneously inherited the inability to grow on both fumarate and malate.

For more precise mapping of *S* and *FM*, recombinants from the above conjugations were used as recipients in interrupted mating experiments. With HfrH as donor and a *lip*$^+$ recombinant AB1325 *S*$^-$, *proA*$^-$, *gal*$^-$, *str*$^-$ as recipient, the *S*$^+$ marker entered 8 min. after *proA*$^+$ and 3 min. before *gal*$^+$ (Fig. 1*a*). Attempts to map the *FM* locus proved difficult due to its instability. However, with a *tsx*$^-$ derivative of a *mtl*$^+$ recombinant AB1325 *FM*$^-$, *metB*$^-$, *xyl*$^-$, *str*$^-$, *tsx*$^-$ as recipient and by selecting for joint transfer of *metB*$^+$ and *FM*$^+$, the *FM*$^+$ marker appeared to enter 12·5 min. after *metB*$^+$ and 2·5 min. after *xyl*$^+$ (Fig. 1*b*). Linked transfer of *FM*$^+$ and *metB*$^+$ was not obtained with the Hfr strain P13 which transfers its genome with the order *o*...*ilv*...*met*.

Transduction

The position of the *S*$^-$ site was further investigated in transduction studies using phage P1kc and recipients possessing markers in the *purE* to *gal* region of the *Escherichia coli* chromosome. The results indicated that *S* was not cotransduced with either *purE* or *lip*, but cotransduction of *S* with *gal* (40 to 50 %), *sucA* (90 %) and *gltA* (97 %) was observed (Table 2). These linkages suggest that the *S* locus is very close to

Table 2. *Tests for cotransduction of S and FM with other markers*

P1kc lysates of donor strains were used to infect stationary phase recipient cultures at a multiplicity of 2. *Gal*$^+$ and *xyl*$^+$ transductants were selected on galactose and xylose minimal medium respectively and other transductants were selected on appropriate glucose media. Transductants were purified by single colony isolation and the distribution of non-selective markers was determined by replica plating.

Donor	Recipient	Selective marker	Transductants per ml. × 10^3	Distribution of non-selective donor marker: No. scored	Marker	Cotransduction (%)
AB257 *FM*$^-$, *S*$^-$	XG3 *lip* 32	*lip*$^+$	5·1	100	*S*$^-$	< 1
		purE$^+$	8·5	100	*S*$^-$	< 1
AB257 *FM*$^-$, *S*$^-$	AB1325 *suc*1	*sucA*$^+$	4·3	100	*S*$^-$	90
		gal$^+$	3·7	100	*S*$^-$	50
AB257 *FM*$^-$, *S*$^-$	W620	*gltA*$^-$	31·7	100	*S*$^-$	97
		gal$^+$	28·0	100	*S*$^-$	40
W1485	AB 1325 *FM*$^-$, *met*$^-$, *mtl*$^-$	*xyl*$^+$	98·2	123	*FM*$^+$	5
AB257 *FM*$^-$, *S*$^-$	AB1325	*xyl*$^+$	12·4	126	*FM*$^-$	0·8

gltA and closer to *sucA* than to *gal*. To determine the order of *S* and *sucA* relative to *gal*, the reciprocal crosses shown in Table 3 were performed and the distribution of the non-selective *gal* marker in S^+, $sucA^+$ transductants scored. If the marker order is *S*...*sucA*...*gal*, then the linkage between S^+, $sucA^+$ and the donor gal^+ marker would be normal (40 to 50 %) in the direction I cross but much reduced for S^+, $sucA^+$ transductants obtained in the direction II cross. Opposite predictions would be for the order *sucA*... *S*...*gal*. Difficulty was experienced in finding a medium suitable for direct selection of S^+, $sucA^+$ recombinants because the S^- mutants grew slowly on plates of acetate medium, and $sucA^-$ mutants usually grew on succinate, albeit after a considerable lag. Nevertheless, S^+, $sucA^+$ transductants could be isolated satisfactorily as the large colonies which appeared early on succinate medium with the S^- recipient and on acetate

Table 3. *Order of sucA and S markers relative to gal*

Transduction with P 1 kc was as described in the Methods section, S^+, $sucA^+$ recombinants were selected on acetate minimal medium (I) or succinate minimal medium (II) and purified by single-colony isolation before testing the distribution of the donor gal^+ marker by replica plating

Direction of cross	Donor	Recipient	Distribution of donor gal^+ in S^+, $sucA^+$ transductants: No. scored	Per cent gal^+
I	AB 257 FM^-, S^- (S^-, $sucA^+$, gal^+)	AB 1325 *suc* I (S^+, $sucA^-$, gal^-)	52	59·6
II	W 1485 *suc* I (S^+, $sucA^-$, gal^+)	AB 1325 S^- (S^-, $sucA^+$, gal^-)	75	1·3

medium with the $sucA^-$ recipient. The results indicate that the order is *S*...*sucA*...*gal*. Similar crosses were attempted to order *S* and *gltA* relative to *gal*, and similar difficulties were experienced in finding suitable media for direct selection of S^+, $gltA^+$ transductants. Some success was obtained using acetate medium in a cross between AB 257 FM^-, S^- (donor) and W 620 $gltA^-$, gal^- (recipient) where the distribution of the donor gal^+ in S^+, $gltA^-$ recombinants was low (5/34) and suggested the order *gltA*...*S*...*gal*. However, no satisfactory selection procedure could be found for the reciprocal cross because S^+, $gltA^-$ recombinants grew well enough on succinate medium to make them indistinguishable from S^+, $gltA^+$ recombinants. Consequently, the relative order of *S* and *gltA* could not be deduced with certainty but a tentative order *gltA*...*S*...*gal* may be indicated.

The FM^- mutation was too unstable to permit direct selection of FM^+ in transduction crosses but attempts were made to use it as a non-selective marker. With AB 257 FM^- and AB 257 FM^-, S^- as donors, no linkage between FM^- and any of the genes in the *purE* to *gal* region was observed. A very weak linkage between *FM* and *xyl* was observed (Table 2) but its significance is difficult to assess in view of the instability of this mutation.

Physiological and biochemical studies with S^- and FM^- mutants

In order to investigate the metabolic lesions associated with the S^- and FM^- mutations, two pairs of mutant and wild-type strains were chosen for detailed study.

These were AB257*FM*⁻ and a revertant AB257*FM*⁺ (selected on fumarate medium), and W1485*S*⁻ and W1485. In all these studies cultures of the mutant organisms were tested to ensure that significant reversion had not occurred during growth.

Growth tests. The growth of the test organisms in liquid media with several different carbon sources is shown in Fig. 2. It can be seen that the *S*⁻ mutation prevented growth on succinate and acetate and it delayed growth on glucose, glycerol, lactate and malate

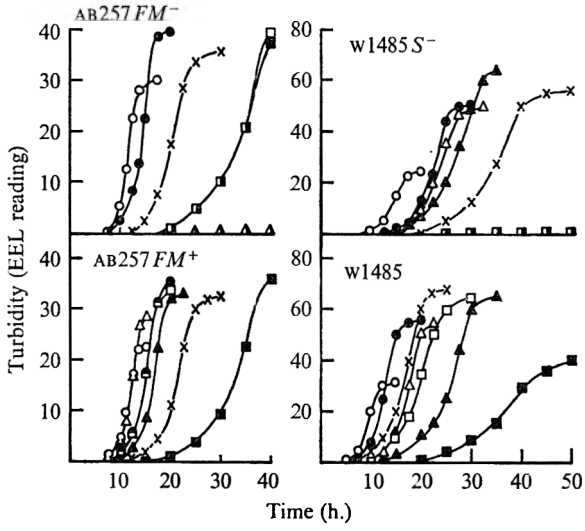

Fig. 2. Growth of *Escherichia coli FM*⁻ and *S*⁻ mutants and the corresponding wild-type strains on various substrates: ○, glucose; ●, glycerol; ×, lactate; △, malate; ▲, fumarate; □, succinate; ■, acetate.

Table 4. *Respiratory activities of washed suspensions of mutant and wild-type strains grown in glucose medium*

Strain	Respiration rates (μl. O_2 consumed/mg. dry wt/h.) with substrate		
	Succinate	Fumarate	Malate
AB257*FM*⁺	114	53	55
AB257*FM*⁻	55	20	35
W1485	63	32	28
W1485*S*⁻	32	31	35

but not fumarate. Supplementing the succinate medium with lactate and glycerol (2 mM) permitted very slow growth of W1485*S*⁻ and full growth was reached only after 70 h. The *FM*⁻ mutation prevented growth on malate and fumarate and growth on succinate was impaired in such a way that it paralleled the response with acetate. Reversion usually occurred on malate after about 45 h. but rarely occurred on fumarate up to 80 h. Supplementing malate and fumarate media with lactate and glycerol (2 mM) promoted rapid growth to a submaximal extent. Neither mutant responded to glutamate, 4-hydroxybenzoate or 2,3-dihydroxybenzoate when added to non-permissive media, but both grew anaerobically with glycerol plus fumarate. The results

obtained with solid media were not as well defined because after prolonged incubation some growth of S^- strains occurred on acetate and succinate. FM^- strains invariably reverted after prolonged incubation and the mutant organisms were fed by the revertant colonies.

Respiratory activities. A study of the oxidation of succinate, fumarate and malate by washed suspensions of the test strains showed that whereas the FM^- mutation affected the oxidation of all three dicarboxylic acids, the S^- mutation only affected the oxidation of succinate (Table 4).

Table 5. *Phosphoenolpyruvate carboxykinase in FM^- and S^- mutants*

Organisms were grown with succinate or fumarate as carbon sources and the enzyme was assayed in cell-free extracts as described in the Methods section.

Strain	Phospho*enol*pyruvate carboxykinase (μmole/mg. protein/h.) after growth on	
	Succinate	Fumarate
W1485	0·48	0·38
W1485 S^-	—	0·37
AB257 FM^+	0·49	0·39
AB257 FM^-	0·45	—

Table 6. *Enzymic activities and uptake of C_4-dicarboyxlic acids*

Organisms were grown in minimal medium with glucose or acetate as carbon sources. Enzymes were assayed in cell-free preparations as described in the Methods section and the results are expressed as μmoles substrate transformed/mg. protein/h. or as μmoles C_2 taken up/mg. protein/h. for particulate malate oxidation. The initial rates of dicarboxylic acid uptake by whole cell suspensions are expressed as nmoles/mg. dry wt organisms/h.

Organism	W1485	W1485 S^-	AB257 FM^+	AB257 FM^-	AB257 FM^+	AB257 FM^-
Growth substrate	Glucose	Glucose	Glucose	Glucose	Acetate	Acetate
Succinate dehydrogenase	4·5	0·9	4·3	3·9	—	—
Fumarase	33	28	32	31	—	—
Malate dehydrogenase	290	282	250	242	564	620
Malic enzyme (NAD)	6·4	3·6	6·3	6·8	6·9	9·5
Malic enzyme (NADP)	6·7	3·9	5·3	2·8	18·1	25·4
Particulate malate oxidation	0·8	0·6	0·7	0·6	—	—
Succinate uptake	119	116	104	86	1460	360
Fumarate uptake	111	124	146	60	1460	380

Enzymic activities. The AB257 FM^-, S^- was originally characterized by a deficiency in phospho*enol*pyruvate carboxykinase activity, so the effects of the individual mutations on this enzyme were examined. Since strains carrying the FM^- or the S^- lesion can grow on at least one C_4-dicarboxylic acid, it could be argued that if the carboxykinase is essential for growth on these substrates both types of mutant must possess adequate amounts of the enzyme. This was confirmed by showing that neither the FM^- nor S^- mutations affected the carboxykinase activity of organisms grown on succinate and fumarate respectively (Table 5).

Several other enzymes concerned with the oxidation and metabolism of C_4-dicarboxylic acids were assayed in an attempt to define the FM^- and S^- lesions; typical results are shown in Table 6. The most striking deficiency in W1485 S^- was the low

succinate dehydrogenase activity. The presence of only 20 % of S^+ activity was confirmed with other S^- derivatives (e.g. AB1325S^- and AB257FM^-, S^-). The specific activities of the malic enzymes were also lower in the S^- mutant, but this could be a secondary effect. The FM^- mutation had no significant effect on the enzymes tested. Several of the enzymes were also assayed in organisms grown on acetate in case a regulatory defect could be detected. However, no differences between FM^+ and FM^- strains were found although the specific activities of malate dehydrogenase and the NADP-linked malic enzyme were considerably higher after growth on acetate (Table 6). The behaviour of this malic enzyme contrasts with the *Pseudomonas putida* enzyme which is repressed by acetate (Jacobson, Bartholomaus & Gunsalus, 1966). The *Escherichia coli* enzyme is also induced by malate (Katsuki, Takeo, Kameda & Tanaka, 1967) and it is thought to supply $NADPH_2$ and acetyl CoA (via pyruvate). Presumably, the provision of $NADPH_2$ is its main function during growth on acetate.

Uptake of C_4-dicarboxylic acids. The S^- mutation had no effect on the initial rate of succinate or fumarate uptake by organisms grown on glucose (Table 6). By contrast, the FM^- mutation decreased the rate of uptake of both substrates and this was particularly apparent with organisms grown on acetate. However, growth of all four test strains on several substrates was inhibited by 3-fluoromalate (0·1 mM), and more precise tests with AB257FM^+ and AB257FM^- growing on acetate indicated that the FM^- strain was only slightly less sensitive over a range of analogue concentrations.

Quinone content. No differences were found between mutant and wild-type strains in tests for the presence of ubiquinone and vitamin K.

DISCUSSION

The genetic studies with AB257^{suc-} and its derivatives clearly indicated the presence of at least two mutations, FM^- and S^-, which primarily affect growth on fumarate and malate and on succinate, respectively. The FM^- mutation mapped in the *xyl* region of the *Escherichia coli* chromosome and it was unstable. The biochemical lesion associated with this mutation is obscure; it had no significant effect on the key enzymes of C_4-dicarboxylic acid metabolism, nor did it appear to affect ubiquinone or vitamin K synthesis. The only deficiency which could be detected was an impaired capacity for dicarboxylic acid transport. Mutants (*dct*$^-$), which apart from being resistant to 3-fluoromalate have the same phenotype, also map in the *xyl* region (Kay & Kornberg, 1969). However, the greatest inhibition of fumarate uptake observed with an FM^- mutant was 74 % and this alone seems unlikely to account for the failure to grow on this substrate. The effect on transport may be secondary to a more fundamental defect such as a regulatory defect or a defect in the provision of either energy or essential metabolites required specifically for growth on malate and fumarate. This is supported by the fact that supplements of lactate and glycerol permit growth on these substrates. Another uncharacterized mutation which affects growth on malate but not on succinate has also been discovered in AB3283, which also carried two mutations affecting ubiquinone biosynthesis (Cox *et al.* 1968). The origin of the FM^- mutation in AB257 is unknown but it is not present in the similar strain HfrC.

The S^- mutation was closely linked to the *gltA* and *sucA* loci and it may be situated between them. It produces a deficiency in succinate dehydrogenase activity, and al-

though the deficiency is not as complete as was found in the succinate dehydrogenase-less mutant of Hirsch *et al.* (1963), both types have many properties in common. The structural gene for succinate dehydrogenase maps in this region of the chromosome (U. Henning, personal communication) and it seems likely that this gene is the site of the S^- mutation. It is interesting to note that all the structural genes for enzymes of the tricarboxylic acid cycle which have been mapped (citrate synthase, α-ketoglutarate and succinate dehydrogenase) are clustered in the *lip* to *gal* region of the chromosome.

To account in part for the results observed by Hsie & Rickenberg (1966) it is necessary to propose that AB257^{suc-} (*FM*$^-$, *S*$^-$) was prepared by single-step mutation of AB257*FM*$^-$ but was subsequently compared with an *FM*$^+$ revertant of the parent strain. Since neither of the two mutations affects phospho*enol*pyruvate carboxykinase activity, the deficiency observed in AB257^{suc-} may be a secondary consequence of combining the two mutations, or alternatively it may be due to a third mutation which was not detected during the present work. No evidence concerning either the location of the carboxykinase (*pck*) gene or the question of whether this enzyme is essential for growth on intermediates of the tricarboxylic acid cycle was obtained.

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Induction of Cellulase (C_x) in *Verticillium albo-atrum*

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SUMMARY

Of a large number of sugars and polysaccharides, only cellulose substrates or cellobiose induced C_x (B-1,4-glucan glucano hydrolase; Enzyme Commission no. 3.2.1.4) in *Verticillium albo-atrum*. Many of the sugars tested inhibited the induction of C_x in the presence of carboxymethyl-cellulose (CM cellulose); this is explained on the basis of a balance between growth and enzyme production such that a low growth rate is usually coupled with high C_x levels and vice versa. One per cent glucose completely repressed C_x induction in 0·1 % cellobiose medium. Cellobiose caused slight inactivation of C_x by end-product inhibition. If the mycelium has no physical contact with insoluble cellulose in liquid cultures, virtually no growth occurs and C_x values are insignificant. These results are discussed in relation to the postulated C_1 enzyme involved in the initial stages of cellulose degradation.

INTRODUCTION

The breakdown of naturally occurring cellulose in plant cell walls by fungal cellulases is not yet well understood (Wood, 1960; Mandels & Reese, 1965) but it is generally believed that a C_1 enzyme acts upon insoluble cellulose to produce linear chains which are broken down by one or more of a second type of enzyme termed C_x (B-1,4-glucan glucano hydrolase; Enzyme Commission no. 3.2.1.4). The activity of the latter can conveniently be studied using various forms of soluble cellulose, including the Na^+ salt of carboxymethyl-cellulose (CM cellulose), as substrates.

It is known that in many fungi the production of cellulases is adaptive so that in the absence of cellulose the enzyme is not formed in detectable quantities. Whether or not actual contact between the fungus and cellulose is necessary for the initiation of this process is conjectural. Since the substrate is highly insoluble it is difficult to ascertain how the fungus 'recognizes' its presence, but Mandels & Reese (1960), working with *Trichoderma viride* and other fungi, have suggested that sufficient traces of enzyme are formed to start cellulose hydrolysis. Once begun, the soluble products are thought to induce the production of larger amounts of enzyme. Cellobiose, one of the common products of hydrolysis, has been shown by these workers to induce C_x; other disaccharides which also contain a β-glycosidic linkage such as lactose and sophorose act in a similar way (Mandels, Parrish & Reese, 1962). In an investigation with *Verticillium albo-atrum*, a vascular wilt organism, Talboys (1958) reported that cellobiose or cellulose would induce C_x whereas glucose, sucrose, lactose and starch all inhibited the formation of this enzyme. In a further study of this fungus, Whitney, Chapman & Heale (1969) showed that three main C_x components were produced when cellulose or

cellobiose was present in the culture medium. The approximate molecular weights of these components were 75,000, 32,000 and 16,000. They also found that incubation of C_x with CM cellulose produced only cellobiose and some medium length polymers. Glucose was absent in the culture filtrates and cellobiase (β-glucosidase) activity was not detectable.

In the present paper, we report on an investigation into the following aspects of the C_x system in *Verticillium albo-atrum*: the nature of the inducing molecule, inhibitory substances, inducer/inhibitor reactions and substrate/fungus contact. An accompanying paper (Heale & Gupta, 1970) deals with the utilization of cellobiose as the soluble product of cellulose degradation.

METHODS

Cultural procedures and carbon sources

A strain of *Verticillium albo-atrum*, isolated from wilted lucerne (*Medicago sativa*) in Norfolk during 1964, was used throughout this investigation. Modified Dox medium contained (g./l.); $NaNO_3$, 2; KCl, 0·5; KH_2PO_4, 1; $FeSO_4.7H_2O$, 0·01; $MgSO_4.7H_2O$, 0·5. The following carbon sources were used: D(−) fructose (glucose-free B.D.H.), D(+) galactose (B.D.H.), D (−) glucose (Analar), α-Me-D-glucoside (Sigma Chemical Co.), β-Me-D-glucoside (Sigma Chemical Co.), cellobiose (B.D.H.), gentiobiose (B.D.H.), lactose (B.D.H.), maltose (B.D.H.), melibiose (B.D.H.), sucrose (Analar), trehalose (B.D.H.), cellulose powder (Whatman standard grade, Balston Ltd), CM cellulose (Na^+ salt, D.S. 0·45 to 0·55, B.D.H.), pectin (Sigma Chemical Co.) and starch (soluble, B.D.H.).

The culture media were adjusted with phosphate buffer (0·1 M) to pH 5·5 before autoclaving. Sixteen oz. medicine bottles with cotton-wool plugs were employed as culture vessels containing 100 ml. liquid medium. Five replicate bottles were used for each treatment and were sterilized by autoclaving at 121° for 15 min. except in the case of pectin and those saccharides with a low melting point which were autoclaved at 115° for 10 min. Cultures were inoculated with a 6 mm. disc, cut with a sterile cork borer, from the growing margin of a 3 week parent culture of the fungus grown on potato–glucose agar. The bottles were incubated at 24 ± 1° in the dark for 14 days, except where stated otherwise, and the mycelial mats were separated by centrifugation at 4000 rev./min. for 20 min. in a MSE Super Medium centrifuge equipped with a universal swing-out head. Toluene (1 ml./l.) was added to the supernatant fluid. Growth was estimated by weighing mats that had been dried to constant weight in an oven at 60°.

In the substrate-contact experiments two different methods involving agar and liquid cultures were used. In the agar tests, modified Dox medium was mixed with 1 % agar (Davis), autoclaved, and 15 ml. samples poured into 10 replicate sterile Petri plates for each carbon source. When the medium had solidified, a well was cut on one side of the dish with a sterile no. 6 cork-borer and filled with warm modified Dox medium containing either cellobiose, CM cellulose or cellulose powder (all at 1 %); unsupplemented medium served as a control. The cellulose powder had previously been washed by centrifugation three times in sterile distilled water to remove traces of sugars. The agar was inoculated 40 mm. from the well and the cultures were incubated at 24 ± 1°. Growth towards the substrate was measured after 3, 6, 9, 12 and 14 days, after which an agar block was removed from about halfway between the well and the leading edge of the hyphae, and homogenized in about 10 ml. acetate buffer (0·2 M, pH 5·2). The

homogenate was centrifuged at 4500 *g* for 30 min. at 0° in a high speed 18 (MSE). The supernatant fluid was tested for C_x activity and for protein levels in the normal way. In the liquid culture tests a special apparatus (Fig. 1) was used; it consisted of two flat flange joints with side arms (Quick Fit, FG 25) bent at right angles and held together with a metal clip. The mouth of each of the vertically positioned side arms was stoppered tightly with a rubber bung joined by glass tubing to a sterile filtration column filled with nonabsorbent cotton wool. The arms were separated centrally at the flange by a membrane filter (size 5 cm., standard grade, Oxoid). One arm was filled with 50 ml. of modified Dox medium and the other with Dox medium supplemented with either 1 % CM cellulose or 1 % cellulose powder (washed carefully as before) and the whole apparatus autoclaved.

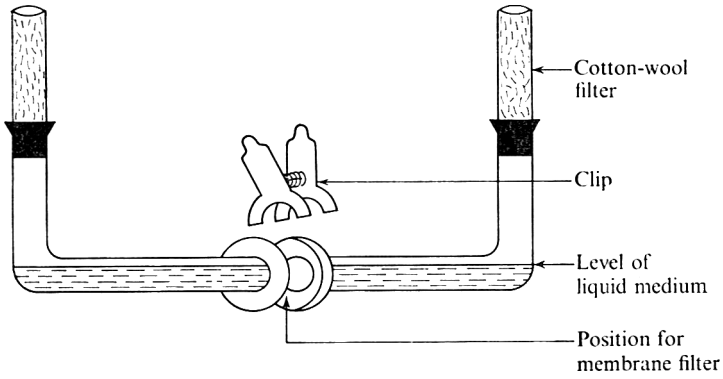

Fig. 1. Apparatus used for the substrate contact experiment.

Protein determination. Extracellular protein production in all supernatant fluids was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). A 5 % final concentration of trichloracetic acid was used to precipitate protein which was then dissolved in 0·1 % (w/v) NaOH solution. Casein standards were used.

CM cellulase (C_x) assay. Ability of the culture supernatant fluid to degrade CM cellulose was tested by using an Ostwald type viscometer (BS/IP/U-BS.188, size D). The enzyme reaction mixture contained 25 ml. of 1 % (w/v) CM cellulose (dissolved by mechanical stirring at 20° for 10 min. in 0·2 M-sodium acetate buffer (pH 5·2); toluene added at 1 ml./l.) and 1 ml. of supernatant fluid. After incubation for 1 h. at 30°, the flow time (sec.) was measured in the viscometer. Enzyme activity was initially calculated as the decrease of viscosity in CM cellulose equivalents. Arbitrary enzyme units (e.u.) were used to standardize the results and allow a quantitative comparison between treatments (Whitney *et al.* 1969).

Sugar determinations. Reducing sugars present in the culture medium before inoculation and in the supernatant fluid after growth were estimated quantitatively as glucose equivalents by a procedure utilizing the potassium ferricyanide–potassium ferrocyanide reduction reaction. The reduction in intensity of the yellow ferrocyanide was measured in an autoanalyser (Technicon) at 420 nm. (Hoffman, 1937). Sucrose was estimated by a modification of the resorcinol method of Roe (Dutton, Carruthers & Oldfield, 1961). Trehalose, α-Me-glucoside and β-Me-glucoside were estimated by the anthrone method (Winder, Brennan & McDonnell, 1967).

RESULTS

The effect of a variety of sugars and polysaccharides on C_x induction

In initial experiments, the fungus was grown for 14 days in modified Dox medium containing the following single carbon sources at 1 %: galactose, glucose, fructose, α-Me-glucose, β-Me-glucose, cellobiose, gentiobiose, lactose, melibiose, maltose, sucrose, trehalose, CM cellulose, cellulose powder, pectin, starch.

After centrifugation, the culture filtrates were assayed viscometrically for C_x activity; sugar and protein in the filtrates were determined and dry weights of mycelial mats calculated.

Table 1. *C_x activity, extracellular protein and dry wt for Verticillium albo-atrum grown on various carbon sources at 1 %*

	C_x activity e.u./ml. of supernatant fluid	μg./ml. Extracellular protein supernatant fluid	Dry wt of mycelium mg./flask
Monosaccharides			
Galactose	0	4·8	270
Glucose	0	2·8	230
Fructose	0	2·0	206
α-Me-glucoside	0	2·3	105
β-Me-glucoside	0	2·3	95
Disaccharides			
Cellobiose	34·0	11·0	550
Gentiobiose	3·0	6·0	338
Lactose	0	6·8	260
Melibiose	0	3·6	258
Maltose	0	5·0	220
Sucrose	0	3·6	228
Trehalose	0	2·5	112
Polysaccharides			
CM cellulose	44·0	21·5	135
Cellulose powder	33·5	10·8	105
Pectin	0	10·2	230
Starch	0	3·5	190

The results in Table 1 showed that *only* cellulosic substrates or cellobiose induced C_x; gentiobiose, however, led to barely detectable quantities of enzyme and so might be considered as having some slight inducing activity. Where C_x was induced, there was a corresponding increase in the levels of extracellular protein. Cellobiose was clearly the best source of carbon in terms of dry weight production. Polysaccharides such as pectin or starch promoted more growth than CM cellulose. It was not possible to measure accurately the yield of cultures grown with cellulose powder because of the residual cellulose, but since the combined weights here were less than the mycelial yields in CM cellulose-grown cultures, it is possible to conclude that cellulose powder was attacked more slowly than soluble CM cellulose. Measurements of sugar uptake showed that cellobiose was completely utilized by the end of the 14 days growth period, i.e. a total uptake of 1·8 mg. of cellobiose/mg. dry wt of mycelium (i.e. dry wt after 14 days). Cultures containing the other disaccharides showed lower levels of sugar uptake (on a dry wt basis) and lower dry weights as compared with cellobiose

media, but sugar uptake was consistently higher than in monosaccharide cultures, the uptake of glucose being 1·3 mg./mg. dry wt. This suggested some mechanism that facilitated the utilization of cellobiose (and other disaccharide molecules to a lesser extent) relative to glucose and other monosaccharides.

Inhibition of C_x by glucose

The fungus was grown for 2 weeks on modified Dox medium containing 1 % CM cellulose and either 0·1, 0·5, 1, 1·5 or 2 % glucose. Control cultures were grown in 1 % cellulose medium without glucose. Fig. 2 shows the inhibitory effect of glucose on C_x induction; even 0·1 % glucose caused a marked decrease in enzyme values as compared with those for 1 % CM cellulose alone. The opposite effect was observed for growth, which increased correspondingly with higher amounts of glucose; at the same time,

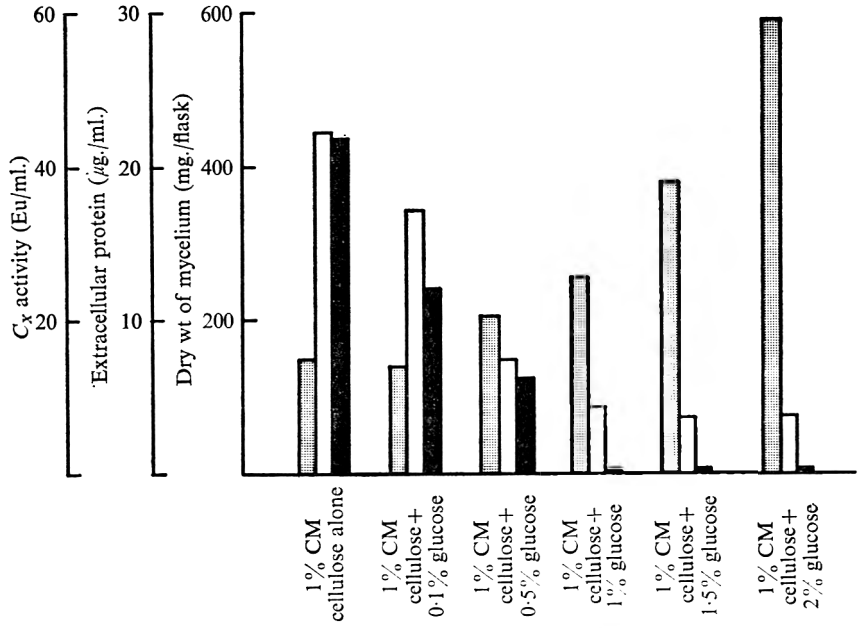

Fig 2. Effect on C_x induction of various glucose concentrations (0·1 to 2 %) in the presence of 1 % CM cellulose. ■, C_x activity (e.u./ml.); □, extracellular protein (μg./ml.); ▦, dry wt of mycelium (mg./flask).

extracellular protein amounts were decreased, reflecting the decrease in C_x. To test whether this effect involved the inhibition of C_x production or some form of enzyme inactivation, glucose was added at various concentrations (0·1 to 2 %) to a series of enzyme preparations and the mixtures assayed, with appropriate controls. In all cases the activity in the presence of glucose was unaltered, showing that its effect was on enzyme production.

The effect of small concentrations (0·1 %) of sugars and polysaccharides on C_x induction in the presence of CM cellulose

The fungus was grown in modified Dox medium containing 1 % cellulose and one of a variety of saccharides at 0·1 %; control cultures contained 1 % CM cellulose.

Glucose had the strongest inhibitory effect of the monosaccharides (Table 2); of the disaccharides, sucrose was very inhibitory and more so than either glucose or fructose, alone. Compared with control cultures C_x values/ml. were greater with cellobiose, with gentiobiose, and to a lesser extent with lactose. Relating enzyme production to mycelial dry wt, however, showed that C_x production was slightly less with cellobiose than in unsupplemented CM cellulose cultures. Of the polysaccharides, starch was strongly inhibitory, pectin slightly so. All the sugars were tested for their possible effects on the activity of C_x. With the exception of cellobiose, which at 2 % caused slight inactivation of C_x, the results were entirely negative.

Table 2. *C_x activity, extracellular protein and mycelial dry wt for Verticillium albo-atrum in 1 % CM cellulose medium in the presence of various sugars and polysaccharides at 0·1 %*

Cellulose supplement	C_x activity e.u./ml. of supernatant fluid	μg./ml. Extracellular protein of supernatant fluid	Dry wt of mycelium mg./flask
Monosaccharides			
Galactose	42·5	27·0	196
Glucose	24·0	17·2	135
Fructose	34·0	25·0	265
α-Me-glucoside	43·4	24·7	185
β-Me-glucoside	45·0	25·3	164
Disaccharides			
Cellobiose	59·2	31·5	253
Gentiobiose	50·0	28·0	305
Lactose	45·9	26·8	434
Melibiose	39·8	25·3	396
Maltose	22·0	12·0	152
Sucrose	10·0	5·9	230
Trehalose	22·9	12·4	200
Polysaccharides			
Pectin	32·0	24·0	159
Starch	15·0	11·5	124
1 % CM cellulose alone (control)	44·0	21·0	133

C_x induction by cellobiose, and interactions with glucose

To study the time course of cellobiose-induced C_x production, the fungus was grown in modified Dox medium containing 0·1, 1 or 2 % cellobiose and harvested after 3, 6, 9, 12 and 14 days. Similar changes in C_x activity per unit dry wt of mycelium were found (Fig. 3) but the higher concentrations led to increased C_x values. The curves at all concentrations increased steeply to maxima at 4 to 6 days, after which there was a decline. This resulted from a slightly increasing rate of growth accompanied by a decrease in the rate of C_x formation, particularly from the 9th day onwards. A close correlation was found between the rates of hexose utilization per unit of mycelial dry wt at 1 and 2 % cellobiose. In 0·1 % cellobiose medium, all of the cellobiose was utilized by the 6th day when C_x values slowly declined until by the 14th day C_x was only just detectable.

To investigate glucose/cellobiose interaction the fungus was cultured in modified

Dox medium containing 0·1 % cellobiose + 1 % glucose, and the cultures harvested at regular intervals. C_x induction was completely inhibited by the glucose and the enzyme was not detected at any time during the growth period.

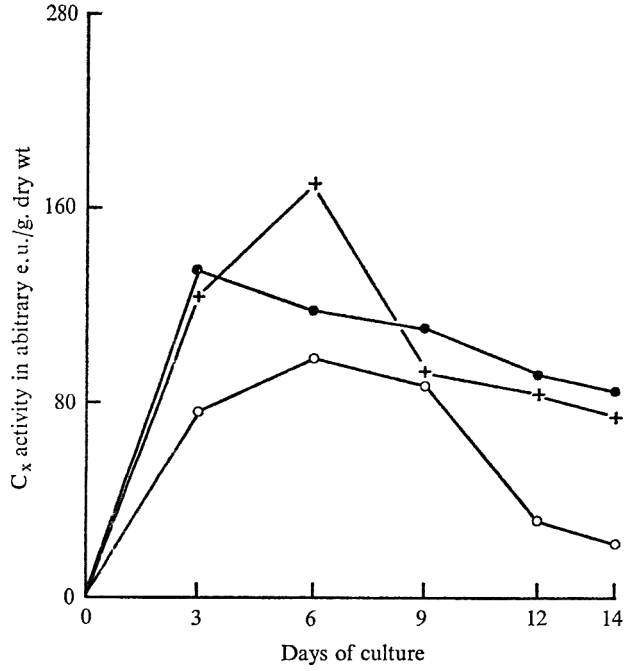

Fig 3. C_x induction in 0·1, 1 and 2 % cellobiose media. +——+, 2 %; ●——●, 1 %; ○——○, 0·1 %.

The effect of substrate contact on the breakdown of cellulose

In the first series of tests to show whether physical contact between the fungus and its insoluble cellulose substrate was essential for degradation to occur, the substrate (cellobiose, CM cellulose, or washed cellulose powder) and inocula were placed 40 mm. apart on plates containing modified Dox agar medium. The fastest growth rate was towards cellobiose (Fig. 4), a somewhat slower rate towards CM cellulose, and there was no difference between the control (unsupplemented agar medium) and washed cellulose powder. Agar blocks from between fungus and substrate showed no C_x activity, even with CM cellulose agar. The C_x in the latter case may have been irreversibly bound to the agar, or alternatively may have been inactivated during the extraction procedure.

In the second series of tests, liquid cultures were used in the apparatus shown in Fig. 1. The fungus was separated from its substrate by a membrane filter. The cellulose was inoculated *in situ* in one treatment, and in a second the modified Dox medium was inoculated in the other arm of the apparatus; CM cellulose as a soluble cellulose and unsupplemented Dox medium were included as controls (Table 3). At intervals of 2 days, liquid was sucked from one arm to the other (in both directions) to ensure that diffusible substances passed across the filter. After 9 days the supernatant fluid from both arms was assayed for C_x and the usual tests made. Growth was negligible and

C_x activity hardly detectable when the fungus was separated from cellulose powder by the membrane filter; when grown in contact with cellulose powder, however, moderate growth and enzyme activity were recorded. As expected, inoculation of Dox medium in the opposite arm to the one containing CM cellulose resulted in similar growth and enzyme activity in both arms.

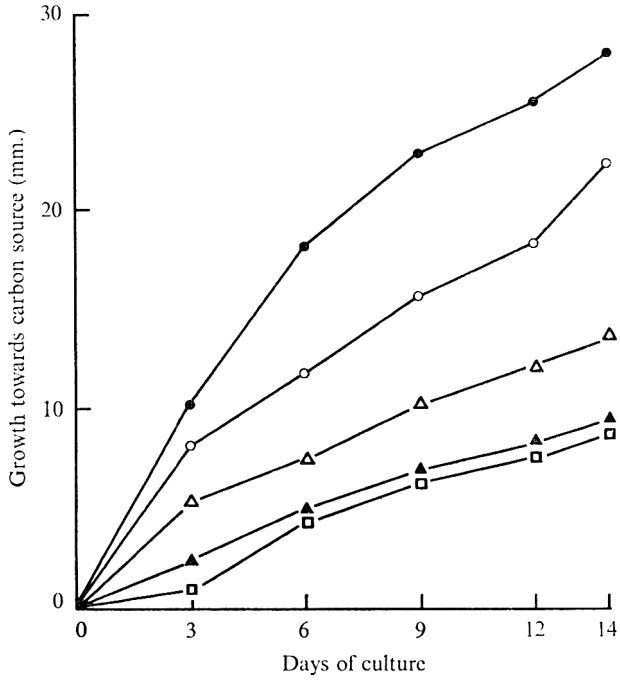

Fig 4. Growth rates in substrate-contact experiment carried out using agar plates (inoculum 40 mm. from substrate). ●——●, Cellobiose; ○——○ CM cellulose; △——△, unwashed cellulose powder; ▲——▲, washed cellulose powder; □——□, control water agar.

Table 3. *Effect on C_x induction of physical contact of Verticillium albo-atrum with cellulose substrate. A membrane filter separated the two arms of the apparatus (Fig. 1)*

Arm of apparatus	Modified Dox medium	Whether inoculated	Dry wt of mycelium (mg.)	Extra-cellular protein (μg./ml.)	C_x activity (e.u./ml.)
Left	Unsupplemented	Inoculated	34	< 1	< 1
Right	Supplemented with cellulose powder	Uninoculated	0·0	< 1	< 1
Left	Unsupplemented	Uninoculated	0·0	8·5	21·0
Right	Supplemented with cellulose powder	Inoculated	98·0	12·0	24·0
Left	Unsupplemented	Inoculated	105·0	18·0	42·0
Right	Supplemented with CM cellulose	Uninoculated	0·0	18·0	38·0
Left	Unsupplemented	Inoculated	14·0	< 1	0·0
Right	Unsupplemented	Uninoculated	0·0	< 1	0·0

DISCUSSION

The data show that the induction of extracellular C_x activity in *Verticillium albo-atrum* depended upon the presence of cellobiose (or cellulose which was degraded to liberate cellobiose units) in the medium. This means that the molecular configuration of the inducing substance is limited to a disaccharide having a β-glycosidic link between C1 and C4 of the two glucose units. Any departure from these characteristics is critical; thus gentiobiose, which has only negligible inducing activity, is very similar to cellobiose except that it has a β-1,6 linkage. Since cellobiose is the main soluble product of cellulose degradation by this fungus (Whitney *et al.* 1969), it follows that the process is a self-maintaining one. Mandels & Reese (1960) reported that cellobiose induced C_x in *Trichoderma viride* and several other fungi but little in comparison with cellulose. They explained this on the basis of the very high growth-promoting characteristics of cellobiose. Under natural conditions (simulated by the use of cellobiose octa-acetate), slow rates of degradation of the insoluble substrate resulted in small amounts of cellobiose being released which were rapidly consumed, accompanied by high values of C_x production. However, with *V. albo-atrum* cellobiose is both an excellent growth source and a potent inducer of C_x. Amongst other disaccharides having inducing activity in *T. viride* are lactose, characterized by a β-1,4 link, and sophorose, a β-1,2 sugar, which is a very powerful inducer, producing 200 times the value of C_x as compared with cellobiose (Mandels *et al.* 1962). When *V. albo-atrum* is grown on 0·1 % cellobiose, the sugar is rapidly utilized and none is detectable after 6 days. Coinciding with the exhaustion of cellobiose, the value of C_x reaches a maximum at about the 6th day and falls thereafter. In *T. viride*, C_x does not appear until after the cellobiose has been consumed, which is why Mandels & Reese (1960) have suggested that the actual inducer is a product of cellobiose metabolism rather than cellobiose itself. We cannot at this stage rule out the possibility that an oligosaccharide (Heale & Gupta, 1970) could act as the internal inducer in *V. albo-atrum*, but it does seem quite clear that the induction mechanism in these two fungi is different in several important respects. These differences might be attributable to the distinct ecological roles involved. *T. viride* being a soil saprophyte, *V. albo-atrum* a vascular wilt pathogen which probably does not begin to degrade cellulose until the death of the host (Whitney *et al.* 1969) and which shows a rapidly declining phase when incorporated into soil along with the host residues (Heale & Isaac, 1963).

Slight end-product inhibition of C_x was demonstrated by adding cellobiose to reaction mixtures during viscometric assays. Similarly, Reese, Gilligan & Norkrans (1952) found that cellobiose inhibited most of the 36 cellulases tested. It is possible, therefore, that the rate of CM cellulose degradation by *Verticillium albo-atrum* could be somewhat limited by inactivation of C_x. It is doubtful, however, whether such an effect could operate as a significant controlling mechanism during cell-wall degradation, when the rate of cellobiose production is probably well below the potential utilization rate.

Cellobiose and some of the other disaccharide sugars such as gentiobiose were much better carbon sources for growth in *Verticillium albo-atrum* than glucose, and measurements of the rates of uptake strongly suggest that some mechanism facilitates the utilization of these disaccharides as compared with glucose.

Monosaccharides such as glucose and fructose, disaccharides such as sucrose,

trehalose and maltose, and the polysaccharide starch all inhibited C_x induction in the presence of CM cellulose. Again, 1 % glucose completely repressed the formation of C_x in the presence of 0·1 % cellobiose. Similar results were reported by Talboys (1958) for glucose, sucrose and starch using cellulose as C_x inducer. The mechanism of such generalized repression is clearly related to the balance between growth and C_x formation. In general, a low growth rate is coupled with high enzyme levels and vice versa; this effect is best seen over a range of glucose concentrations in the presence of 1 % CM cellulose (Fig. 2), but it is also observed during growth on different sugars in the presence of CM cellulose. A similar phenomenon has been reported by Mandels & Reese (1960) when, using *Trichoderma viride*, they showed that rapidly consumed metabolites reduced the levels of C_x in the medium. These findings are generally consistent also with an earlier investigation (Whitney *et al.* 1969) which showed that C_x levels in the diseased host were only three times that of the healthy uninoculated control plants until the terminal stages of the disease are reached. At this time when the host dies, the level of C_x rises to 20 times that of the controls. This sudden increase in C_x was attributed to a derepression effect resulting from the exhaustion of the more easily assimilable sugars present in the cells, and it is only at this stage that generalized cell-wall degradation occurs.

The results of the substrate-contact experiment must be considered in relation to the widely held view that the initiation of the induction mechanism is due to very small amounts of C_x produced 'constitutively' which liberate some cellobiose from cellulose and so induce the enzyme in detectable quantities. Since growth was negligible in the Dox medium (minus carbon source) separated by a porous membrane from the cellulose powder on which fungus grows if inoculated *in situ*, it seems clear that actual contact is necessary for the initiation of the cellulose degrading system. According to Selby (1963), evidence from different sources shows that some enzyme system (C_1) present in the vicinity of the growing micro-organism is necessary for the breakdown of natural cellulose and is missing from extracellular extracts as normally prepared. Until more evidence is forthcoming on the nature and role of the C_1 enzyme in this and other fungi it is difficult to decide whether separation of the mycelium from the insoluble substrate affects the induction of both C_1 and C_x or only C_1. The absence of C_1 would mean that the initial step in cellulose degradation would not occur and following from this the lack of cellobiose liberation would be reflected in insignificant C_x values.

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The Utilization of Cellobiose by *Verticillium albo-atrum*

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SUMMARY

β-Glucotransferase was demonstrated in mycelial extracts and culture filtrates of *Verticillium albo-atrum* grown on 2 % cellobiose as the sole source of carbon. Analysis of the mycelium indicated relatively large amounts of cellobiose and gentiobiose, as well as small amounts of a series of three oligosaccharides (DP 3, 4, 5) and glucose; the same compounds were detected in the culture filtrate but at much lower concentrations (except for cellobiose) and glucose was barely detectable. Cellobiose phosphorylase was also present in mycelial extracts, and it is suggested that the production of glucose-1-phosphate from the phosphorylytic cleavage of cellobiose and its rapid utilization as a respiratory substrate explains why the fungus grows so much faster on cellobiose than on glucose. The level of these two enzyme systems is considered to be an important factor in controlling the rate of growth. The significance of the formation of oligosaccharides by the transferase system is discussed in relation to cellulose degradation.

INTRODUCTION

Whitney, Chapman & Heale (1969) have shown that the degradation of cellulose by *Verticillium albo-atrum* leads to the appearance of relatively large amounts of cellobiose in the culture medium and that no glucose is produced. Three main components with cellulase (C_x) activity were separated from culture supernatants and characterized according to molecular weight, pH activity curves and Michaelis constants. Gupta & Heale (1970) have presented evidence that, apart from being an excellent growth source for this fungus, cellobiose is the specific inducer of C_x. They also found that cellobiose and most other disaccharides were taken up at rates which suggested some special mechanism for utilization as compared with monosaccharides such as glucose. These findings were similar to those obtained by many workers using cellulolytic bacteria, e.g. *Cellvibrio gilvus* (Hulcher & King, 1958; Swisher, Storvick & King, 1964). Faster growth on cellobiose than on glucose is usually attributed to a higher yield of energy per molecule of cellobiose utilized as compared with two free glucose molecules. Phosphorylytic cleavage of cellobiose (mediated by cellobiose phosphorylase) yields free glucose and glucose-1-phosphate, the latter being rapidly consumed in the respiration pathway, while the former is utilized only slowly owing to low levels of hexokinase. As far as we are aware, this system has not been investigated in cellulose-degrading fungi such as Verticillium.

The formation of significant amounts of oligosaccharides and polysaccharides in culture filtrates of *Verticillium albo-atrum* grown on sucrose has been reported by

Caroselli (1954) and Green (1954). Le Tourneau (1961) found oligosaccharides up to the hexacompound in the mycelium and culture filtrate of the same fungus grown on glucose, and she suggested that they were formed by a gluco-fructo-transferase. Buston & Khan (1956) showed that cell-free extracts of *Chaetomium globosum* when incubated with cellobiose produced glucose, gentiobiose, sophorose and laminaribiose as well as β-linked trisaccharides. Mandels & Reese (1960) found glucose, cellobiose and cellotriose in the culture filtrate of *Trichoderma viride* grown on cellobiose. The significance of the glucotransferase system involved in oligosaccharide formation during cellulose degradation in these organisms has not yet been satisfactorily explained. The present paper deals with the possible role of this enzyme and of cellobiose phosphorylase in the utilization of the major soluble product of cellulose decomposition, i.e. cellobiose, in *V. albo-atrum*.

METHODS

Transferase activity and the separation of oligosaccharides

Transferase activity was investigated in mycelial extracts prepared from 14 day cultures grown in modified Dox medium (Gupta & Heale, 1970) supplemented with 2 % cellobiose. Mycelial mats were harvested by centrifugation at 4500 *g*, washed with sterile distilled water and extracted by grinding in 0·1 M-phosphate buffer (pH 6·0) containing merthiolate (a few drops of a 1/1000 dilution) at 4° with purified sand (B.D.H.) in a pestle and mortar. The extract was centrifuged at 24,000 *g* for an hour at 0° in a High Speed MSE 18 and the clear supernatant dialysed against 0·01 M-phosphate buffer (pH 6·0) for 24 h. at 4°. The enzyme reaction mixture consisted of 0·3 ml. of 0·1 % cellobiose, 0·3 ml. of 0·1 % glucose plus uniformly labelled D-glucose ^{14}C (U), specific activity 320 mCi/mM, (Radiochemical Centre, Amersham) and 0·2 ml. of the extract. One drop of merthiolate was added to the mixture, which was incubated at 24° ± 1°. As a control, either 0·1 ml. of the phosphate buffer (0·1 M) or boiled enzyme was used in place of the extract. Fifty μl. was removed at intervals of 30, 60, 90, 120 and 150 min. and applied to Whatman no. 1 filter paper sheets and chromatographed at room temperature for 20 h. using a solvent system containing *n*-propanol, ethyl acetate and water (7:1:2). In addition, glucose and cellobiose (both at 0·1 %) were incubated *separately* with the mycelial extract and tested similarly. For reference spots, a mixture of glucose and cellobiose was employed. Chromatograms were dried, sprayed with acetic benzidene and heated at 110° (Bacon & Edelman, 1951). The location of the sugars separated by chromatography was determined by preparing radioautograms. The activity of each separated sugar was determined by liquid scintillation counting: for this the chromatograms were cut into 2·5 × 6·0 cm. pieces and each piece was placed around the inner horizontal wall of a scintillation vial together with 15 ml. of a solution containing 5 g. of butyl-PBD/l. toluene. For a quantitative determination, similarly chromatographed and dried filter paper pieces containing known amounts of ^{14}C radioactive glucose were employed. Each sample was counted four times for 200 sec. with a Packard Tri-carb scintillation spectrometer. The counting efficiency using this method was found to be 65·5 % and the average mean counts were converted to μCi per sample. The transferase activity was then expressed in terms of the percentage incorporation of D-glucose ^{14}C into oligosaccharides. In addition, both the mycelial extract and the supernatant were examined chromatographically over the 14 day culture period in order to correlate the results of the *in*

vitro assay with the levels of cellobiose and oligosaccharides actually formed during growth.

Cellobiose phosphorylase. Cellobiose phosphorylase activity was investigated in mycelial extracts of the fungus prepared in the same way as described above, except that 0·05 M-Na citrate buffer was used (pH 6·0). The reaction mixture consisted of 1 ml. of buffer containing 100 μmoles cellobiose, 3 μmoles NaF, $4{\cdot}5 \times 10^{-3}$ μmoles $MgCl_2$, 18 μmoles of inorganic phosphate and 1 ml. of enzyme preparation (Sato & Takashi, 1967). One drop of merthiolate was added and the mixture incubated at 30° for 2, 4, 6 and 8 h. As a control, boiled enzyme was used. The reaction was stopped by placing the tubes in boiling water for 5 min. and the disappearance of inorganic phosphate was determined according to a modification of the procedure of Fiske & Subbarow (1925) using an auto analyser (Technicon). Enzyme activity was expressed as μmoles of inorganic phosphate consumed in the presence of 1 ml. of enzyme preparation. The products of the reaction were analysed by paper chromatography using a pyridine–butanol–water (2:3:1·5) solvent system. Glucose-1-phosphate was detected by a spray containing 3 % perchloric acid, 1 % ammonium molybdate and 0·1 % versene in 0·1 N-HCl. The chromatogram was air dried before examining the phosphate spots under u.v. irradiation (Hanes & Isherwood, 1949).

The formation of cellobiose from glucose and glucose-1-phosphate as a result of cellobiose phosphorylase activity was also investigated. The reaction mixture consisted of 0·05 ml. of 0·1 M-$MgCl_2$, 0·05 ml. of 0·1 M-NaF, 0·3 ml. of 0·1 M-glucose-1-phosphate, 0·3 ml. of 0·1 M-glucose plus uniformly labelled glucose (^{14}C (U)), specific activity 320 mCi/mM), 0·3 ml. of 0·05 M-Na citrate buffer (pH 6·0) and 1 ml. of enzyme preparation. One ml. of this mixture was placed in each of two tubes; one was immediately immersed in boiling water for 5 min. and served as a control. After addition of merthiolate both tubes were incubated for 8 h. at 30°. Enzyme activity was stopped by placing the tubes in boiling water. The mixtures were brought to room temperature and approximately 50 mg. of a 1:1 mixture of Permutit de-Acidite, FF, SRA 64, anion exchange resin, and Zeocarb 225 SRC 13, cation exchange resin added to each tube. Shaking the tubes with the resin removed salts that otherwise interfered with chromatographic separation of the sugars. After standing, the supernatant was decanted and 50 μl. samples were applied to Whatman no. 1 filter paper sheets. Autoradiograms were prepared as described above. Enzyme activity was expressed as the percentage incorporation of glucose ^{14}C into cellobiose.

RESULTS

Oligosaccharide formation and transferase activity

Chromatographic analysis over the growth period showed that both cellobiose and gentiobiose accumulated in large amounts within the mycelium. These substances were not well separated in the solvent system used but could be distinguished by the use of reference markers (cellobiose Rg 50, gentiobiose Rg 45). Small amounts of glucose were positively identified by correspondence to a glucose reference. Three oligosaccharides were also detected with Rgs of 35, 23 and 12; they showed a good linear relationship between their logarithmic partition function α (defined as log 1-Rg/Rg and molecular size (Chesters & Bull, 1964) assuming them to be of DP 3, 4 and 5 respectively. It was not possible, however, to decide definitely whether they were β-1,4 or β-1,6 linked oligosaccharides. The amounts of oligosaccharides (as measured by their

benzidene reaction) of DP3 and above decreased with increasing molecular size. Similar compounds were detected in the culture filtrate but at much lower concentrations and glucose was barely detectable.

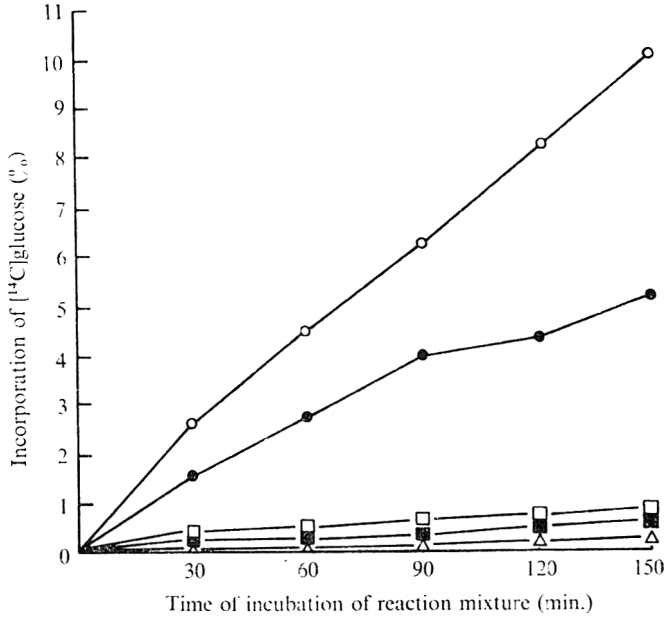

Fig. 1. Percentage incorporation of [^{14}C]glucose into various compounds* in the presence of a mycelial extract showing β-glucotransferase activity. ○——○, Unidentified compound (5); ●——●, gentiobiose (4); □——□, gentiotriose (3); ■——■, gentiotetraose (2); △---△, gentiopentaose (1).

* The numbers correspond to those numbers on the autoradiograph in Pl. I.

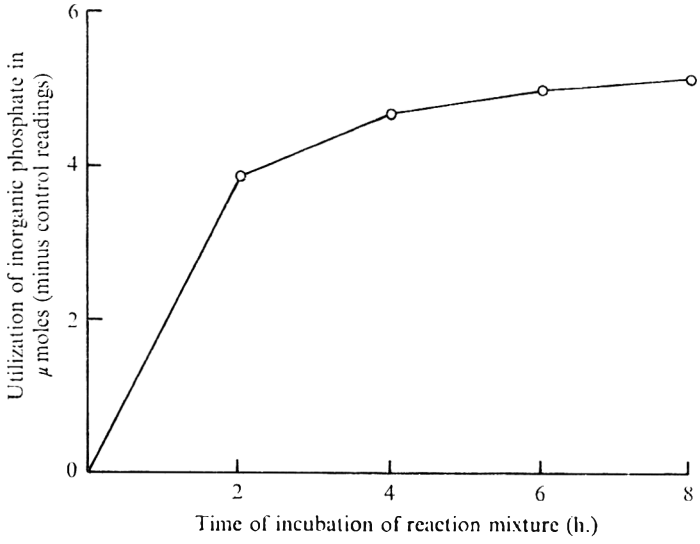

Fig. 2. Utilization of inorganic phosphate in the phosphorylation of cellobiose to glucose-1-phosphate and glucose in the presence of a mycelial extract showing cellobiose phosphorylase activity.

When cell-free mycelial extracts from 14 day cultures of the fungus were incubated with a mixture of cellobiose and labelled glucose followed by autoradiography (Pl. 1), evidence of glucotransferase activity was obtained. Carbon 14 was rapidly incorporated into two compounds, one of which (spot 4) was identified as gentiobiose; the other compound (spot 5) was of mobility intermediate between cellobiose and laminaribiose and remains unidentified. Three labelled oligosaccharides were also detected (spots 3, 2, 1) and a plot of their logarithmic functions suggested that gentiobiose and these substances were in the same series, i.e. they were gentiodextrins of DP 2 to 5 containing β-1,6 linkages. The extent of incorporation (Fig. 1) decreased with increasing DP, possibly reflecting the relative amounts of these oligosaccharides detected in the mycelial analyses. Similar indications of glucotransferase activity were obtained when concentrated culture supernatant (reduced to $\frac{1}{10}$th volume by dialysis against polyethylene glycol at 4°) was used instead of the mycelial extract.

Cellobiose phosphorylase. The rates of utilization of inorganic phosphate (Fig. 2) shown by mycelial extracts suggested a high phosphorylase activity. The production of glucose-1-phosphate and glucose during phosphorylytic cleavage of cellobiose was confirmed by chromatography. When glucose-1-phosphate and glucose with radioactive glucose were incubated with the mycelial extract for 8 h. labelled cellobiose was detected.

DISCUSSION

The radioactive incubation tests demonstrated β-glucotransferase activity both in mycelial extracts and, to a lesser extent, in the culture filtrate of *Verticillium albo-atrum*. The mechanism of formation of labelled gentiobiose is almost certainly one involving direct transfer of the glucosyl moiety from cellobiose to the labelled glucose acceptor and the formation of a β-1,6 linkage as follows:

(*a*) Cellobiose + enzyme → glucosyl enzyme complex + glucose,

(*b*) Glucosyl-enzyme complex + labelled glucose* → labelled gentiobiose*.

Chesters & Bull (1964) working with several different fungi reported that glucosyl transfer to the $-1, 6$ position predominated possibly because of the greater availability of the $-OH$ group at carbon atom 6 since it lies out of the plane of the hexose ring.

The subsequent transferase reactions are envisaged as follows:

(1) Gentiobiose* + gentiobiose ⇌ gentiotriose* + glucose,

(2) Gentiotriose* + gentiobiose ⇌ gentiotetraose* + glucose,

(3) Gentiotetraose* + gentiobiose ⇌ gentiopentaose* + glucose.

At each of the steps (1) to (3) the gentiobiose involved could be labelled from (*a*) and (*b*) so that with increasing times of incubation, the proportion of label in the gentiodextrins increases. At the same time, however, the level of incorporation would decrease with DP. The analysis of the mycelial extracts confirmed that cellobiose and gentiobiose were present in large amounts but synthesis of gentiodextrins of DP 3 and above is less important. There also seems to be slight transferase activity in the culture supernatant which explains why some oligosaccharides are formed there (Caroselli, 1954; Green, 1954; le Tourneau, 1961), but whether extracellular transferase plays any part in cellulose utilization is problematical.

The high cellobiose phosphorylase activity demonstrated in the present work may help to explain why cellobiose is a much better carbon source for growth than is glucose for this fungus. The production of glucose-1-phosphate which can be readily utilized in the respiration pathway as already suggested in *Cellvibrio gilvus* and other

cellulose-decomposing bacteria (Hulcher & King, 1958; Swisher *et al.* 1964) could be the important factor here.

The levels of the phosphorylase and the transferase systems, must play an important part in controlling the rates of growth and cellulose degradation. It has already been established (Whitney *et al.* 1969; Gupta & Heale, 1970) that exogenous cellobiose induces the production of three major extracellular cellulase (Ca) components. These components mediate the degradation of β-1,4 linked cellulose chains to cellobiose units. Once inside the mycelium cellobiose can be metabolized by at least two reactions: in the first, cellobiose phosphorylase cleaves the disaccharide producing glucose phosphate; alternatively, transferase reactions outlined above result in the formation of gentiobiose and β-1,6 linked gentiodextrins. The phosphorylase system would overcome any 'metabolic bottleneck' caused by the lack of a cellobiase (Whitney *et al.* 1969) so perhaps it is best to regard the production of gentiodextrins as a mechanism for maintaining a pool of relatively simple carbon compounds that could be utilized if cellobiose uptake becomes limiting, i.e. when the cellulose substrate is depleted.

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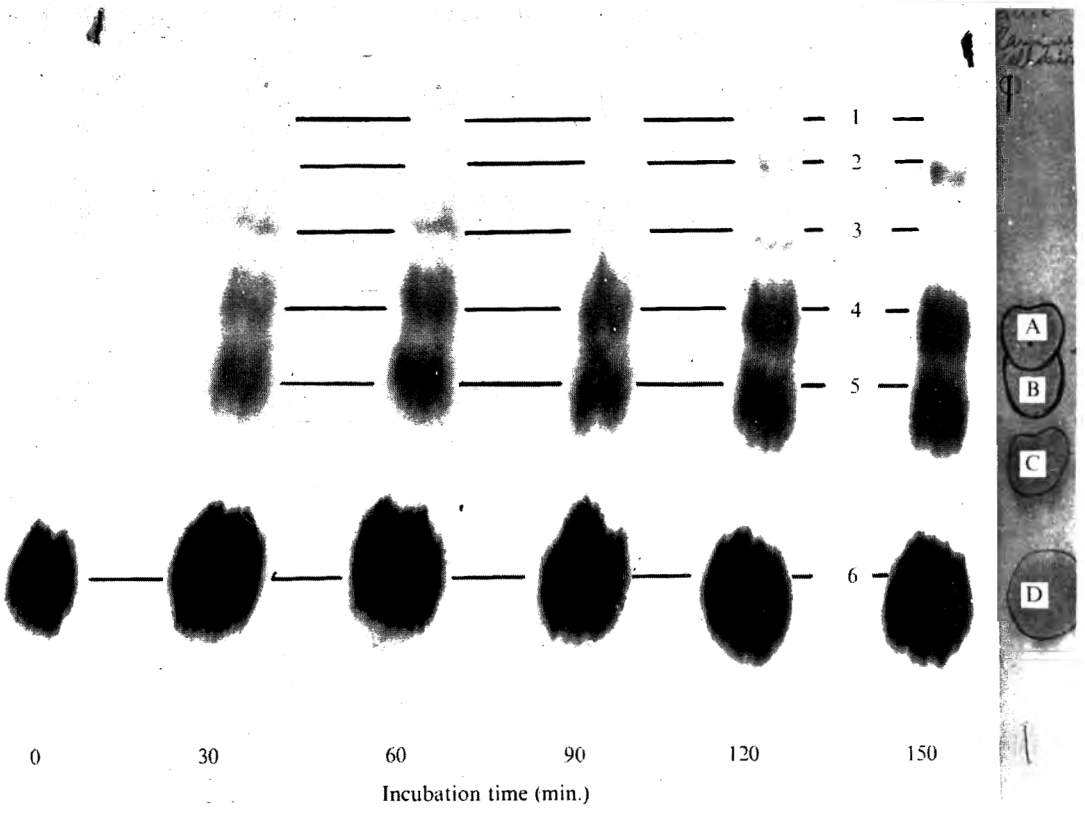
1
2
3
4
5
6
A
B
C
D
0
30
60
90
120
150
Incubation time (min.)

EXPLANATION OF PLATE

Autoradiograph showing increasing incorporation of [^{14}C]glucose label into various oligosaccharides during incubation of a mycelial extract of *Verticillium albo-atrum* showing β-glucotransferase activity. A benzidene-sprayed chromatograph strip is shown on the right with several reference compounds: A, gentiobiose; B, cellobiose; C, laminaribiose; D, glucose. Tentative identifications (see text) of labelled compounds: 1, gentiopentaose; 2, gentiotetraose; 3, gentiotriose; 4, gentiobiose; 5, unidentified oligosaccharide; 6, glucose.

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Purine Catabolism in *Fusarium moniliforme*

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SUMMARY

Fusarium moniliforme utilized the purines hypoxanthine, xanthine and uric acid as sole nitrogen sources. Allantoin and urea supported relatively more growth. The methylated purines caffeine and theobromine were not utilized. Uric acid, allantoic acid, glyoxylic acid and ammonia were detected in culture filtrates. Xanthine dehydrogenase, uricase, allantoinase, allantoicase and urease were present in cell homogenates and cell-free extracts. Uric acid was degraded to allantoic acid via allantoin.

INTRODUCTION

Penicillium chrysogenum can utilize several purines as sole sources of nitrogen (Allam & Elzainy, 1969*a*). Cell-free extracts contained xanthine dehydrogenase, uricase, allantoinase, allantoicase and urease. Uric acid was degraded to allantoic acid by way of allantoin, and allantoin was degraded to glyoxylic acid via allantoic acid. These results indicated that purine breakdown by this fungus is similar to that shown in aerobic bacteria (Campbell, 1955; Franke & Hahn, 1955). In the present work the pattern of purine catabolism was investigated in another fungus, *Fusarium moniliforme*. A preliminary report of this work has appeared (Allam & Elzainy, 1969*b*).

METHODS

Cultures and preparation of cell-free extracts

A local strain of *Fusarium moniliforme*, no. 13, was obtained from the National Research Centre of Egypt. Surface cultures of the organism were used and were prepared as previously described (Allam & Elzainy, 1969*a*). After 3 days incubation at 30°, the mycelium was harvested and cell-free extracts prepared as described by Allam & Elzainy (1969*a*).

Cell-homogenates. The freshly harvested mycelium was homogenized with 0·1 M-potassium phosphate buffer, pH 7·0, for 5 min. in a cold Waring blender.

Potassium allantoate. This was prepared by the alkaline hydrolysis of allantoin by the method of Young & Conway (1942).

Chemical methods. Xanthine was determined by the method used by Litwack, Bothwell, Williams & Elvehjem (1953); uric acid by the method of Blauch & Koch (1939); allantoin, allantoic acid and glyoxylic acid by the method used by Franke, Taha & Krieg (1952); ammonia by Nessler's reagent or by vaccuum distillation (Varner, Bullen, Vanecko & Burrell, 1953); and protein by the method of Sutherland, Cori, Haynes & Olsen (1949).

Enzymatic assays. Xanthine dehydrogenase was assayed by conventional manometric techniques with air as the gas phase; uricase by the same techniques and by measuring the disappearance of uric acid and the formation of allantoin; allantoinase, allantoicase and urease as described in a previous publication (Allam & Elzainy, 1969*a*).

Chromatography. Allantoin and allantoic acid were identified by paper chromatography as previously described (Allam & Elzainy, 1969*a*).

RESULTS

Growth of Fusarium moniliforme on different purines and other nitrogenous compounds

As shown in Table 1, *Fusarium moniliforme* can grow on the chemically defined Czapek–Dox medium in which hypoxanthine, xanthine or uric acid replaced $NaNO_3$ as sole source of nitrogen. The growth on xanthine or uric acid was approximately equal to that obtained on $NaNO_3$. However, no growth was obtained with the methylated purines caffeine and theobromine. Similar results were reported by Allam & Elzainy (1969*a*) and by Kim & Wolf (1961) using *Penicillium chrysogenum.* Table 1 also shows that allantoin and urea supported slightly better growth than that on hypoanthine, xanthine or uric acid. The poor growth with NH_4Cl may be due to the fall in pH value following ammonium uptake.

Table 1. *Growth of Fusarium moniliforme on different purines and other nitrogenous compounds*

Triplicate culture flasks contained 50 ml. of Czapek–Dox medium in which $NaNO_3$ was replaced by a nitrogen-equivalent amount of each N source, and were incubated 8 days at 28°.

Nitrogen source	Mean mycelial dry wt (mg./flask)
Hypoxanthine	210·0
Xanthine	271·9
Uric acid	242·6
Caffeine	0·0
Theobromine	0·0
Allantoin	284·1
Urea	295·5
$NaNO_3$	266·1
NH_4Cl	143·0

Detection of some intermediates in the media during growth on xanthine

Table 2 shows the occurrence of uric acid, allantoic acid, glyoxylic acid and ammonia in the media in which xanthine served as sole N source. Allantoin and urea could not be detected; the rate of their utilization may have equalled the rate of their formation, since cell-free extracts of this organism contain fairly active allantoinase and urease. Glyoxylic acid could be derived either from the purine or from the sugar of the medium. In subsequent experiments, extracts of *Fusarium moniliforme* were found to exhibit a weak allantoicase activity, suggesting that at least part of the glyoxylic acid might be formed from the purine. Similar results were reported by Sukhenko & Podgainaya (1958), who studied the utilization of uric acid by *Microsporum lanosum*, *M. ferrugineum* and *Fusarium* sp.

Xanthine dehydrogenase and uricase in cell homogenates

Figure 1 shows xanthine dehydrogenase and uricase activities in cell homogenates of *Fusarium moniliforme* grown with xanthine as sole source of nitrogen. Oxidation rates were very similar. Also the homogenates clearly had a fairly high endogenous rate of oxygen uptake, about 50 % of that when either xanthine or uric acid was added to the reaction mixture, as estimated after $3\frac{1}{2}$ h. Similar results were reported by Taha, Krieg & Franke (1955), who studied the oxidation of xanthine and uric acid in several fungal species.

Table 2. *The occurrence of intermediary metabolites during the utilization of xanthine*

Pooled samples from triplicate culture flasks were analysed; each flask contained 50 ml. of the medium in which 48·6 mg. of xanthine replaced $NaNO_3$. Values are in mg./culture flask.

Time in days	Xanthine	Uric acid	Allantoic acid	Glyoxylic acid	NH_3
2	26·70	4·70	0·08	0·09	0·466
4	12·40	3·00	0·08	0·18	0·232
6	3·30	1·06	0·15	0·12	0·216
8	1·16	0·13	0·04	0·16	0·186

Uricase and combined uricase-allantoinase activities

In this experiment 7·2 μmoles of uric acid were incubated with 16 mg. of extract protein of xanthine-grown mycelia in the presence of 90 μmoles of sodium pyrophosphate buffer, pH 8·6. This reaction mixture (4 ml.) was incubated at 37° for 4 h. Uric acid disappearance and allantoin and allantoic acid formation were followed. One-half of the uric acid disappeared in the first 65 min. and almost all of it after 150 min. Analysis of the reaction mixture at the end of the incubation period showed the formation of 4·6 μmoles of allantoin and 3·0 ·moles of allantoic acid. However, glyoxylic acid was not detected in this reaction mixture. The formation of allantoic acid indicates the presence of allantoinase which hydrolysed part of the allantoin formed to allantoic acid. Allantoin therefore appears to be an intermediate in the degradation of uric acid to allantoic acid. Comparable results were reported by Allam & Elzainy (1969*a*) using extracts of *Penicillium chrysogenum*.

Allantoinase, allantoicase and urease activities

The enzyme allantoinase was demonstrated in extracts of xanthine-grown mycelia by following the formation of allantoic acid when allantoin was incubated with these extracts at 37°. The reaction mixture (4 ml.) contained 34 μmoles of allantoin, 6·6 mg of extract protein, and 100 μmoles of potassium phosphate buffer, pH 7·0. Over the first 15 min. allantoic acid was produced at the rate of 13 ± 1 μmole/h.

Table 3 shows the low allantoicase activity found in these extracts. No activity was obtained in the absence of neutral phenylhydrazine. This allantoicase activity is considerably weaker than that demonstrated in *Penicillium chrysogenum*, for which the addition of phenylhydrazine was not required.

Urease activity in the same extracts was determined by following the formation of ammonia when 60 μmoles of urea were incubated at 37° with 6·6 mg. of extract pro-

tein in the presence of 100 μmoles of potassium phosphate buffer, pH 7·0 (total volume 3 ml.). The initial rate of ammonia formation was 170 $\pm$ 10 μmole/h. Comparable urease activity was reported in extracts of *Penicillium chrysogenum* (Allam & Elzainy, 1969*a*). The presence of an active urease points to the important role of this enzyme: it seems that any urea formed as a result of the serial degradation of the purine skeleton, acting as the sole source of nitrogen, is rapidly hydrolysed to ammonia.

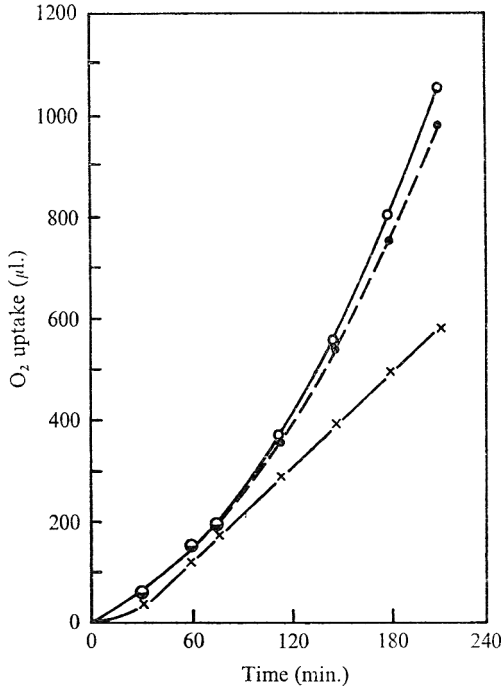

Fig. 1. Xanthine and uric acid oxidation by cell homogenates of *Fusarium moniliforme* grown with xanthine. Each manometric flask contained: xanthine or uric acid, as indicated, 40 μmoles (side arm); potassium phosphate buffer (pH 7·0), 100 μmoles; cell homogenates, 1·0 ml.; 20 % KOH, 0·2 ml. (centre well); total volume, 3 ml.; temp. 30°. O——O, Xanthine; ●——●, uric acid; × —— ×, endogenous.

Table 3. *Allantoicase in extracts of Fusarium moniliforme*

Reaction mixture contained: potassium allantoate, 32 μmoles; potassium phosphate buffer (pH 7·4), 50 μmoles; neutral phenylhydrazine, 30 μmoles; extract, 5·94 mg. of protein; total volume, 3·3 ml.; temp. 37°.

Time (min.)	μmoles glyoxylic acid formed
0	—
30	0·2
55	0·55

DISCUSSION

The degradation of xanthine by this organism follows almost the sequence reported for *Penicillium chrysogenum* (Allam & Elzainy, 1969*a*), involving the oxidation of

xanthine to uric acid. The latter is then converted to allantoic acid via allantoin. Allantoic acid is in turn degraded to glyoxylic acid and urea. Urea is finally hydrolysed to ammonia and CO_2.

The principal difference between the enzymes of *Fusarium moniliforme* and those of *Penicillium chrysogenum* is the comparatively low allantoicase activity of the former organism, which may be interpreted on the basis of the equilibrium of the reaction being in favour of allantoate formation. Trijbels & Vogels (1967) reported that the reaction catalysed by allantoicase is reversible in *Pseudomonas aeruginosa*. It is also possible that allantoate is degraded via more than one pathway in *F. moniliforme*.

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Comparison of Serological and Physiological Classification of the Genus *Saccharomyces*

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SUMMARY

The physiological and morphological properties of 28 species of the genus *Saccharomyces* were subjected to numerical analysis. Ten groups were distinguished, which agreed well with serological groupings, thus: (1) *S. bailii*, *S. bisporus*, *S. mellis* and *S. rouxii*, of serological group A; (2) *S. carlsbergensis* and *S. cartilaginosus* (A); (3) *S. cerevisiae* (AB); (4) *S. oviformis*, *S. steineri* and *S. veronae* (AC) and *S. bayanus* and *S. pastorianus* (C); (5) *S. acidifaciens* and *S. elegans* (AD); (6) *S. fragilis* and *S. marxianus* (B); (7) *S. chevalieri* (BC); (8) *S. cerevisiae* var. *ellipsoideus*, *S. diastaticus*, *S. logos*, *S. uvarum*, *S. willianus* and a further group of strains of *S. carlsbergensis* (all of group C); (9) *S. fermentati*, *S. microellipsodes* and *S. rosei* (D); (10) *S. delbrueckii* (D).

Serological classification offers results sufficiently close to those obtained by morphological and physiological tests to provide a rapid and reliable method of identification of Saccharomyces species.

INTRODUCTION

A simple serological classification of the genus *Saccharomyces*, using four absorbed sera, has been described previously (Campbell, 1968). However, in some instances the serological classification does not coincide with the identification by the standard morphological and physiological tests of Lodder & Kreger-van Rij (1952). For example, there are two antigenically distinct subgroups of *S. carlsbergensis*, termed I and II by Campbell & Brudzynski (1966), and of *S. cerevisiae*, but in the latter case the antigenic variant closely corresponds to the morphological variant *ellipsoideus* (Campbell & Allan, 1964). Another discrepancy between generally accepted classification and the serological method is the large group of species, including *S. carlsbergensis* II, *S. cerevisiae* var. *ellipsoideus*, *S. logos*, *S. uvarum* and *S. willianus*, which are antigenically identical (Campbell, 1968).

Kockova-Kratochvilova & colleagues (1966*a*, *b*, 1967, 1968) have demonstrated by numerical taxonomy of many species of Saccharomyces that certain species recognized by Lodder & Kreger-van Rij (1952) do not on full analysis justify specific rank. *Saccharomyces pastorianus*, *S. bayanus* and *S. willianus* were sufficiently similar to be grouped as one species, *S. heterogenicus* was significantly different from that group, but *S. steineri* formed a transition between them (Kockova-Kratochvilova, Vojtkova-Lepsikova, Sandula & Pokorna, 1968).

In the present paper an analysis of 28 Saccharomyces species is presented in order to examine fully the compatability of our serological identification scheme with the Lodder & Kreger-van Rij system, and with the condensed classification proposed by Kockova-Kratochvilova *et al.* (1968).

METHODS

Yeast cultures. Cultures of 28 Saccharomyces species were obtained from the National Collection of Yeast Cultures (NCYC), Nutfield; additional strains were isolated in this Department or obtained from other sources. The total of 136 cultures were identified on isolation or receipt by the system of Lodder & Kreger-van Rij (1952). Cultures were normally grown at 25° for 3 days; those for preparation of antisera and inoculation of identification tests were prepared on Sabouraud glucose agar (Oxoid).

Characters for numerical analysis. The 48 tests described below were performed on each strain.

Morphology (9 characters). Cells, grown in malt extract broth, were assessed as spherical (length up to $1\frac{1}{2}\times$ breadth), oval ($1\frac{1}{2}$ to 3×1) or long-oval (over 3×1); small (width up to 4 μm.) or large (width over 4 μm.). Production of pseudomycelium (slight, up to 5 cells long; extensive, over 5 cells) on Oxoid cornmeal agar was assessed without regard to morphology; for this and other tests on solid media nine cultures were conveniently spotted on one 10 cm. diameter plate. Colony morphology on malt extract agar was assessed after 3 days as smooth (circular outline, glossy surface) or rough (irregular outline, matt or rough surface).

Physiology (35 characters). Suspensions of cells from Sabouraud agar were prepared in 0·1 M-phosphate buffer, pH 6, washed by centrifugation and incubated at 25° in the same buffer for 2 h. to deplete intracellular reserves of nutrients. After two further washings the suspensions were adjusted to approximately 10^7 cells/ml. and the test media inoculated with 0·02 ml. of suspension. All tests were performed in duplicate or triplicate on separate occasions. Fermentation and assimilation of galactose, sucrose, maltose, raffinose, lactose, melibiose and maltotriose (Lodder & Kreger-van Rij, 1952; Gilliland, 1956) were tested by the method of Campbell & Brudzynski (1966). Maltotriose, added at 3 % to the basal medium, was prepared by hydrolysis of pullulan (Ueda, Fujita, Komatsu & Nakashima, 1963) by pullulanase (Bender & Wallenfels, 1961). Assimilation alone of sugars was scored + −. Fermentation, which included assimilation, was scored + +, except in the case of maltotriose when only fermentation was scored as +. No distinction was made between partial and complete fermentation of raffinose. Assimilation of ethanol, glycerol or L-arabinose, each at 2 %, as sole carbon source, and of 0·25 % glycine, L-histidine, L-lysine, L-methionine or L-tryptophan as sole nitrogen source (Wickerham, 1952) were tested on the appropriately supplemented Difco yeast nitrogen or carbon medium, solidified with 1 % Oxoid agar no. 1. In addition to growth on tryptophan, cultures were examined for producton of a brown halo which was observed to surround the colonies of certain species on tryptophan agar. Requirements for vitamins (Schultz & Atkin, 1947) were assessed in terms of 'bios factors' 1, 2, 3 and 6 (inositol, pantothenate, biotin and pyridoxine + thiamine) on agar based on the formula for Difco yeast vitamin medium but prepared from salts and amino acids supplied by B.D.H. Chemicals Ltd (Poole, Dorset) and vitamins by Koch-Light Ltd (Colnbrook, Buckinghamshire).

As additional identification tests, the following were examined: growth in malt extract broth containing 8, 12 or 16 % (v/v) ethanol (added aseptically to sterile broth) and growth under conditions of high osmotic pressure in a medium of 20 g. glucose, 5 g. NaCl and 1 g. Oxoid mycological peptone per 100 ml. ('Osmophilic medium')

(Kockova-Kratochvilova *et al.* 1966*a*) and at 30 and 37° over 2 days on malt extract agar. Lipolysis was detected on Oxoid tributyrin agar and acid production on the chalk agar described by Lodder & Kreger-van Rij (1952). Strains were also tested for growth in the presence of 20 p.p.m. crystal violet (Kato, 1967).

Flocculation and fining (4 characters). These properties were assessed as described by Campbell, Robson & Hough (1968) and each scored − −, + − or + +.

Table 1. *Antigenic structure of various Saccharomyces species*

Species and NCYC* numbers	Antigenic structure
Saccharomyces bailii 385, 580	A
S. bisporus 171, 697	A
S. cartilaginosus 76	A
S. mellis 418	A
S. oviformis 374, 482	AC
S. steineri 406, 701	AC
S. fructuum 410, 609, 620	C
S. heterogenicus 415, 484	C
S. italicus 108	C
S. microellipsodes 698	D
S. rosei 566, 585	D

* National Collection of Yeast Cultures, Nutfield, Surrey.

Numerical analyses. Properties, other than serological, were recorded on 80-column punched cards and the percentage similarity between strains calculated on an I.C.L. 4130 computer. Both positive and negative matches were assessed as similarity, using the formula for S_s of Sneath (1962). Similarity matrices and thence dendrograms were prepared as described by Sneath (1962), plotting results at 5 % intervals. Relationships between groups of strains were drawn at the highest mutual similarity level, as at lowest or mean similarity level the differences between groups were unjustifiably enhanced. Normally the percentage similarity between strains in a cluster fell within a 5 % range.

Since the large number of strains involved in the project could not be handled conveniently in a single run of the computer, sets of up to 20 strains of similar serological properties were processed together, and compared in turn with a similar number of strains of other serological groups. Thus all strains of species of serological group A (*Saccharomyces bailii*, *S. bisporus*, *S. carlsbergensis* I, *S. cartilaginosus*, *S. mellis* and *S. rouxii*) (Campbell, 1968; see also Table 1) were compared in successive analyses with groups of strains of serological groups AB, AC, AD, B, BC, C and D, which were compared in turn with all other groups. A small number of strains were chosen of each species to confirm in a final survey the relationships indicated by the preliminary findings.

RESULTS

Serological properties of species. All available strains of 28 Saccharomyces species were tested by absorbed antisera A, B, C and D (Campbell, 1968). Agglutination patterns of species not classified in the earlier communication are listed in Table 1. In addition, sera were prepared against at least one strain of each species not tested previously. On analysis by a full programme of absorption tests with strains of various antigenic groups, no antigenic factors additional to those already listed in Table 1 were

Table 2. *Properties of strains*

Saccharomyces species	No. of strains	Assimilation of: Glycine	Histidine	Lysine	Methionine	Tryptophan	Arabinose	Ethanol	Glycerol	Bios number	Alcohol tolerance (%)	Osmotic tolerance	Growth at 30°	Growth at 37°	Hydrolysis of tributyrin agar	Clearing of chalk agar	Growth on C.V.A.	Maltotriose fermentation	Flocculence	Fining
S. acidifaciens	6	−	×	+	+	×	−	+	+	0	8 to 12	×	+	+	×	−	−	−	−	± +
S. bayanus	1	−	−	−	+	+	−	−	+	236	8	−	+	−	−	−	−	+	−	−
S. bailii	3	×	×	×	−	×	−	−	−	×	8 to 12	−	+	−	+	−	−	−	×	+
S. bisporus	3	×	+	×	+	+	−	−	−	0	8 to 12	−	+	−	+	−	−	−	−	+
S. carlsbergensis I	3	−	−	−	+	+	−	−	−	0	8 to 12	−	+	−	−	−	+	×	−	+ +
S. carlsbergensis II	12	−	×	−	×	×	−	×	×	×	8 to 12	−	+	−	−	−	×	×	×	×
S. cartilaginosus	1	−	−	−	+	+	−	−	−	2	8 to 12	−	+	−	−	−	±	−	−	+
S. cerevisiae	12	−	−	−	×	×	−	−	−	×	8 to 12	−	+	+	−	−	−	×	×	+ +
S. cerevisiae var. *ellipsoideus*	12	−	×	−	×	×	−	×	×	×	12 to 16	−	+	×	−	−	+	×	×	×
S. chevalieri	5	−	−	−	+	+	−	−	−	×	8	−	+	×	−	−	+	−	−	−
S. delbrueckii	3	+	+	−	+	×	−	−	−	×	8	−	+	−	+	+	+	−	−	+ +
S. diastaticus	4	−	−	−	+	×	−	+	×	3	12 to 16	−	+	±	−	−	×	+	×	×
S. elegans	1	−	+	−	+	+	−	+	+	36	8	−	+ +	+	−	−	−	−	−	+ +
S. fermentati	3	−	+	+	+	± ±	−	×	×	×	12	+	+	−	+	−	−	−	−	+
S. fragilis	5	×	+	+	+	+ ±	+	+	−	236	8 to 12	+	+	+	+	+	−	−	−	+
S. fructuum	3	×	−	−	+	±	−	−	−	3	12 to 16	+	+	±	−	−	+	−	−	×
S. heterogenicus	3	−	−	−	+	×	−	+	±	0	≤8	−	×	−	−	−	−	−	−	−
S. italicus	1	−	−	−	+	+	−	+	−	3	8	+	+	+	−	−	−	−	−	+
S. logos	3	−	−	−	×	−	−	−	−	236	8 to 12	−	+	+	−	−	+	×	×	+
S. marxianus	2	×	+	+	+	±	±	+	−	236	8	+	+	±	+	+	−	−	−	×
S. mellis	1	−	+	+	−	+	−	−	−	2	< 8	+	−	−	−	−	−	−	−	+ +
S. microellipsodes	1	−	+	+	+	+	−	−	−	0	8	−	+	−	−	+	−	−	−	+
S. oviformis	12	−	−		±	×	−	×	±	×	12 to 16	×	+	×	−	−	×	−	−	−
S. pastorianus	3	−	−	−	×	+	−	−	±	236	8	−	+	−	−	−	−	×	×	+
S. rosei	3	−	+	+	+	+ +	−	+	±	×	12 to 16	+	+	−	±	−	−	−	−	+
S. rouxii	6	−	−	+	+	+	−	−	±	0	8	+	×	−	−	−	+	−	×	+ +
S. steineri	8	−	−	−	±	×	−	−	±	×	8 to 12	+	±	+	−	−	×	−	−	+
S. uvarum	5	−	−	−	±	±	−	×	×	236	8 to 12	−	+	×	×	−	±	−	−	+
S. veronae	3	−	×	±	+	± +	−	−	±	236	8	+	+	−	−	−	−	−	−	×
S. willianus	8	−	×	−	±	+	−	+	±	0	8 to 12	+	×	×	−	−	−	−	−	+ +

Tryptophan: + = growth; + + = brown halo round colony; − = no growth.
Flocculence, fining: — = absent; +, + + = increasing fining or flocculence.

Tributyrin and chalk agars: + = zone of clearing round colony; − = no effect.
Other tests: + = growth; − = no growth; × = various reactions between strains, or between tests with same strain.

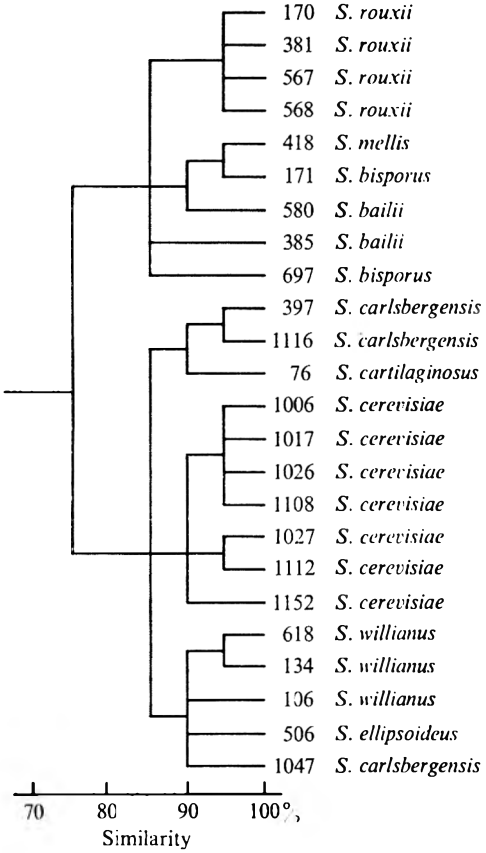

Fig. 1

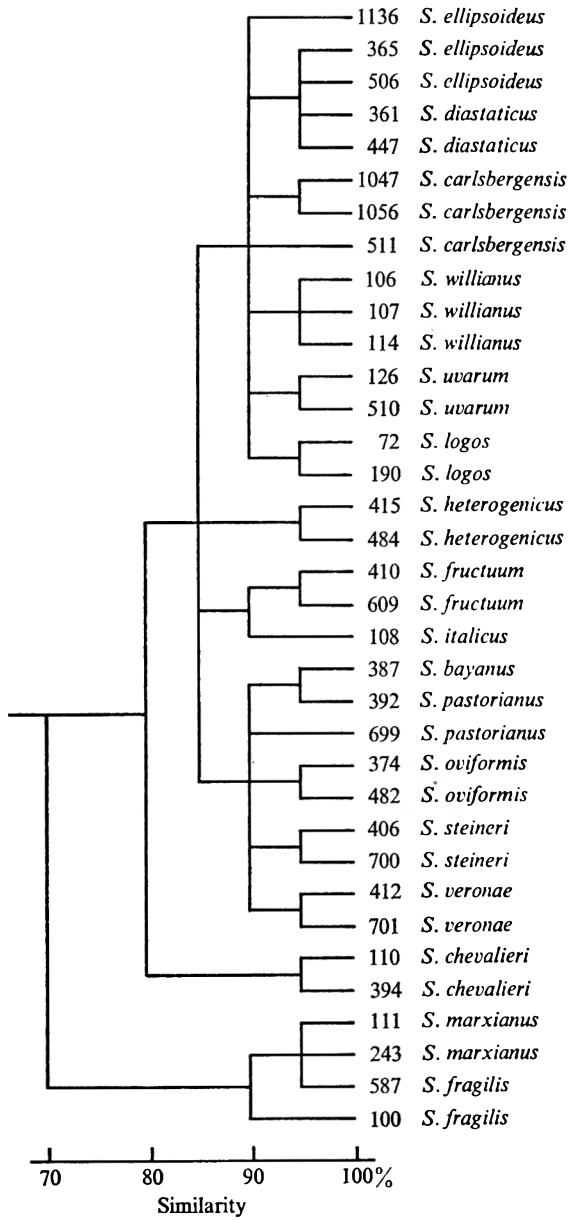

Fig. 2

Fig. 1 Dendrogram of typical NCYC strains reacting with serum A, and selected group C strains (*Saccharomyces carlsbergensis* 1047, *S. ellipsoideus* 506 and *S. willianus* 106) for comparison.

Fig 2. Dendrogram of typical NCYC strains reacting with serum B (*Saccharomyces marxianus* and *S. fragilis*) and serum C (other species).

found. Sera to *Saccharomyces oviformis* and *S. steineri* agglutinated cultures of both groups A and C; absorption by a culture of one group left agglutinating activity to all cultures of the other group. All species agglutinated by serum A, other than *S. rouxii*, were also agglutinated by the serum 1 described previously (Campbell, 1968). No reactions were noted between the newly examined strains and sera 2, 3, 4 and 5.

Numerical analyses. The identification tests of Lodder & Kreger-van Rij (1952) accounted for 20 morphological and physiological characters. Some tests, e.g. fermentation of glucose, were not applicable because of consistently positive results in the genus *Saccharomyces*; other tests, e.g. pellicle formation, were consistently negative. The remaining properties of each species are summarized in Table 2. Typical examples of

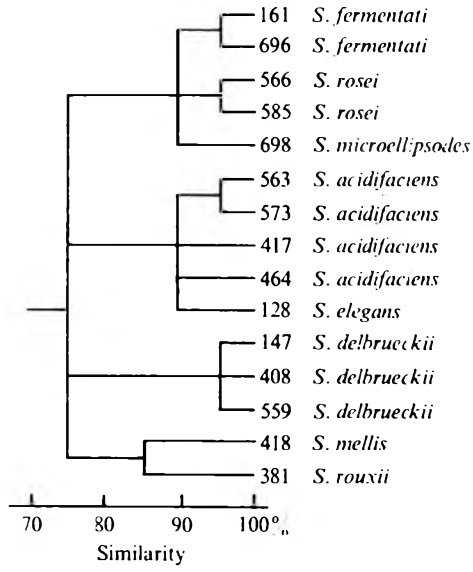

Fig 3. Dendrogram of typical NCYC strains reacting with serum D, and selected group A strains (*Saccharomyces mellis* 418 and *S. rouxii* 381) for comparison.

dendrograms are presented in Fig. 1 to 3, which show only NCYC strains, although additional strains of most species, from the NCYC and elsewhere, were examined. The total number of strains tested of each species is shown in Table 2.

The relationship between *Saccharomyces cerevisiae*, *S. carlsbergensis* I, two strains of *S. willianus* and the one strain available of *S. cartilaginosus*, all of which react with serum A, is illustrated in Fig. 1. The 90 to 97 % mutual similarity of all strains of *S. rouxii*, and the more distant relationship of other species of serological group A is also shown. However, the small-celled species *S. bailii*, *S. bisporus*, *S. mellis* and *S. rouxii* show a percentage similarity of 75 or less to the large-celled species *S. carlsbergensis* and *S. cartilaginosus* which similarly react only with serum A. The '*S. carlsbergensis* group' shows more affinity to *S. cerevisiae* and the two strains, 134 and 618, of *S. willianus* reacting with sera A and B, but these *S. willianus* strains in turn show a closer similarity to the group C strains of their own species, of which strain 106 appears as an example in Fig. 1. *Saccharomyces willianus*, irrespective of serological properties, is taxonomically closer to *S. cerevisiae* var. *ellipsoideus* and those strains of *S. carlsbergensis* which react with serum C, than to *S. cerevisiae*.

Fig. 2 illustrates the close relationship between the strains of groups AC and C, e.g. the 80 to 85 % similarity between *Saccharomyces oviformis* (AC) and *S. cerevisiae* var. *ellipsoideus* (C). *Saccharomyces fructuum*, *S. heterogenicus* and *S. italicus*, serologically of group C, occupy an intermediate position on the dendrogram between the '*S.*

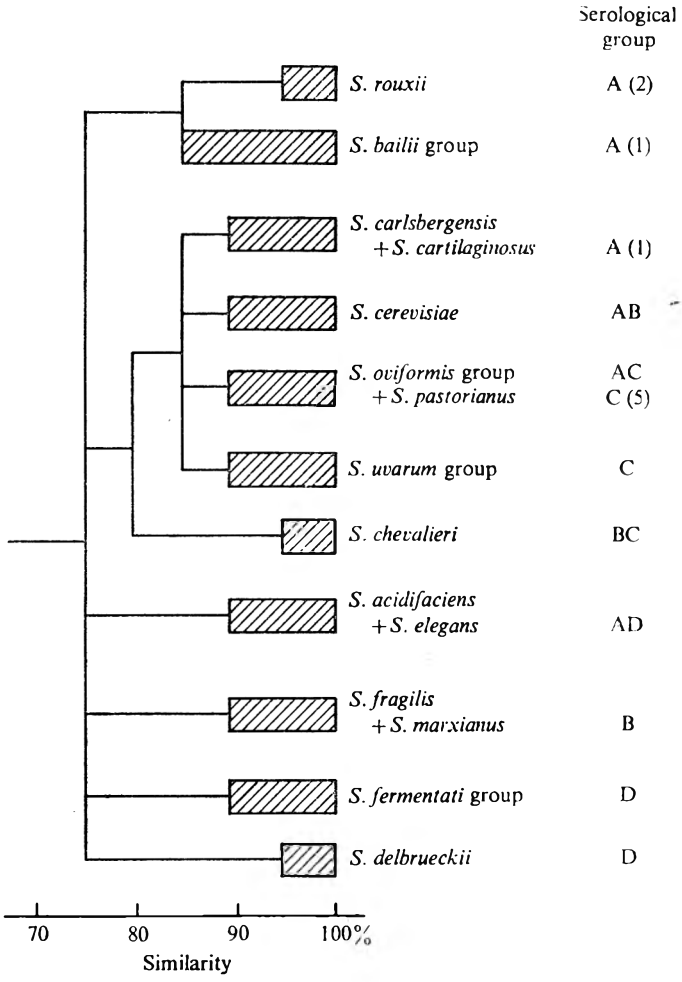

Fig. 4. Dendrogram of all species tested, from NCYC and other sources. Spread of proposed groups is denoted by shading.

carlsbergensis-S. willianus group' (C) and the '*S. oviformis group*' (AC), thus producing a large cluster of species with no significant subgrouping. Only *S. chevalieri* and the *S. fragilis–S. marxianus* group showed a significant difference from other species shown in Fig. 2, which agrees well with their different serological properties, BC and C respectively.

Fig. 3 shows all NCYC strains which reacted with the specific group D serum. *Saccharomyces mellis* and *S. rouxii* are included for comparison with organisms of serological group A; both groups are mainly composed of species which ferment few of the sugars tested, or only glucose, but on taking into account all 48 properties the

similarity between species of serological groups A and D is no more than 75 %. *Saccharomyces acidifaciens* and *S. elegans* react with both sera A and D, the serological properties reflecting a marked taxonomic difference.

Comparison of serological and physiological groupings. Strains were allocated to distinct groups according to all suitable physiological and morphological tests (Fig. 1 to 3). In Fig. 4, compiled from a final analysis of representative NCYC strains of each species, or taxonomic group as determined above, the observed groups are compared with our serological classification (Campbell, 1968; and Table 1 above). Distinct groups, corresponding to antigenic properties, include *Saccharomyces acidifaciens* and *S. elegans* (group AD), *S. fragilis* and *S. marxianus* (B), *S. chevalieri* (BC), and *S. fermentati*, *S. microellipsodes* and *S. rosei* (D). *Saccharomyces rouxii* forms a taxonomically and serologically distinct subgroup of a group of small-celled poorly fermentative species of serological group A. *Saccharomyces delbrueckii*, although also of group D, is only distantly related to *S. fermentati*.

DISCUSSION

Many antigenically identical species (Campbell, 1968) have been shown by Kockova-Kratochvilova *et al.* (1966*a*, 1968) to be very similar in general properties. The present report describes an investigation by numerical taxonomy of a full range of Saccharomyces species of which the antigenic structure has been determined by our system using four sera A, B, C and D.

The largest group of Saccharomyces species, reacting with sera A, A and B, A and C, or C alone, contains the large-celled species actively fermenting a wide range of sugars: glucose, sucrose, maltose, often galactose and raffinose, and occasionally maltotriose. These species are important in the fermentation industries, both as culture yeasts and as contaminants. Antigenically they form four distinct groups, as shown in Fig. 4, but numerical analyses (above, and Kockova-Kratochvilova *et al.* 1966*a*, 1968) show that they are very closely related; all show a similarity by our method of calculation of 80 to 85 % between antigenically different strains and 85 to 90 % between strains of the same antigenic group. The species of antigenic group C, *S. carlsbergensis*, *S. cerevisiae* var. *ellipsoideus*, *S. diastaticus*, *S. uvarum* and *S. willianus*, show a particularly close interrelationship, and instances were noted of strains more closely related to strains of other species than to other strains of their own species, e.g. between *S. cerevisiae* var. *ellipsoideus* and *S. diastaticus* (Fig. 2).

In Fig. 2 and 4, *Saccharomyces pastorianus*, *S. bayanus* and the species reacting with sera A and C are grouped separately from those reacting with serum C only. Although *S. pastorianus* is not agglutinated by serum A, it possesses a more complex antigenic structure than species of the *S. carlsbergensis–S. willianus* 'C-only' group. Campbell (1968) described five minor sera as an aid to species classification; one of these, serum 5, agglutinated *S. pastorianus*, but the 'C-only' group does not react with any of the sera 1 to 5. Thus the species of simplest antigenic structure are distinguishable by numerical taxonomy from those species reacting with sera A and C or C and 5.

Saccharomyces carlsbergensis I (of serological group A), *S. cerevisiae* (group AB) and groups AC, including C5, and C may be valid subdivisions, separable at about 85 % similarity, but the results of Kockova-Kratochvilova *et al.* (1966*a*, *b*, 1967, 1968) obtained from a similar but not identical series of tests, combined with the above

observations suggest that the serological groups overlap to form a single large group. It is nevertheless useful, for diagnostic and epidemiological purposes, to subdivide serologically. In our experience, two strains showing a similarity of over 85 % are, almost invariably, serologically identical; conversely, two isolates which are serologically identical are normally over 85 % similar in other respects. The two antigenic types of *S. willianus*, and the difference between *S. delbrueckii* and the other species of serological group D, provided the only exceptions, and we have found the rapid serological method a reliable preliminary identification system for Saccharomyces yeasts isolated from spoilt beers, wines and foods. Although our basic system (Campbell, 1968), using only four sera A, B, C and D, gives more limited information than the more comprehensive antigenic analyses of Tsuchiya *et al.* (1965), it is sufficient for identification within the genus *Saccharomyces*. Thus any yeast isolate which ferments glucose actively, i.e. overnight at 25°, and is therefore presumed to be of the genus *Saccharomyces*, is quickly allocated to the appropriate antigenic group by a slide agglutination test. In most cases we found it was sufficient simply to record the isolation of '*S. oviformis* type' (group AC), '*S. bailii* type' (group A), etc. Following the precedent of Beech, Davenport, Goswell & Burnett (1968), no distinction was attempted between Saccharomyces species and the corresponding species of Candida or Torulopsis, and sporulation was not considered in our numerical analyses.

For more accurate identification, a set of seven sera was necessary: A, B, C and D, and the sera 1, 2 and 5. Serum 2 distinguished *Saccharomyces rouxii* from other species reacting with serum A; also, all other group A species were agglutinated by serum 1. Serum 5 separated *S. pastorianus* from the various species which reacted only with serum C. Thus, by agglutination test and a morphological examination of cells, e.g. to distinguish *S. carlsbergensis* or *S. cartilaginosus* from the small-celled species reacting with serum A, the various serological subgroups of the genus *Saccharomyces* may be rapidly distinguished. The numerical analyses of Kockova-Kratochvilova *et al.* (1966*a*, *b*, 1967, 1968) and the above results suggest that serological methods reliably identify groups of Sacharomyces species to the limit of taxonomic validity.

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Electron Microscopic Observations on Growing and Dividing Protoplasts of *Bacillus megaterium*

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SUMMARY

Protoplasts of *Bacillus megaterium* grew well and divided in 1 % casein hydrolysate (enzymic) medium containing 0·5 M-NaCl as the stabilizer. A fibrous layer of coat, possibly composed of murein, developed around the growing protoplast. A small protuberance then formed on the protoplast and grew until a symmetrical dumbbell-shaped body was formed. The coat did not develop around the 'daughter' protoplast.

Division was completely inhibited in the presence of penicillin, and a coat did not develop around the protoplast. Materials which might otherwise have formed the coat in the absence of penicillin were released into the medium.

After 12 h. of growth in the absence of penicillin, normal cell wall was formed around the cell, although reversion to bacillary form was not observed. The cells divided into 4 to 6 cells simultaneously by transverse septa, and intracellular membranous organelles (mesosomes) appeared within.

INTRODUCTION

A previous report (Kusaka, 1967) indicated that protoplasts of *Bacillus megaterium* grew well and divided in 1 % casein hydrolysate (enzymic) medium containing 0·5 M-NaCl as the stabilizer, and a part of the murein which was known to be completely lost in protoplast formation was formed on the cytoplasmic membrane during growth. When murein formation was disturbed by penicillin, division of protoplasts was completely inhibited. These facts suggested that formation of murein around the protoplasts might have been essential for their division. To obtain more detailed information about the changes in the surface structures of protoplasts during division and growth, the fine structure of the protoplasts has been examined by electron microscopy.

METHODS

Organism and culture conditions. Bacillus megaterium IAM 1166 was used throughout the study. Media, methods for formation of protoplasts and growth conditions were as described earlier (Kusaka, 1967).

Electron microscopy. Cells were harvested by centrifugation and suspended in 0·5 M-NaCl in 0·1 M-phosphate buffer (pH 6·5) containing 1 % OsO_4 (Merck) and kept at 4° for 15 h. After fixation, cells were washed several times with 50 % ethanol by gentle decantation and stained with saturated uranyl acetate in 50 % ethanol at 4° for 2 h. The pellets were then dehydrated in a graded series of ethanols, transferred to acetone and embedded in an Epon 812 mixture (Shell Chemical Co.). Sections were cut on a Porter–Blum MT-2 ultramicrotome (Ivan-Sorvall Co.) with a glass knife, picked upon

formvar-coated grids reinforced with carbon, stained with uranyl acetate and lead citrate (Frasca & Parks, 1965) and examined with a JEOL JEM–7A electron microscope operating at 80 kV.

RESULTS

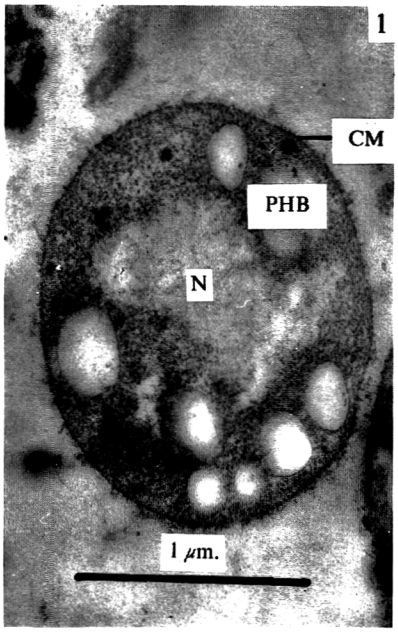
Initial protoplasts were almost completely free from cell wall and about 1·6 μm. in diameter. Small fibrous strands were attached to the surface of the cell, being perhaps remnants of cell-wall materials (Pl. 1, fig. 1). After 2 h. growth, the cell enlarged about 2·5 times in diameter and a coat-like material appeared all around the cell surface to form the outermost layer. A protuberance appeared on the coated protoplasts (Pl. 1, fig. 2) and enlarged during a further 1 h. of growth (Pl. 1, fig. 3*a*, *b*). until a symmetrical dumbbell-shaped body had formed (Pl. 2, fig. 4). In dividing cells, the coat did not form over the protuberance, so after division two kinds of daughter cells—one with and one without a coat—appeared. The coat was loosely packed and consisted of fibrous material (Pl. 2, fig. 5). The large nuclear region, containing sparse filaments, can be seen in the growing protoplasts (Pl. 1, fig. 3*b* and Pl. 2, fig. 5).

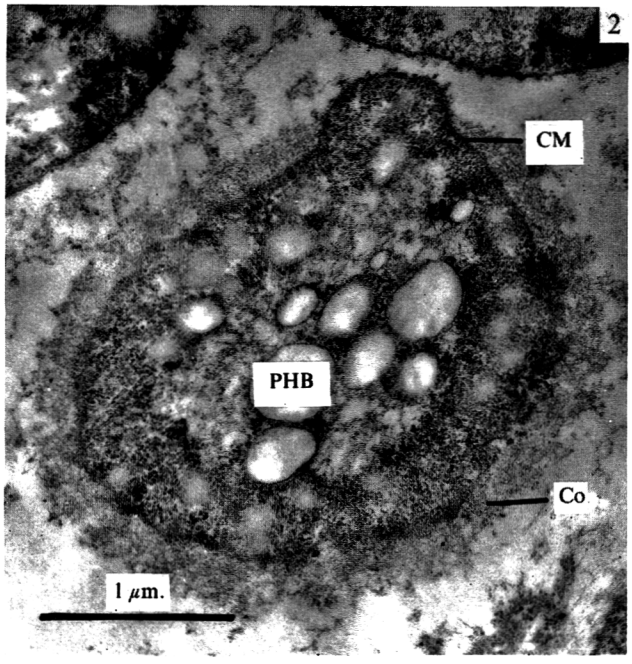
Protoplasts in the presence of penicillin. In the presence of penicillin (1000 units/ml.) division was suppressed but the cells enlarged to about 6 μm. in diameter. Thin sections of these cells d emonstrated that the coat did not develop around the protoplasts but fibrous strands which might otherwise have formed the coat were released into the medium (Pl. 2, fig. 6).

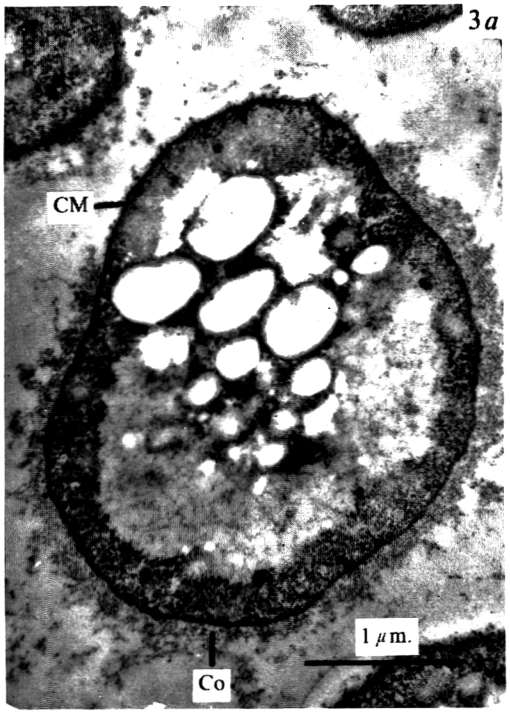
Cells after 12 h. of growth in the absence of penicillin. After about 12 h. of growth in the absence of penicillin, cell-wall formation was complete, and normal-looking, compact cell walls about 60 nm. thick were formed. These walls appeared to have two components, an inner, uniform layer and an outer, granular or fibrous layer, similar to the coat observed on protoplasts after a few hours growth. The cells were still spherical, and never revert to normal rods. Within these cells, several septa formed simultaneously, and intracellular membranous structures (mesosomes) were observed (Pl. 3, fig. 7, 8).

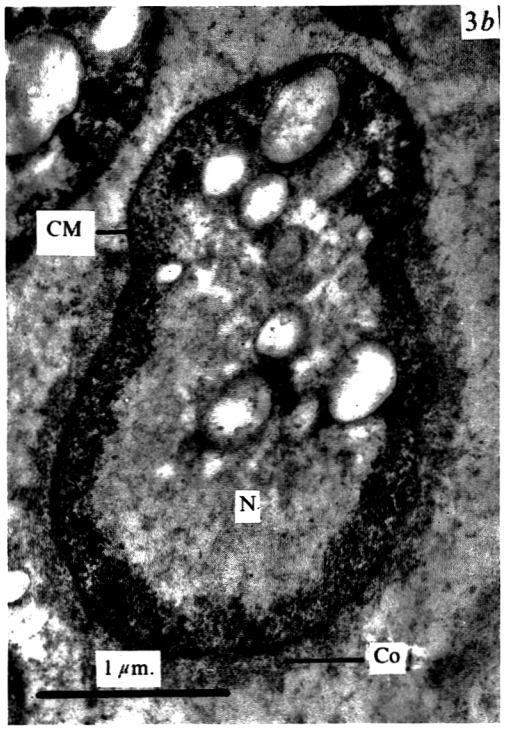
DISCUSSION

It was previously shown Kusaka (1967) that protoplasts of *Bacillus megaterium* grew well and divided in casein hydrolysate medium containing 0·5 M-NaCl as the stabilizer. The sequence of growth and division was similar to the results reported by McQuillen (1955). During the multiplication of protoplasts, the formation of cell wall was resumed and a part of the wall appeared to be formed on the protoplasts. When wall formation was prevented by penicillin, division was prevented although growth was not influenced. Observations presented in this paper revealed that the fibrous coat was formed around growing and dividing protoplasts. The coat did not develop in the presence of penicillin. Under these circumstances fibrous materials, which might otherwise have formed the coat, appeared to be released into the medium. These results indicate that the coat may have represented the new wall (murein) and that wall formation may have resumed during protoplast growth. They also indicate that the fibrous materials which were released into the medium may have represented unorganized murein synthesized in the presence of penicillin, since penicillin is known to prevent the cross-linking reaction in the synthesis of bacterial murein (Wise & Park,









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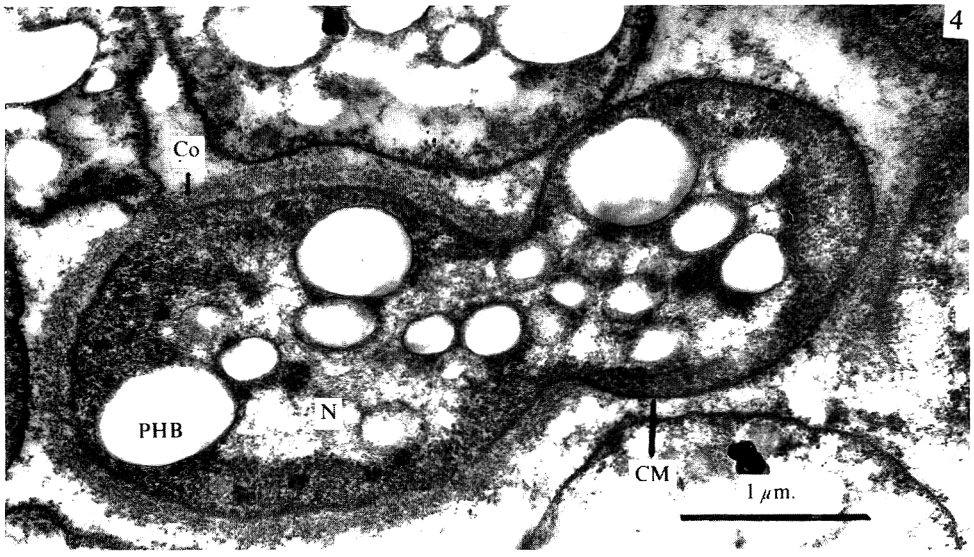
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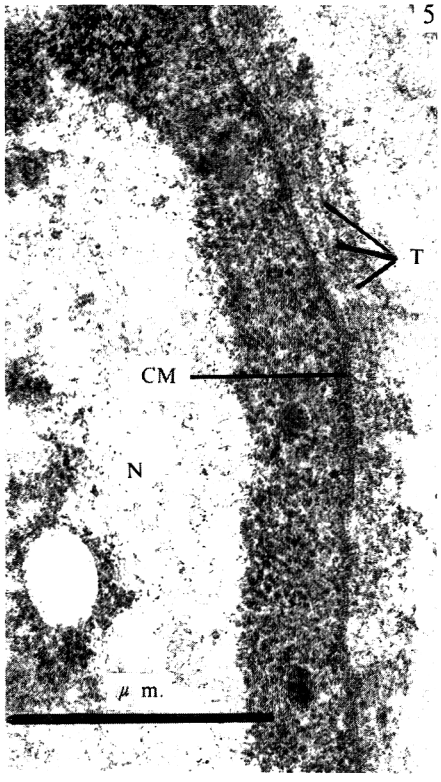
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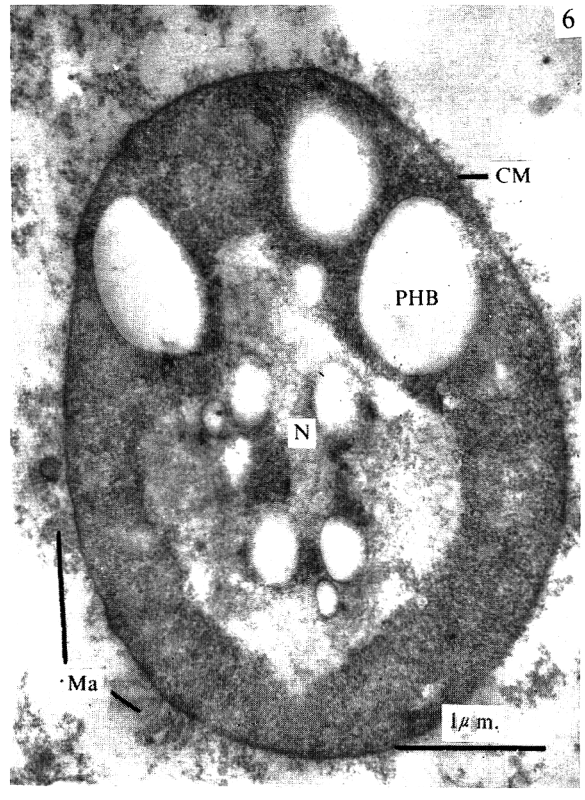
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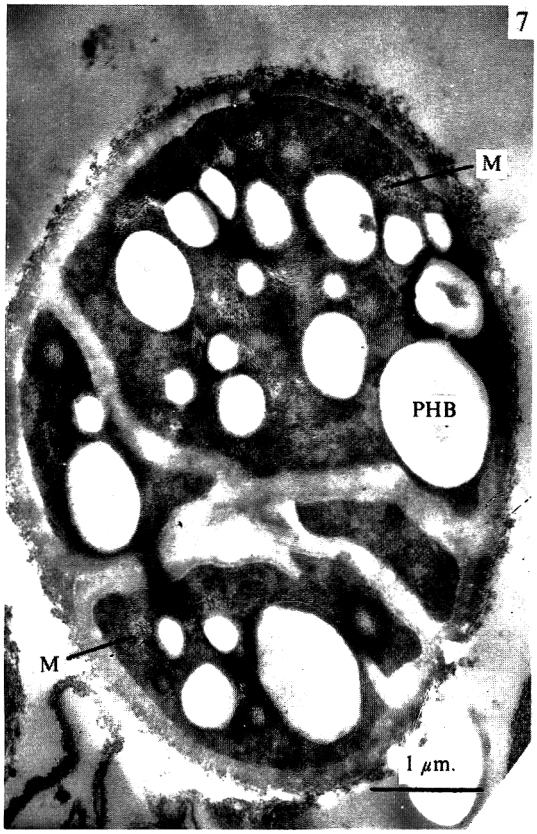
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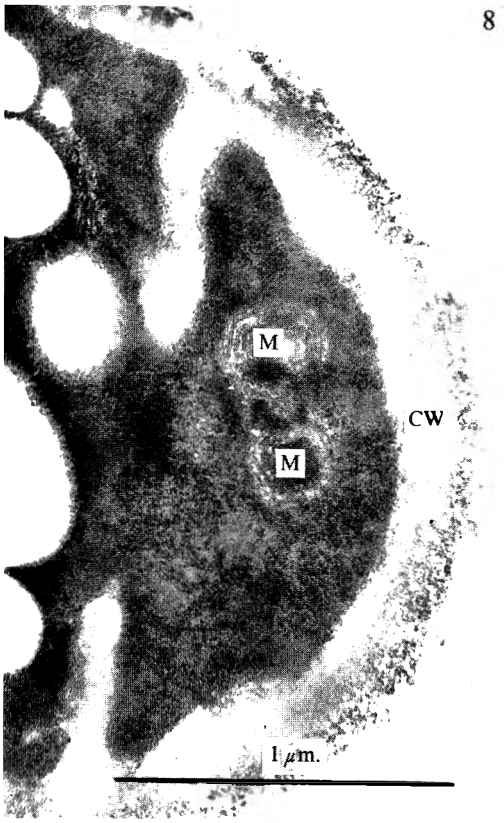
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1965). In dividing protoplasts the protuberant area lacked a coat and may have been extruded from the 'parent' which was still surrounded by one.

After about 12 h. growth in the absence of penicillin, wall formation by protoplasts was complete, a thick compact cell wall having been formed. A number of septa were then formed simultaneously, one cell dividing into 4 to 6 although reversion to a normal rod was never found. Recently, Landman, Ryter & Fréhel (1968) reported that protoplasts of *Bacillus subtilus* reverted to bacillary form when grown in gelatin media. In their study the reverting cells possessed a wide array of shapes, including branched and very irregular ones. These irregular forms, especially branched cells, might easily have led to rod-shaped cells. In liquid culture, as used here, cells were only spherical. These differences in morphology might be caused by the difference in the physical milieu surrounding the cell.

Mesosomes, which are expelled from the cells during protoplast formation (Fitz-James, 1964), were found in cells grown for 12 h. but their participation in septum formation was not observed. However, the strain used in the study contained poly-β-hydroxybutyrate granules which interfered with exact observations of the mesosomes. Mesosomes appeared to develop after normal cell walls were formed.

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EXPLANATION OF PLATES

CM, cytoplasmic membrane; PHB, poly-β-hydroxybutyrate granules; N, nuclear region; Co, coat; Ma, materials; M, mesosome; CW, cell wall.

PLATE 1

Fig. 1. Initial protoplast of *Bacillus megaterium*. × 30,000.

Fig. 2. Budding protoplast of *B. megaterium* showing a small protuberance breaking through the coat (Co). × 25,000.

Fig. 3 (*a*, *b*). Dividing protoplasts of *B. megaterium* showing a growing protuberance. Coat does not form on the protuberance. (*a*), × 20,000; (*b*), × 25,000.

PLATE 2

Fig. 4. Dumbbell-shaped protoplast of *Bacillus megaterium*. × 25,000.

Fig. 5. Organized fibrous materials in the coat (T) formed around the protoplast. × 70,000.

Fig. 6. Protoplast of *B. megaterium* grown in the presence of penicillin. The materials which might be the coat material appear to be secreted into the medium. × 20,000.

PLATE 3

Fig. 7. Multiseptate cell of *Bacillus megaterium*. × 15,000.

Fig. 8. Multiseptate cell of *B. megaterium* showing a typical mesosome × 45,000.

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Caproic Acid Metabolism and the Production of 2-Pentanone and Gluconic Acid by *Aspergillus niger*

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SUMMARY

Vegetative hyphae of *Aspergillus niger* rapidly converted caproic acid to 2-pentanone whereas germinating spores carried out the transformation slowly and ungerminated spores not at all. Glucose stimulated ketone production by germinating spores, but suppressed it in hyphae; the degree of stimulation and suppression varied with glucose concentration. This explains earlier reports that spores but not vegetative hyphae convert fatty acids to methyl ketones, since hyphae were earlier tested with high sugar concentrations, conditions where ketone formation was inhibited. Glucose disappeared from cultures containing caproate even though ketone production was inhibited, and glucose disappearance was paralleled by gluconic acid accumulation in the medium. These findings suggest that free fatty acids may play an important role in the regulation of metabolic pathways in *A. niger*.

INTRODUCTION

Some filamentous fungi produce methyl ketones from fatty acids (Stärkle, 1924; Stokoe, 1928; Acklin, 1929). The process, in which the methyl ketone formed is one carbon atom shorter than the fatty acid substrate, is specific, and fatty acids longer than 14 carbon atoms are not attacked (Thaler & Stählin, 1949).

Gehrig & Knight (1958) reported that spores of *Penicillium roqueforti* but not vegetative hyphae, were able to convert fatty acids into methyl ketones. Franke, Platzeck & Eichorn (1962) reported that vegetative hyphae of *Aspergillus niger* could not form methyl ketones from fatty acids, whereas spores carried out the transformation very efficiently. The process is widespread among filamentous fungi as indicated by the work of Franke & Heinen (1958), who found that 30 out of 38 fungi tested converted fatty acids into methyl ketones, and Gehrig & Knight (1961) who found that 9 out of the 11 Aspergillus species and 9 out of the 12 Penicillium species tested also carried out the transformation. Both these reports, however, indicated that members of the Mucorales were unable to produce significant amounts of methyl ketones from fatty acids.

Lawrence (1966, 1967) reported that both oxygen uptake and methyl ketone production by spores of *Penicillium roqueforti* were greatly increased by certain sugars and amino acids. He interpreted this to mean that these compounds were readily oxidized to yield either energy or metabolic products essential for activation of dormant spores.

The present study establishes that vegetative hyphae of *Aspergillus niger* rapidly

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convert caproic acid to 2-pentanone, while germinating spores carry out the transformation more slowly, and examines the relationship of the process to glucose catabolism.

METHODS

Stock cultures. Aspergillus niger van Tieghem was isolated from old caproic acid solutions which had a strong ketone odour. A single spore isolate was obtained by dilution and plating on Czapek's solution agar supplemented with 0·1 % yeast extract (Difco). Stock cultures were subsequently maintained on this medium.

Production of spores and vegetative hyphae. A medium of the following composition (%, w/v) was employed: D-glucose, 4·0; yeast extract, 0·5; Neopeptone (Difco), 1·0. Excellent sporulation was obtained on slants (Difco Bacto agar, 1·5 %, w/v) of this medium after 4 to 5 days incubation at 30°. The spores were harvested by adding 10 ml. sterile 0·85 % NaCl to the slant and rubbing the surface with a stiff inoculating needle. The spore suspension was adjusted to 1×10^9 spores/ml. and 5 ml. of this suspension were added to each experimental flask. For experiments with vegetative hyphae, spores were obtained as described above, the spore suspension adjusted to 5×10^7 spores/ml. and 1·0 ml. of the suspension added to 50 ml. liquid medium in 250 ml. Erlenmeyer flasks. These flasks were incubated at 30° on a rotary shaker (New Brunswick Scientific Co., Psychrotherm) at 180 rev./min. After 24 h. the vegetative hyphae were harvested, washed three times by centrifugation and resuspended in sterile 0·85 % NaCl under aseptic conditions. The hyphal density was adjusted to 230 μl. packed mycelium/ml. suspension by either removal or addition of sterile NaCl (0·85 %) solution (Conway & Downey, 1950). Ten ml. hyphal suspension were used as an inoculum for each experimental flask.

Experiments on caproate utilization and 2-pentanone production. Experiments were carried out with the following solution (%, w/v): caproic acid, 0·5; $NaNO_3$, 0·2; K_2HPO_4, 0·1; KCl, 0·05; $MgSO_4.7H_2O$, 0·001; distilled water). This basal solution was supplemented with 0·01, 0·1, 0·5 and 1·0 % D-glucose, adjusted to a final pH of 6·9 with 0·1 N-KOH, and 110 ml. of each formulation including unsupplemented solution, placed in 500 ml. Erlenmeyer flasks. These solutions and culture media were sterilized in an autoclave at 121° for 15 min.

Both spores and vegetative hyphae were incubated on a reciprocating shaker (Eberbach) at $28 \pm 2°$ and 100 strokes/min. for 98 h. Four ml. samples were removed when required.

Analytical methods. 2-Pentanone and caproic acid were determined by gas–liquid chromatography in an Aerograph model A–600–D chromatograph equipped with a hydrogen flame ionization detector and a Leeds and Northrup Speedomax H recorder. The carrier gas was nitrogen flowing at 25 ml./min. The detector was operated at the same temperatures as the column, since it was mounted in the column oven.

2-Pentanone was resolved on a 5ft × $\frac{1}{8}$ in. stainless steel column containing as a stationary phase 20 % phenyl diethanolamine succinate on Chromosorb W (acid-washed and treated with dimethyl-dichlorosilane, DMCS). The column was operated at 100° and the injector block at 150°. A sample of the culture fluid was introduced on to the column by flash vaporization, and amounts of 2-pentanone determined by comparing peak heights with those for pure 2-pentanone standards. Caproic acid was resolved on a 5 ft × $\frac{1}{8}$ in. stainless steel column containing as a stationary phase 10 %

FFAP (Varian Aerograph Corp.) on Chromosorb W, acid-washed and DMCS-treated. The column was operated at 175° and the injector block at 225°. A sample of the culture fluid was introduced on to the column by flash vaporization, and amounts of caproic acid determined by comparing the product of the peak height and its width at half height with the values obtained for pure caproic acid standards.

After ketone and fatty acid analyses were complete, the remaining culture fluids were evaporated to dryness in a flash evaporator at 90°, dissolved in distilled water and their sugar contents determined by the method of Folin & Malmros (1929).

Oxygen uptake measurements were made with a constant volume respirometer (Braun Instrument Co.) using standard manometric techniques (Umbreit, Burris & Stauffer, 1964). Three ml. samples from the ketones production media were added to Warburg vessels containing 0·1 ml. of 20 % KOH in the centre wells, oxygen uptake was measured over 1 h. at 30°. Dry weights were then determined for each sample.

Gluconic and citric acids in culture fluids were detected by paper chromatography (Koepsell, Stodola & Sharpe, 1952). Quantitative estimation of gluconic acid from paper chromatograms was done as described by Fisher, Parsons & Holmes (1949).

RESULTS

Spore preparations of *Aspergillus niger* lacking glucose converted caproic acid to 2-pentanone at a low rate and did not yield detectable ketone until after 50 h. (Fig. 1). When glucose was added to the basal medium, 2-pentanone production was stimulated in proportion to the amount of sugar added (Fig. 1), and it commenced much earlier although none was produced before 12 h. The onset of ketone formation was correlated with the emergence of germ tubes. Ungerminated spores were bound very tightly to hyphae and to germ tubes, indicating that spores differed in surface charge from the other two forms.

Vegetative hyphae produced 2-pentanone from caproic acid at a rapid rate, yielding detectable ketone after 3 h. Addition of glucose to the basal medium, except in very small amounts, caused a suppression of ketone production in proportion to the amount of glucose added (Fig. 2).

Spore preparations did not utilize detectable quantities of caproate until germination had occurred (Fig. 3). Glucose greatly stimulated caproate disappearance, which paralleled 2-pentanone formation. On the other hand, vegetative hyphae utilized caproate immediately, the utilization was suppressed by the addition of glucose (Fig. 4). This suppression paralleled the depression of 2-pentanone production.

Manometric measurements showed that large amounts of O_2 were taken up by vegetative hyphae in the presence of caproate alone, and in the presence of caproate and 0·01 or 0·1 % glucose (Fig. 6). At higher glucose concentrations (0·5, 1·0 %) O_2 uptake was drastically reduced after 12 h. (Fig. 6). In all instances the initial rate of O_2 uptake was increased by glucose. Spore preparations did not begin O_2 uptake until after 12 h. and the rate of O_2 uptake was proportional to the amount of glucose added (Fig. 5). The initial lag in O_2 uptake coincided with the time required for germination of spores.

The pH values of spore cultures, with and without added glucose, increased steadily (Fig. 7). At low glucose concentrations the pH values of vegetative hyphal cultures also increased, but decreased at higher glucose concentrations (Fig. 8). The drop in

pH values coincided with the suppression of fatty acid utilization and ketone production and the decrease in respiratory activity. The pH of both spore and hyphal cultures containing 1 % glucose but no caproate fell rapidly owing to accumulation of organic acids (Fig. 7, 8).

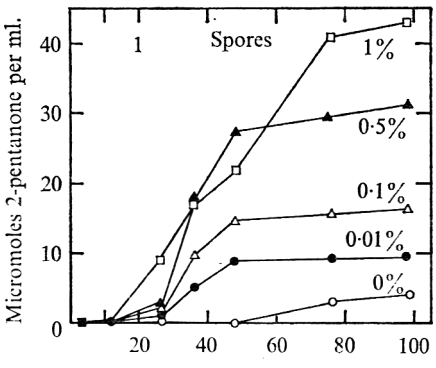

Fig. 1

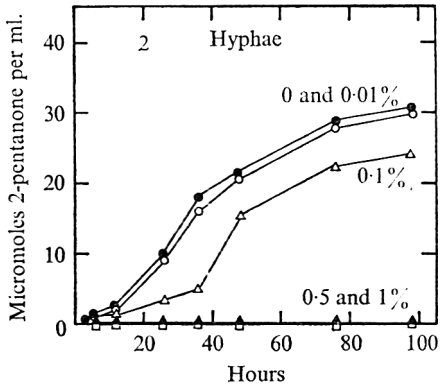

Fig. 2

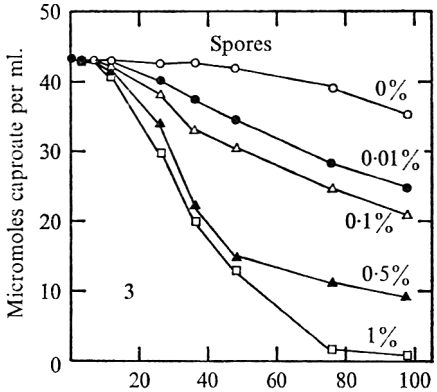

Fig. 3

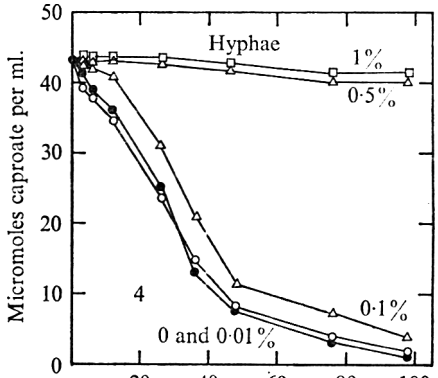

Fig. 4

Fig. 1 to 4. Effect of glucose concentration on 2-pentanone production (Fig. 1, 2) and caproate utilization (Fig. 3, 4) by spore and hyphal cultures of *Aspergillus niger* containing 0·5 % caproate. No glucose, ○—○; 0·01 % glucose, ●—●; 0·1 % glucose, △—△; 0·5 % glucose, ▲—▲; 1·0 % glucose, □—□.

Both spore and vegetative hyphal cultures containing caproate utilized glucose, but the latter utilized considerably more than the former. With 1·0 % glucose, vegetative hyphae utilized 25 % of the sugar during the first 26 h. with the Q_{O_2} never exceeding 4.

Hyphal cultures with caproate but with glucose absent or in low concentrations did not accumulate detectable quantities of gluconic acid. At higher glucose concentrations gluconic acid accumulated in the culture fluid. The quantity of gluconic acid present increased with time of incubation and increasing glucose concentration (Fig. 9). Vegetative hyphal cultures with 1·0 % glucose but no caproate accumulated gluconic acid during the first 12 h. and utilized it during the next 24 h. (Fig. 9). Spore cultures never accumulated detectable quantities of gluconic acid.

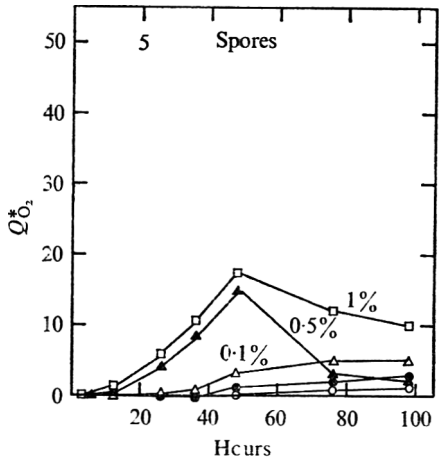

Fig. 5

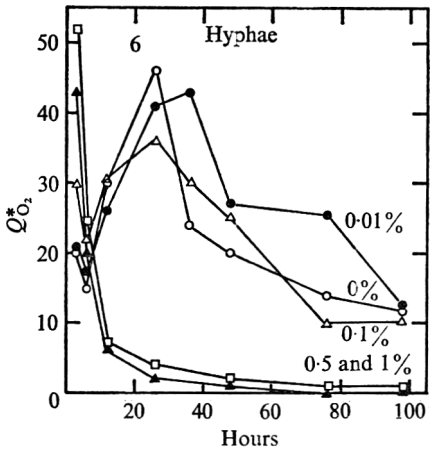

Fig. 6

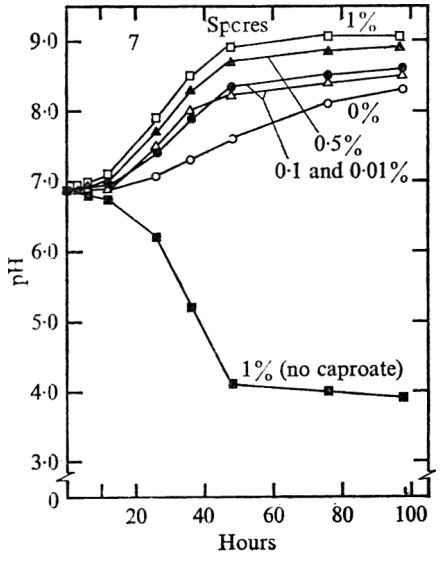

Fig. 7

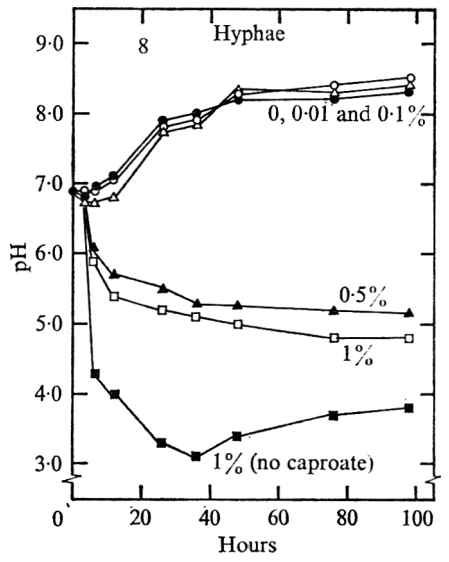

Fig. 8

Fig. 5 to 8. Effect of glucose concentration on respiratory activity (Fig. 5, 6) and pH values (Fig. 7, 8) of spore and hyphal cultures of *Aspergillus niger* containing 0·5 % caproate. No glucose, ○—○; 0·01 % glucose, ●—●; 0·1 % glucose, △—△; 0·5 % glucose, ▲—▲; 1·0 % glucose, □—□; cultures without caproate but containing 1·0 % glucose, ■—■.

DISCUSSION

Several investigators (Gehrig & Knight, 1958; Franke *et al.* 1962; Lawrence, 1966) have maintained that only ungerminated spores of filamentous fungi can convert fatty acids to methyl ketones. My studies have shown (Fig. 1) that ungerminated conidia of *Aspergillus niger* convert caproic acid to 2-pentanone at an extremely low rate, if at all. Moreover, the course of ketone production by conidia was directly related to the course of germination and formation of young hyphae. This agrees with

the report of Franke, Platzeck & Eichorn (1961) that the β-keto acid decarboxylase activity of *A. niger* was greatest in young mycelia.

The production of 2-pentanone from caproic acid by vegetative hyphae of *Aspergillus niger* is contrary to the report of Franke *et al.* (1962), which stated that vegetative hyphae of this organism were unable to form methyl ketones from fatty acids. However, good ketone production by spore preparations depends upon a supplementary growth substrate such as glucose, as found in the present study (Fig. 1) and by other workers

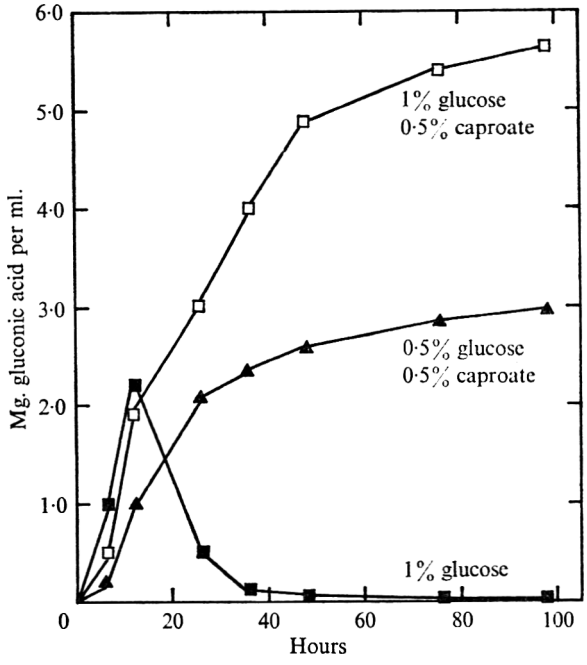

Fig. 9. Effect of caproate (0·5 %) on gluconic acid production from glucose by hyphal preparations of *Aspergillus niger*. One % glucose alone, ■—■; 0·5 % glucose + 0·5 % caproate, ▲—▲; 1·0 % glucose + 0·5 % caproate, □—□.

including Franke *et al* (1962) and Lawrence (1966). Under these conditions, ketone production by vegetative hyphae is practically nil (Fig. 2). It is therefore not surprising that reports of the inability of vegetative hyphae to form ketones from fatty acids have appeared, since the hyphae were usually tested under conditions for maximal ketone production by spore preparations.

The suppression of the conversion of caproate to 2-pentanone in vegetative hyphae by glucose (Fig. 2) may be caused by the oxidation of glucose to gluconic acid with the consequent fall in pH, since this would result in an increased concentration of undissociated as compared with dissociated caproic acid, and as shown by Lewis & Johnson (1967), high concentrations of undissociated caproic acid can inhibit terminal respiratory enzymes, and thus fatty acid oxidation. Glucose oxidase did not, however, appear to be inhibited since glucose disappearance and gluconic acid production continued even though fatty acid oxidation and ketone formation had ceased.

Activation of fatty acid oxidation in *Aspergillus niger* spores by glucose (Fig. 1) is consistent with the report of Lawrence (1965). Very little carbon or energy could have

been derived from fatty acid oxidation by spores, since caproate was converted almost exclusively to 2-pentanone. Increases in pH and Q_{O_2} values with increasing glucose concentrations indicated that spores and young mycelia oxidized glucose completely. Gluconic acid never accumulated in caproate containing cultures of germinating spores, and only small amounts of other organic acids were detected. These results are consistent with the report by Bhatnager & Krishnan (1960) that activated spores and vegetative hyphae of *A. niger* contain different enzymes.

Moyer, Umberger & Stubbs (1940) found that gluconic acid production by *Aspergillus niger* was affected by the pH value of the culture medium. At pH values above 6·5, glucose was converted almost exclusively to gluconic acid, whereas citric acid was the predominant acid produced at pH values below 3·0. Butkewitsch (1924) pointed out that gluconic and citric acid formation proceeded sequentially in fungal cultures with initial pH values near neutrality. Gluconic acid was produced when the pH value was above 6·5, but as the pH value fell below 6·5 citric acid accumulated. In the present study this occurred in vegetative hyphal cultures with 1·0 % glucose and no caproate but not when 0·5 % caproate was present in addition to glucose. Gluconate production then continued after the pH value fell below 6·5 (Fig. 8, 9).

Ehrlich & Lewis (1968) found that fatty acids (C_3–C_{13}) did not inhibit glucose oxidase from *Aspergillus niger* although succinic dehydrogenase activity was completely inhibited and many of the fatty acids stimulated glucose oxidase activity, the effect being maximal at pH values below 6·5. Selective inhibition of enzymes involved in glucose dissimilation by free fatty acids under conditions where gluconeogenic enzymes were not affected had been reported for bacterial (Ferdinandus & Clark, 1969) and animal (Weber, Convery, Lea & Stamm, 1966) systems.

The present study shows that caproic acid can alter the mode of glucose catabolism in *Aspergillus niger*; and since the lipid fraction of *A. niger* may contain high concentrations of free fatty acids (Bernhauer & Posselt, 1937; Cochrane, 1958), these compounds may be of importance for the control of metabolic pathways in this fungus.

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A Simple Chemically Defined Medium for the Production of Phase I *Bordetella pertussis*

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SUMMARY

A simple, chemically defined medium is described consisting of sodium glutamate, proline, cystine, salts, and growth factors, which is suitable for the large-scale production of phase I *Bordetella pertussis*. More than 30×10^9 organisms/ml. were produced in 48 to 72 h. growth in shake flasks and fermentors. The cultures were detoxified by the addition of 0·14 % formalin to yield vaccines which were non-toxic to mice and guinea pigs and had good mouse-protective antigen levels. The antigenic stability was satisfactory after storage of the final vaccines at elevated temperatures.

INTRODUCTION

Hornibrook (1939) first described the use of a liquid medium for the propagation of phase I *Bordetella pertussis*, and since that time many modifications of his original formulation have been published (Verwey & Sage, 1945; Wilson, 1945; Cohen & Wheeler, 1946; Verwey, Thiele, Sage & Schuchardt, 1949; Sutherland & Wilkinson, 1961). These liquid media have consisted of a casein hydrolysate to which were added various salts, growth factors, and either starch, charcoal, or anionic resins. Studies on the growth requirements of *B. pertussis* have resulted in the publication of several formulae for chemically defined media (Ungar *et al.* 1950; Jebb & Tomlinson, 1955, 1957; Wilson, 1963; Goldner, Jakus, Rhodes & Wilson, 1966), and an excellent review of the problem by Rowatt (1957). In a series of metabolic studies on *B. pertussis* in our laboratories, Wilson (1963), Goldner *et al.* (1966), Vajdic, Goldner & Wilson (1966), used a synthetic medium in rocked L-tubes and obtained good growth of phase I organisms. The composition of their optimal medium is given in Table 1. This is a completely defined medium apart from the liver co-enzyme preparation, but would be unsuitable for large-scale production due to its relative complexity. This paper describes further modifications which have resulted in greatly improved yields of phase I bacteria. The properties of vaccines prepared from these cultures were also examined.

METHODS

Seed culture preparation and growth conditions

The strain of *Bordetella pertussis* used in most of our investigations was one of the Connaught Laboratories' routine vaccine production strains, obtained originally from Dr Pearl L. Kendrick, and designated no. 18334. Two other strains were also used in the large-scale production experiments, Kendrick's no. 10536, and a strain obtained

from Dr R. J. Wilson, no. 1494. The lyophilized cultures were grown on a Bordet–Gengou plate for 4 to 5 days, subcultured to Bordet–Gengou slants in $8 \times 1\frac{1}{2}$ in. tubes, and grown for a further 48 h. All Bordet–Gengou media contained 30% citrated sheep's blood. The growth from one slant was washed off into three 500 ml. Erlenmeyer flasks, containing 100 ml. liquid medium (see below), and grown on a rotary

Table 1. *Composition of chemically defined media for Bordetella pertussis*

Comparison between Goldner's formula and basic 'glutamate + proline' medium ('1 G + 1 P' medium).

Component	Goldner's formula* (mg./l.)	Basic 'glutamate + proline' medium ('1 G + 1 P') (mg./l.)
DL-Alanine	100	—
L-Arginine	40	—
DL-Aspartic acid	130	—
L-Glutamine	400	—
Glycine	50	—
DL-Histidine	50	—
DL-Serine	130	—
L-Proline	240	240
L-Glutamic acid	620	670†
L-Cystine	10	40
NaCl	2500	2500
KH_2PO_4	500	500
KCl	200	200
$MgCl_2.6H_2O$	100	100
$CaCl_2$	20	20
$FeSO_4.7H_2O$	10	10
$CuSO_4.5H_2O$	5	—
Adenine	10	—
ATP (Di-Na salt)	0·3	—
Guanine	0·3	—
Hypoxanthine	0·3	—
Thymine	0·3	—
Uracil	0·3	—
Xanthine	0·3	—
Activated Charcoal (B.D.H.)	15	—
Tris buffer 121	6075	6075
Liver co-enzymes (CoA, DPN, TPN)	100	—
Asorbic acid	5	20
Niacin	1	4
Glutathione	25	100
2-Deoxyribose	0·5	—

* Compiled from Goldner *et al.* (1966).
† Present as sodium glutamate.

action shaker for 48 h. (Model V, New Brunswick Scientific Co., New Jersey, U.S.A.; this model has a 1 in. circular orbit and was used at 350 rev./min). In the early work a casein hydrolysate medium (Wilson, 1945) was used for the growth of seed cultures, but the chemically defined medium was substituted in the shake flask and small fermentor experiments, following certain modifications, which will be discussed later. All growth temperatures were between 35 and 37°. Five ml. inocula were taken from these seed flasks and used to seed each 100 ml. experimental medium in 500 ml. flasks. In the large-scale production work, three sizes of fermentors were used: a Pyrex

jar (18 × 10 in.), containing 9 l. medium, and stainless steel tanks containing either 60 or 140 l. medium. All vessels were aerated by vortex stirring with air blown over the surface at the rate of 0·1 l. of air/l. of medium/min. Approximately 5 % seed was employed for the fermentors, which were grown for 48 to 72 h.

Basic medium preparation

In very early work it was found that the medium described by Goldner *et al.* (1966) could be simplified without interfering with the final yields of bacteria obtained. The nucleic acid derivatives, liver co-enzymes, 2-deoxyribose, $CuSO_4$, and all of the amino acids except glutamic acid, proline, and cystine could be left out. The addition of charcoal, starch or resin was also found to be unnecessary. The formula of the simplified basic medium is given in Table 1, in which it should be noted that the concentration of cystine, ascorbic acid, niacin, and glutathione have each been increased fourfold on those specified in Goldner's formula. Sodium glutamate is used in place of glutamic acid since it is more soluble and less expensive. This basic medium is termed the 'glutamate + proline medium' or '1 G + 1 P medium', and contains 670 mg. sodium glutamate and 240 mg. proline/l. The many other combinations of glutamate and proline used are expressed in terms of the concentrations shown in Table 1, e.g. the medium designated '16 G + 2 P' would contain 10,720 mg. glutamate and 480 mg. proline/l., etc. In the preparation of media for the shake flask experiments, the amino acids, salts and tris buffer were dissolved in the necessary amount of water, the pH adjusted to 7·6 with 2·5 N-HCl, and autoclaved. In the preparation of the large volumes of medium for the fermentors, the amino acids, salts and tris buffer were dissolved in water at 70°, and sterilized by steaming for 40 min. and autoclaving for 60 min. at 260° (17 to 18 p.s.i.). The cystine, glutathione, ascorbic acid, niacin and ferrous sulphate were sterilized by filtration and added to the autoclaved medium. When the ferrous sulphate was added before autoclaving a hazy precipitate of ferrous phosphate appeared and presumably the Fe^{2+} ions were no longer available to the organism since growth was diminished.

Bacterial concentrations

Bacterial concentrations were estimated by photometric comparison with the Opacity Standard for pertussis vaccine and challenge suspension, supplied by the National Institutes of Health, Bethesda, Maryland, U.S.A. Ten opacity units (o.u.) were considered as being equivalent to 10×10^9 organisms/ml.

Toxicity testing of finished vaccines

The cultures were detoxified by the addition of formalin (37 % solution of formaldehyde) at concentrations and for times described below. The treated cultures were centrifuged, the vaccines resuspended in 0·85 % NaCl and tested using the standard N.I.H. mouse toxicity test: ten mice (CMRL strain, random-bred), 14 to 16 g., were injected intraperitoneally with 10 o.u. vaccine and group weights assessed after 7 days. A further test for freedom from toxicity was carried out by injecting three guinea pigs (320 to 340 g.) with 100 o.u. vaccine contained in a volume of 5 ml. Two of the animals were injected intraperitoneally, and one subcutaneously, and observed for 16 days with individual weights being recorded.

Potency assays

The protective potencies of the vaccines were determined by the method set out in *Minimal Requirements for Pertussis Vaccine* (U.S., N.I.H. 1948, revised 1968) and the results calculated by the Worcester–Wilson procedure (Worcester & Wilson, 1943). For the stability studies, samples were made up to contain 10 or 20 o.u./ml., either as straight pertussis vaccine or in combination with 40 Lf Diphtheria toxoid and 8 Lf Tetanus toxoid, and stored for varying periods of time at 4, 25, or 37°. These results were assayed by Probit analysis with 95 % confidence limits. The antigenic potencies of the diphtheria and tetanus toxoids in the Diphtheria–Pertussis–Tetanus preparations were determined as described previously (Stainer, 1968), and the results expressed in International Units (i.u.)/ml. compared to the W.H.O. International Standards for diptheria and tetanus toxoids.

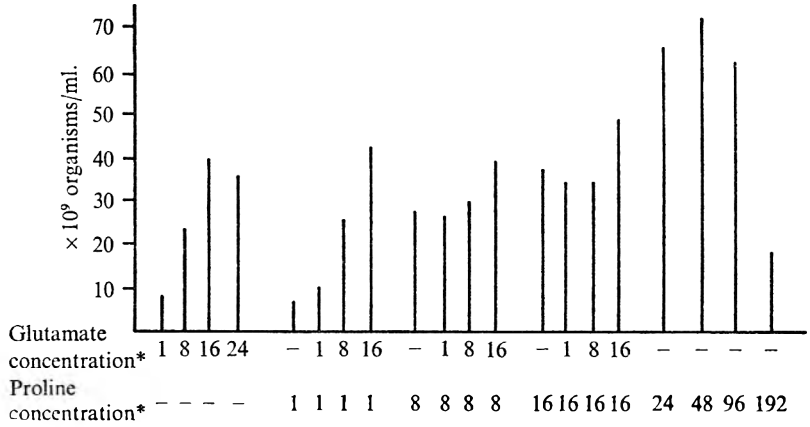

Fig. 1. Effect of various glutamate and proline concentrations on the opacity of *Bordetella pertussis* in shake flasks after 48 h. growth. *Concentrations of the amino acids are expressed relative to the basic 'glutamate + proline' medium ('1 G + 1 P') given in Table 1 (1 G, 670 mg. glutamate/l.; 1 P, 240 mg. proline/l.).

RESULTS

Growth in shake flasks

Using the '1G+1P' medium given in Table 1, yields of approximately 10×10^9 organisms/ml. were obtained after 48 h. growth. Fig. 1 illustrates the results of numerous experiments in shake flasks in which an increased concentration of either glutamate or proline in '1G+1P' medium is shown to have a significant effect on the final opacities attained. The seed cultures for these studies were grown in '16G+1P' medium and not in the casein digest medium (Wilson, 1945). This was found to be possible, if as previously mentioned, the ferrous sulphate was added *after* the medium had been autoclaved and also provided that the seed cultures were taken in the early exponential phase of growth. The medium designated '48P' gave the highest opacity values, followed by '24P', '96P', '16G+16P' and '16G+1P'.

Shake flasks containing '12G' medium (no proline) or '12P' (no glutamate) were sampled at 0, 30 and 48 h. after growth, the cultures centrifuged at 10,000 *g* for 1 h. and portions of the resultant supernatants chromatographed on Eastman Cellulose thin-layer chromatography sheets (no. 6065), using *n*-butanol + acetic acid + water

(4 + 1 + 1). In view of the high level of seeding employed (4 %) and to avoid the carry-over of extraneous amino acids, the flasks used for the chromatography experiments were seeded in the following way: the flask containing the '12G' medium was seeded with a culture grown in the '12G' medium, and similarily the flask containing the '12P' medium was seeded with a culture grown in the '12P' medium.

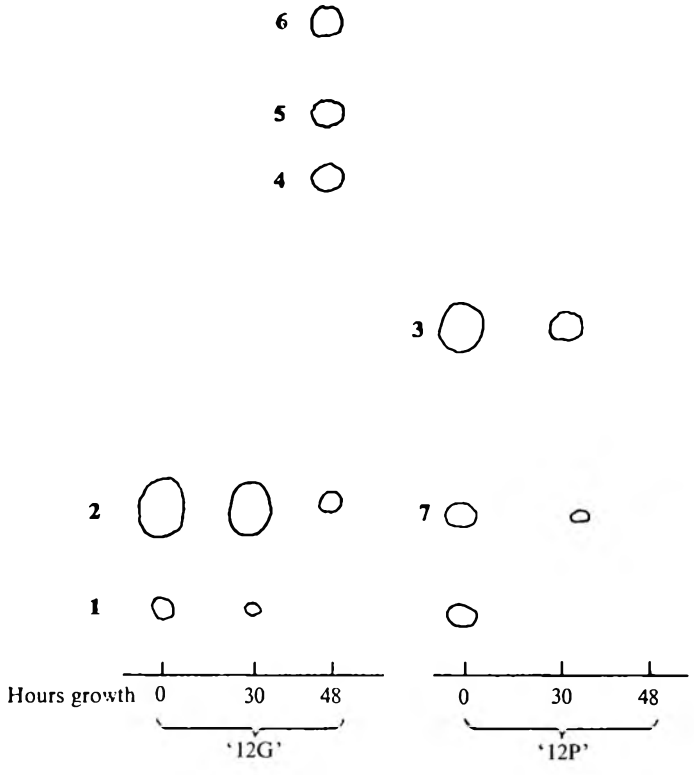

Fig. 2. Thin-layer chromatography of cell-free *Bordetella pertussis* culture supernatants on Eastman Cellulose paper (6065), using *n*-butanol + acetic acid + water (4 + 1 + 1).
1, cystine and glutathione; 2, glutamic acid; 3, proline; 4, threonine, 5, alanine; 6, valine; 7, glutamic acid (from glutathione).

Some interesting differences were noted as depicted in Fig. 2. Forty-eight h. after growth in the '12G' medium, three ninhydrin-positive spots were produced which were not present in the original medium. Subsequent analysis with the Spinco automatic amino acid analyser showed them to be threonine, alanine and valine. These amino acids were not present in the culture supernatants from the '12P' medium.

Growth in fermentors

Concurrent with the shake flask experiments, attempts were made to grow *Bordetella pertussis* in various chemically defined media in vessels of large capacity. Seed for the fermentors was prepared by inoculating 100 ml. of a 40 h. culture of *B. pertussis*, grown in a shake flask containing casein digest medium (Wilson, 1945), into 8 l. of the same medium in a 20 l. bottle. Air was bubbled through the bottle of medium at 1 l./min. and the culture grown for 40 h. Approximately 5 % seeding was used in these

studies. Table 2 shows the results obtained in three sizes of fermentors. Four different combinations of glutamate and proline concentrations were compared and gave essentially the same results as those from the shake flask experiments. The medium which gave the highest yields in shake flasks, namely '48P', was not used in large-scale production since proline is an expensive amino acid. The medium designated '16G+1P' gave good yields and was used to prepare numerous lots of culture suitable for conversion to vaccines.

Table 2. *The growth of Bordetella pertussis in chemically defined media in large volumes*

Volume (l.)	No. of experiments	Relative concentrations* G	Relative concentrations* P	Average yield after 48 to 72 h. growth† ($\times 10^9$ org.)
9	2	4	8	29·1
9	6	4	16	43·0
9	3	8	2	28·2
9	10	16	1	42·0
60	6	16	1	38·0
140	5	4	8	24·3
140	15	16	1	36·1

* Compared to basic '1G+1P' medium ('1G', 670 mg. glutamate/l.; '1P', 240 mg. proline/l.).
† The lag phase was extended depending on the age of the seed culture (see text).

Table 3. *Detoxification studies on Bordetella pertussis grown in '16G+1P' chemically defined medium*

No. of tests	Formalin (%)	No. days incubation at 36 to 37°	Av. wt gain per mouse (g.)	Av. no. deaths/test
3	0·07	3	3·8	2
5	0·07	5	4·4	1·4
1	0·07	7	6·8	0
5	0·1	5	5·5	1
3	0·1	6	5·6	0·3
5	0·1	7	7·5	0·5
3	0·12	7	7·7	0
6	0·14	5	7·4	0
		Conventionally-grown vaccines		
12	0·07	3	7·1	0
		Saline		
10	—	—	8·9	0

Detoxification studies

Cultures were detoxified by the addition of formalin as indicated in Table 3. After centrifuging, the vaccines were tested as described previously. Several experiments indicated that the most suitable conditions for producing a nontoxic vaccine were the addition of 0·12 % formalin and incubation for 7 days, or 0·14 % formalin and incubation for 5 days at 36 to 37°. Vaccines prepared in this manner gave satisfactory weight gains in mice. Vaccines produced by growing *Bordetella pertussis* in casein digest

medium (Wilson, 1945) require 0·07 % formalin and incubation for 3 to 4 days. The reason for the extra formalin needed to detoxify the cultures grown in the chemically defined medium could be related to the greater bacterial densities attained in this medium (30 to 40 o.u. compared to 20 to 30 o.u./ml. with conventionally grown cultures). Injection of the finished vaccines into guinea pigs elicited no toxic reactions and good weight gains were noted.

Table 4. *Potency of Bordetella pertussis vaccines prepared from cultures grown in '16G+1P' chemically defined medium*

Lot no.	Potency*	Lot no.	Potency*
P55	4·2	1057	19·4
	4·3	—	10·1
	—	—	15·2
P56	21·4	—	—
	16·2	1059	13·1
	—	—	11·0
P57	8·0	—	18·2
	7·6	—	—
	—	1065	5·3
1005	16·8	—	—
	13·4	1066	5·3
1006	18·2	1068	16·7
1055	10·4	1069	13·5
	13·6	—	—
	13·7	1071	10·4

* i.u./20 o.u. tested against U.S. Pertussis Vaccine Lot 6.

Table 5. *Adjuvant effect of Bordetella pertussis vaccines prepared from cultures grown in '16G+1P' chemically defined medium*

Sample	Time (months)	Temperature (°C)	Diphtheria potency (i.u./ml.)	Tetanus potency (i.u./ml.)
Fluid diphtheria toxoid	7	4	6 (3 to 12)*	—
Fluid tetanus toxoid	7	4	—	22 (11 to 45)
Diptheria toxoid—40 Lf	7	4	20 (10 to 44)	46 (23 to 87)
Tetanus toxoid—8 Lf	7	25	34 (16 to 73)	56 (28 to 108)
Pertussis vaccine—20 o.u.	7	37	29 (14 to 61)	120 (64 to 230)
Diphtheria toxoid—40 Lf	7	4	16 (7 to 39)	69 (26 to 180)
Tetanus toxoid—8 Lf	7	25	53 (22 to 154)	102 (36 to 364)
Pertussis vaccine—10 o.u.				

* 95 % Limits given in parentheses.

Antigenicity studies

Agglutination tests with vaccines prepared by growing *Bordetella pertussis* in the '16G+1P' medium showed that they all agglutinated with phase I antiserum, and further testing proved the presence of appreciable quantities of agglutinogens 1, 2 and 3 (Preston, 1963, 1965).

The mouse-protective potency of numerous lots of vaccine (each diluted to contain 20 o.u./ml.) is shown in Table 4. All lots tested had protective activity of more than 4 i.u./ml.

The adjuvant effect of pertussis vaccine on the response to diphtheria and tetanus toxoids is well known (Fleming, Greenberg & Beith, 1948). Samples of two combined

Table 6. *Antigenic stability of Bordetella pertussis vaccines prepared from organisms grown in '16G+1P' chemically defined medium*

Storage time (weeks)	Temp. (°C)	Pertussis samples at 20 o.u./ml. (i.u./ml.)	Pertussis samples at 10 o.u./ml. (i.u./ml.)
		(A) Pertussis vaccine	
0	—	13·4 (6·6 to 26·1)*	—
4	4	8·6 (4·4 to 16·6)	6·3 (3·3 to 12·3)
26	4	4·6 (2·0 to 10·7)	7·0 (3·1 to 15·7)
44	4	9·4 (4·8 to 18·2)	—
4	25	9·4 (4·7 to 19·6)	5·9 (3·0 to 11·5)
26	25	5·6 (2·4 to 13·0)	4·7 (2·1 to 10·8)
8	37	8·8 (4·6 to 17·0)	6·4 (3·3 to 12·3)
26	37	1·2 (0·5 to 2·8)	2·4 (1·0 to 5·4)
		(B) Pertussis component of D.P.T.	
0	—	13·4† (6·6 to 26·1)	—
4	4	13·2 (6·9 to 25·4)	9·5 (4·9 to 18·2)
26	4	7·8 (3·4 to 18·1)	3·6 (1·5 to 8·3)
4	25	21·9 (11·4 to 42·3)	5·8 (3·0 to 11·2)
26	25	1·4 (0·6 to 3·4)	3·4 (1·4 to 8·2)
8	37	10·0 (5·2 to 19·3)	5·2 (2·7 to 10·0)
26	37	1·3 (0·5 to 3·7)	2·7 (1·2 to 6·2)

Note: vaccines contained either 20 or 10 o.u./ml. and were stored and tested separately against U.S. Pertussis Vaccine Lot 6. * 95 % Limits given in parentheses. † This is a theoretical calculation.

vaccines, containing a mixture of two lots of *Bordetella pertussis* grown in the chemically defined medium ('16G+1P') and diphtheria and tetanus toxoids, were each stored for seven months at three temperatures. The resulting potencies of the tetanus and diphtheria components were compared with the original potencies of the fluid toxoids, and the results are given in Table 5. The combined vaccines contained 40 Lf diphtheria, 8 Lf tetanus toxoids, and either 20 or 10 o.u. pertussis vaccine/ml. The antigenic potencies of the toxoids were significantly increased ($P = 0{\cdot}01$) when combined with the pertussis vaccine. An unexpected finding was the additional adjuvant effect on the potencies of the tetanus component of the combined vaccine when stored at elevated temperatures. This is to be further investigated. The stability of the pertussis component used in the combined vaccines was also determined, and these results are given in Table 6. More information is needed but it appears that the new pertussis vaccine is stable for at least 44 weeks at 4°, and 8 weeks at 37°, when stored as either a plain vaccine or in combination with diphtheria and tetanus toxoids. Three of the four samples stored at 25° were stable for 26 weeks.

DISCUSSION

The use of a chemically defined medium in which all the ingredients essential for the growth of bacteria are known is obviously very desirable, especially in relation to vaccine production. In the case of *Bordetella pertussis* many such media have been described and every author has stressed the importance of certain amino acids, notably glutamic acid, proline and cystine. We have confirmed these findings. Since most of the chemically defined media published were developed for basic metabolic studies, the yields have not been sufficient to warrant their use in the routine production of *B. pertussis* in large volumes. In the present case, however, very good yields were obtained.

In the experiments reported here, the cultures used for seeding the large fermentors were grown in casein digest medium (Wilson, 1945) and could be stored at 4° for 4 weeks. The lag phase became extended, however, as the time of storage of the seed culture progressed. Similar results were obtained using '16G+1P' chemically defined medium for the growth of seed cultures, but the effects of storage of these cultures on the resultant lag phase was much more pronounced unless the seed was taken during the early exponential phase of growth. This could imply that the '16G+1P' chemically defined medium is incomplete and that before growth can start, specific intermediates must be synthesized. We do not feel that this is the case since we have evidence that *Bordetella pertussis* will grow very well in the '16G+1P' medium in shake flasks using a fresh seed culture grown in the same medium, and that the flasks can be serially subcultured for up to 29 transfers. Antigenic analyses of these serially transferred cultures showed no loss in either mouse-protective antigen levels or the ability to agglutinate phase I *B. pertussis* antiserum for up to eight transfers. Beyond this number of transfers, antigen levels declined, although there was still 5 to 10 % of the original mouse-protective activity present after 21 transfers.

The absence of high molecular weight material (e.g. starch), its ease of preparation, and the fact that vaccines produced from it have low toxicity and high antigenicity, make the proposed '16G+1P' chemically defined medium very attractive from the production standpoint. The interesting differences noted when *Bordetella pertussis* was grown in the absence of either glutamate or proline could indicate that different

metabolic pathways exist for these amino acids, and the extreme simplicity of these media makes possible further, more detailed biochemical studies of the organism. Preliminary investigations have already been reported (Stainer & Scholte, 1969), and further work is in progress.

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Nutrition and Chemotaxis in the Myxomycete *Physarum polycephalum*: the Effect of Carbohydrates on the Plasmodium

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(*Accepted for publication 10 July 1970*)

SUMMARY

The ability of a range of carbohydrates to support the growth of plasmodia of the myxomycete *Physarum polycephalum* was investigated and a method for the objective study of the chemotaxis of plasmodia was developed. Plasmodia showed positive chemotaxis to solutions of glucose, maltose, mannose and peptone, and to galactose after a delay. They did not respond to sucrose, fructose or ribose. The chemotactic effectiveness of sugars paralleled their ability to support growth.

INTRODUCTION

There are a number of early reports of the attraction (positive chemotaxis) of myxomycete plasmodia to nutrient solutions and to extracts of micro-organisms. Many of these reports—cited and briefly reviewed by Hawker (1952) and Ziegler (1962)—are of limited value. Assessment of chemotaxis was commonly subjective and, since pure cultures were not available, the agents studied could have acted indirectly through their effects on microbial contaminants. The lack of pure cultures and soluble media also prevented quantitative nutritional studies, which alone could determine whether the attractants were themselves nutrients or acted in some other manner. One of the more convincing of the early studies was that of Coman (1940), who found that plasmodia of *Physarum polycephalum* Schweinitz responded to glucose solutions by positive chemotaxis but were indifferent to sucrose solutions. Since this study, the pure culture of plasmodia of *P. polycephalum* on liquid media has been achieved (Daniel & Rusch, 1961). It therefore seemed desirable to confirm Coman's finding with pure cultures, to investigate the effects of other carbohydrates and to ascertain whether there was a correlation between the chemotactic effectiveness of carbohydrates and their ability to support growth.

METHODS

Organism and routine culture. Physarum polycephalum strain $a+i$ (Dee, 1962) was maintained in pure culture on liquid and agar media. The standard medium was a modification of the semidefined medium advocated by Daniel & Baldwin (1964) and contained the following constituents (%, w/v): glucose, 1·0; bacteriological peptone (Oxoid), 1·0; citric acid.H_2O, 0·354; $CaCl_2.6H_2O$, 0·06; Na_2 EDTA, 0·022; $FeCl_2.4H_2O$, 0·006; $ZnSO_4.7H_2O$, 0·003; thiamin hydrochloride, 0·004; biotin, 0·0005; haem, 0·0005; distilled water: pH adjusted to 4·6. Details of the preparation and sterilization of this medium and of culture methods are given elsewhere (Carlile,

1970). All experiments were carried out at 24° and aseptic procedures were employed throughout.

Nutritional studies. Cultures (50 ml. medium in 500 ml. Erlenmeyer flasks) were shaken at 200 rev./min. on a rotary shaker with radius of gyration 4·5 cm. Plasmodia were centrifuged, the culture medium decanted, the plasmodia resuspended in distilled water, centrifuged again, dried overnight at 110° and weighed.

Assessment of chemotaxis. Tests on the chemotactic effectiveness of sugars were carried out on Petri plates of sugar-free standard agar medium from which two wells and a central trough had been excised (Pl. 1). Distilled water was placed in one well and an equal volume of sugar solution in the other and the dish left for 1 to 4 days for the sugar to diffuse through the agar to the edge of the trough. A massive plasmodial inoculum was allowed to spread for 1 to 4 days on sugar-free standard medium to deplete carbohydrate reserves, and a square of plasmodium with underlying agar was excised and deposited in the centre of the trough. Migration of the plasmodium was observed daily, or more frequently if necessary; slime tracks indicated the path taken by the plasmodium. Chemotaxis to peptone was studied on peptone-free standard medium with plasmodia that had migrated on that medium, and chemotaxis to the complete medium on plain agar (pH 4·6) with plasmodia from plain agar.

An individual test was recorded as being positive if the plasmodium had reached the well containing the test solution but had not reached the control well, and negative if the control well was reached first. On occasions when a plasmodium came into contact with both wells or with neither well, the individual test was regarded as unsuccessful and was not included in the total count. In a set of ten tests, the most probable outcome of random migration is five positive results whereas positive or negative chemotaxis may yield ten or zero positive results respectively. The significance of an experiment or group of experiments was assessed by means of the binomial distribution (Snedecor & Cochran, 1967).

RESULTS

Nutritional studies

A typical experiment on the growth of plasmodia on standard medium is illustrated in Fig. 1. Virtually no lag phase occurred, and growth rate (doubling time about 12 h.) and yield were comparable to, or greater than, the highest reported by other workers (Daniel & Rusch, 1961; Daniel & Baldwin, 1964; Brewer, Kuraishi, Garver & Strong, 1964). When glucose was omitted from the standard medium, little growth occurred (Table 1; Fig. 1), although plasmodia remained active (showed streaming) for at least 2 weeks. On agar media lacking glucose, plasmodia eroded and ultimately burrowed into the agar, behaviour not seen on cultures with adequate glucose. The omission of peptone from standard medium resulted in little or no growth and rapid sclerotium formation, whether or not glucose was present. The omission of citric acid (S. Barnes, personal communication) did not affect growth rate and final yields, but affected morphology, plasmodia becoming very long and tangled. Citric acid was not utilized by plasmodia; its role in the standard medium appears to be that of a pH buffer and metal chelator.

The effect of replacing glucose by other carbohydrates is indicated in Table 1. Maltose, glycogen, soluble starch and cellobiose were closely similar to glucose in

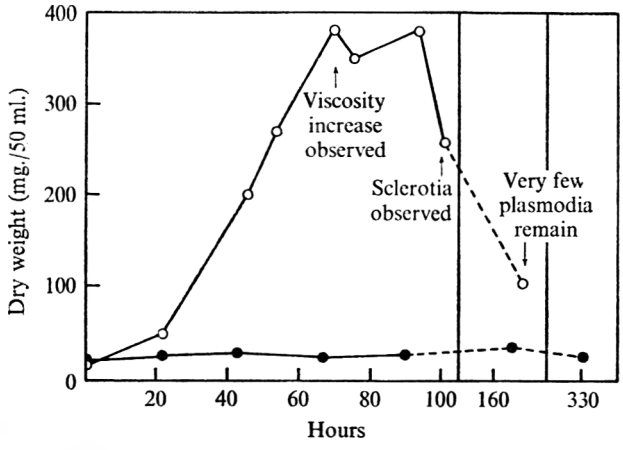

Fig. 1. Growth of plasmodia of *Physarum polycephalum* in shaken liquid culture. The inoculum (5 %, 2·5 ml.) was from a vigorously growing culture. Standard medium, —○—○—; sugar-free standard medium, —●—●—.

Table 1. *Growth of plasmodia on standard media in which glucose was replaced by other carbohydrates*

Carbon source*	Time of harvest (days)†	Yield (% of dry wt obtained with glucose)‡
Glucose	4	100
Maltose	4	98
Cellobiose	4	117
Glycogen (oyster)	4	98
Soluble starch	4	107
Glucan§	5	94
Mannose	5 to 6	103
Trehalose	6	89
Galactose	10 to 11	69
Lactose	10	65
Edifas B‖	10	19
Arabinose	10	2
Ribose	11	2
Xylose	10	2
Sorbose	10	3
Rhamnose	11	1
Fructose	12 to 13	3
Sucrose	12	2
Dextran	10	4
Glucosamine HCl	9	1
None	9 to 12	2

* Carbohydrate solutions were autoclaved separately from the other components of the medium and aseptically added to them when cool to give a medium containing 1 % (w/v) carbohydrate.

† Harvesting was carried out at the time of maximal growth (estimated visually).

‡ Average of at least three cultures. Controls (*a*) with glucose and (*b*) lacking any carbohydrate were included in all experiments. To minimize the carry-over of glucose the inoculum was taken from cultures that had already attained maximum growth on standard medium. A yield of 1 % represents no growth, as 1 % inocula were employed.

§ A β 1 → 3 linked glucan with β 1 → 6 branches, from *Claviceps fusiformis* (Buck, Chen Dickerson & Chain, 1968).

‖ Sodium carboxymethylcellulose (Imperial Chemical Industries, Ltd). The average degree of substitution is 0·7 to 0·8 (14 to 16 %), a value giving good solubility but permitting partial utilization by organisms able to utilize cellulose.

their value as carbon sources, giving similar yields after the same number of days. Mannose, trehalose and a glucan gave comparable yields but rather more slowly. Galactose or lactose gave good growth after a lag phase of some days, but the increase in viscosity observed with other media giving good growth did not take place. Yields remained roughly constant for many days with sclerotia and plasmodia co-existing; the clearcut sequence of phases seen on media containing glucose did not occur. Lower but still appreciable yields were obtained with a soluble cellulose derivative. Little or no growth took place with arabinose, ribose, xylose, fructose, rhamnose, sorbose, sucrose, dextran or glucosamine hydrochloride.

Studies on chemotaxis

The chemotactic effectiveness of glucose was repeatedly demonstrated (Table 2) with both filter-sterilized and autoclaved glucose solutions. In all instances the plasmodia reached the experimental well without having come into contact with the control well. Strong positive chemotaxis was normally evident within 1 day of inoculation and the experiment could be terminated in 2 days. Maltose, mannose, peptone and standard medium (which contains glucose and peptone) gave similar results. Chemotaxis to both filter-sterilized and autoclaved galactose solutions occurred, but with a delay not experienced with maltose and mannose solutions. Observations at 1 day indicated apparently random initial migration and experiments were not terminable until the third or fourth day, by which time positive chemotaxis had taken place.

Table 2. *Summary of experiments on chemotaxis*

Substance*	Tests successfully completed	Positive responses obtained	Probability of result (%)†	Conclusion‡
Glucose	30	30	5×10^{-6}	+
Maltose	10	10	0·2	+
Mannose	9	9	0·4	+
Galactose	8	8	0·8	+
Sucrose	18	10	51	0
Fructose	10	5	100	0
Ribose	10	4	75	0
Peptone	30	30	5×10^{-8}	+
Standard medium	10	10	0·2	+

* Sugar solutions (1 %, w/v) were filter-sterilized. Oxoid bacteriological peptone (1 %, w/v) and standard medium were autoclaved.

† Probability (%) of the experimental finding being due to random migration.

‡ + = Positive chemotaxis; 0 = random migration.

Chemotaxis to sucrose, fructose and ribose solutions did not occur, although strong positive chemotaxis was observed in control experiments carried out simultaneously with glucose.

DISCUSSION

Nutritional requirements. The omission of glucose from standard medium results in growth yields of as low as 2 % of those given by control cultures (Table 1); this establishes that peptone cannot act as sole carbon source for the growth of *Physarum polycephalum*. The high yields obtained in the absence of added carbohydrates by earlier workers, 29 % (Daniel & Rusch, 1961) and 36 to 49 % (Daniel & Baldwin, 1964)

of control cultures, are probably due to the presence of large amounts of yeast extract in their media, as are the high yields (52 % of control cultures) obtained with *P. rigidum* (Henney & Henney, 1968).

The report (Daniel & Baldwin, 1964) that glucose can be replaced by maltose or soluble starch, but not by sorbose, xylose or arabinose, has been confirmed. Their claim that fructose can be utilized was not confirmed (and is not supported by their own table showing a yield with fructose which differed little from that of their sugar-free controls); nor was their statement that galactose is not utilized supported by our findings. Results on another myxomycete, *Physarum rigidum*, are in complete agreement with those reported here; Henney & Henney (1968) found that glucose, mannose, lactose and galactose were utilized and that fructose, sucrose, arabinose, ribose and xylose were not.

Chemotaxis. Coman (1940) found that plasmodia of *Physarum polycephalum* respond to glucose solutions by positive chemotaxis but are indifferent to sucrose. The present study confirms Coman's findings, and demonstrates that with the seven sugars studied there is a complete correlation between their ability to support growth and their chemotactic effectiveness. Positive chemotaxis to peptone also occurs, thus taxis to nutrients is not limited to sugars. Taxis to standard medium is presumably attributable to glucose, peptone and possibly other nutrients.

If a nutrient is lacking, or is available only in a growth-limiting concentration, it will be advantageous for the plasmodium to respond by positive chemotaxis to fresh supplies of that nutrient. If other nutrients are also lacking, or are only present in low concentrations then the requirement for the test nutrient will soon be satisfied and chemotaxis will not continue. Hence the agar-solidified medium which was employed both for the preparation of the plasmodial inoculum and the actual assay, contained in abundance all the nutrients required by the organism except the one being tested. Preliminary experiments indicated that it was this feature of the assay that led to the achievement of highly consistent results. The principle is one generally applicable to the design of assays for the study of nutritional chemotropism and chemotaxis (Harris 1961; Rosen, 1962, Adler, 1966; Carlile, 1966) and is likely to be crucial wherever the response takes hours or days rather than minutes.

I wish to thank Dr J. Dee for providing strain $a+i$, Mr S. Barnes, Dr K. W. Buck and Dr A. G. F. Dickerson for advice on carbohydrate biochemistry and metabolism, Dr A. L. Cooper for access to calculations on glucose diffusion, Miss S. Ford for photography, Mr C. E. Groome for skilled technical assistance, and Professor Sir Ernst Chain, F.R.S. for helpful discussion.

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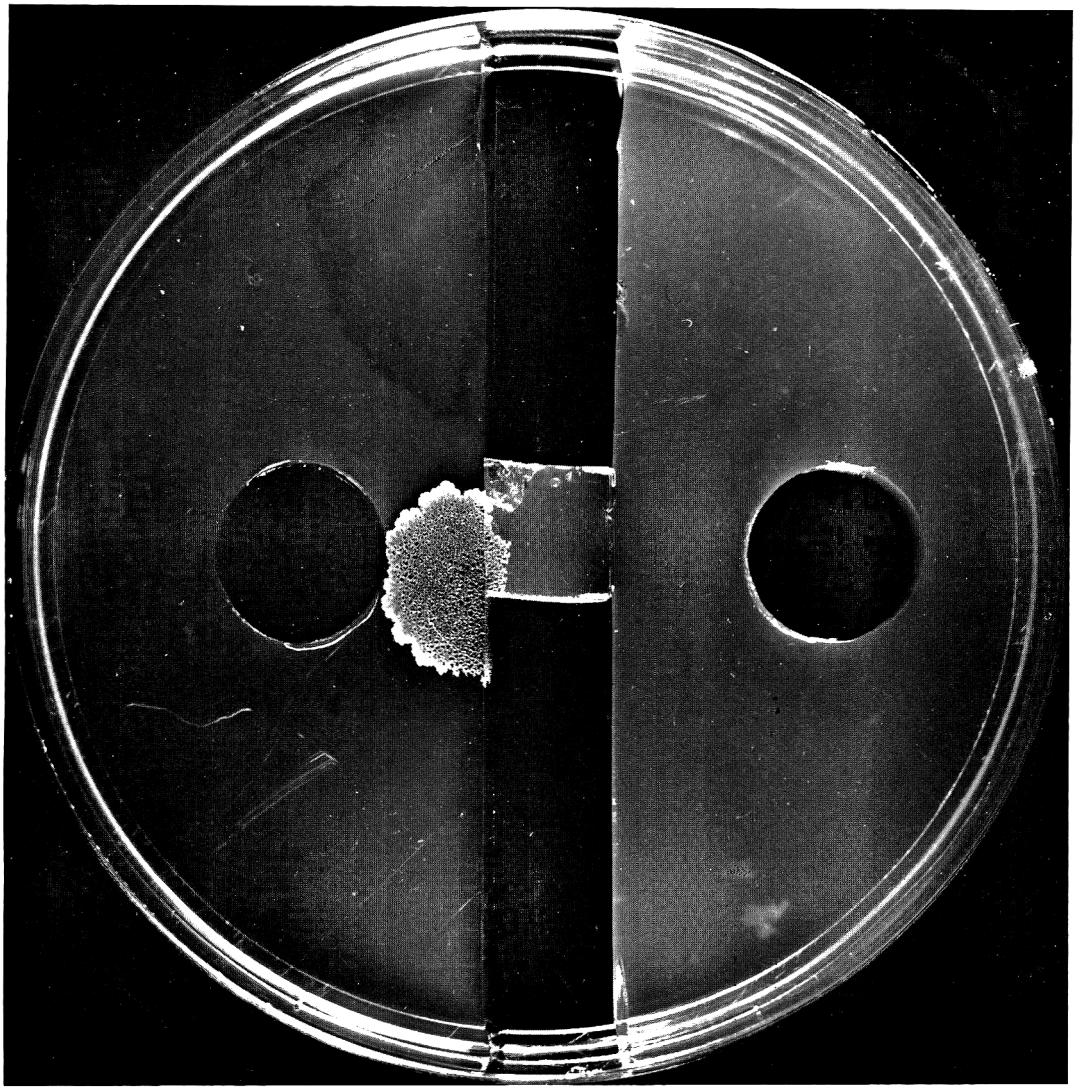
EXPLANATION OF PLATE

Positive chemotaxis of plasmodium of *Physarum polycephalum*. Glucose solution on left, distilled water on right. × 1·5.



M. J. CARLILE

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Comparison of Galactokinase Induction in Protoplasts and Intact Bacteria of *Bacillus megaterium* 216

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SUMMARY

Bacillus megaterium 216 was induced with D-galactose to form galactokinase (EC 2.7.1.6) in amounts comparable with those induced in *Escherichia coli* K12. When compared with the basal level, the increase was 20 times greater than in *E. coli*.

The greater the concentration of D-galactose, the greater the production of galactokinase in both protoplasts and whole bacteria. In each case the maximum amount of enzyme was induced by 5mM D-galactose. D-Fucose, a gratuitous inducer, produced approximately half the specific activity of enzyme in protoplasts as D-galactose at any particular concentration. The yield of enzyme in the presence of an optimal concentration of D-galactose was increased when the medium was fortified with low concentrations of casein hydrolysate. The specific activities of galactokinase induced in protoplasts and in whole bacteria were respectively 148% and 37% greater in the presence of 0·01% casein hydrolysate.

The maximum specific activity of galactokinase achieved after induction of protoplasts for 60 min. was 3·7 units/mg. protein compared with 12·5 units/mg. protein in whole bacteria. However, over the first 15 min. after the addition of inducer the same specific activity of galactokinase was achieved in protoplasts as in whole bacteria.

INTRODUCTION

In 1955 the induced formation of arabinokinase in *Bacillus subtilis* protoplasts (Wiame, Storck & Vanderwinkel, 1955) and β-galactosidase in *Bacillus megaterium* protoplasts (Landman & Spiegelman, 1955; McQuillen, 1955) were reported. Subsequently, a few isolated reports of enzyme induction in *Escherichia coli* spheroplasts appeared (McQuillen, 1960). However, over the years surprisingly little work has been done on these potentially useful sources of cell-free enzyme-forming systems. The only such study has been made by Kiho & Rich (1964), who isolated a cell-free system for β-galactosidase formation after induction of *E. coli* spheroplasts with methyl-β-thiogalactopyranoside.

The present study was undertaken with the object of developing a model system in which the factors which limit the cell-free synthesis of a specific protein could be studied. It was considered desirable to have an intact organized system which could be converted into a cell-free extract with the minimum delay and by the gentlest possible treatment. These conditions were fulfilled by a *Bacillus megaterium* system which readily forms stable protoplasts which can be induced to form galactokinase (EC 2.7.1.6), a relatively low molecular weight enzyme for which there is a sensitive

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and simple assay procedure. Further, these galactokinase-forming protoplasts can be readily and rapidly disrupted by osmotic lysis to give cell-free preparations. The only detailed studies on galactokinase induction in bacteria have been restricted to whole cells of *Escherichia coli* (Paigen, 1963; Buttin, 1968; Wilson & Hogness, 1969).

EXPERIMENTAL

Organism. The bacterial strain used was *Bacillus megaterium* 216 from the collection of Professor G. Ivanovics, The Medical University, Szeged, Hungary. Samples of the freeze-dried bacteria were subcultured at 3 month intervals on basal medium (see below), containing 20 mM D-glucose, streaked on nutrient agar slopes and incubated at 30° for 24 h. The resulting cultures were used as sources of day-to-day inocula. *Escherichia coli* K12 strain W1485, used in the comparative studies, was kindly provided by Dr J. R. Guest, Department of Microbiology, University of Sheffield. It was subcultured on to nutrient agar and incubated at 30° for 24 h. immediately before use.

Growth conditions. The bacteria were grown in a medium which was essentially that of Buttin (1963), and consisted of a defined salts solution (referred to as the 'basal medium') together with 20 mM D-glucose unless otherwise stated. The basal medium consisted of KH_2PO_4, 0·1 M; $MgSO_4 . 7H_2O$, 0·8 mM; $(NH_4)_2SO_4$, 15 mM; $FeSO_4 . 7H_2O$, 0·01275 mM; 'trace metal ion' solution, 0·5 ml./l. (Coleman & Elliott, 1965); pH 7·2.

The medium (50 ml./250 ml. conical flask) was inoculated from a slope and incubated for 18 h. at 30° in a Gyrotory incubator–shaker (model G25, New Brunswick Scientific Co. Inc.) operating at 240 oscillations/min. The resulting culture was then diluted tenfold with fresh nonsterile medium in a 2 l. conical flask and incubated (*ca.* 4 h.) until $E_{600}^{1\,cm.} = 0{\cdot}8$.

Washed bacteria and protoplast experiments. Cultures at $E_{600}^{1\,cm.}$ of 0·8 were centrifuged at 4500 *g* for 2 min. and the bacterial pellet washed with fresh basal medium by centrifuging. For the experiments with whole bacteria, the organisms were resuspended to the initial density in basal medium plus the required carbon and complex nitrogen sources, and samples of the suspension (40 ml./250 ml. flask) were shaken at 240 oscillations/min. at 30°.

Protoplast suspensions were prepared from the washed bacteria resuspended to the initial density in basal medium containing sucrose (20 %, w/v) and lysozyme (B.D.H. Chemicals Ltd; 5 mg./100 ml.) and shaken (200 ml. suspension/2 l. flask) at 30° at 120 oscillations/min. Protoplast formation was completed in 15 min. and the preparation was used immediately.

Preparation of cell-free extracts. Bacteria and protoplasts were washed prior to disruption; this procedure removed D-galactose, which if present interfered in the assay of galactokinase. Bacteria from 40 ml. of suspension were harvested by centrifuging at 4500 *g* for 2 min., resuspended in fresh basal medium and then centrifuged again. The pellet was resuspended in 2·5 ml. of supplemented buffer solution; this contained K_2HPO_4, 0·02 M; dithiothreitol, 1·5 mM; EDTA, 1 mM; bovine serum albumin 100 μg./ml.; adjusted to pH 7·4 with glacial acetic acid (Gulbinsky & Cleland, 1968). The bacteria were lysed by adding 1·25 mg. lysozyme and allowing to stand at room temperature for 30 min. Protoplasts were washed by layering 25 ml. batches of suspension on to 25 ml. ice-cold 30 % (w/v) sucrose in basal medium and centrifuging at 4500 *g* for 10 min. The protoplast pellet was lysed by shaking with

2·5 ml. of the supplemented buffer solution. The lysed bacteria or protoplasts were centrifuged at 110,000 *g* for 45 min. at 0° and the supernatant fractions taken for the assay of galactokinase and protein.

Determination of galactokinase. Galactokinase was determined by a radiometric method adapted from the procedure of Sherman & Adler (1963) by Gulbinsky & Cleland (1968). Extracts of fully induced washed bacterial suspensions were diluted up to 120-fold and extracts of protoplasts tenfold with supplemented buffer solution to give preparations whose activity was in the range where there was a linear relationship between velocity of reaction and enzyme concentration.

Determination of protein. Extracts from washed bacteria contained sufficient protein to be estimated by the biuret method of Layne (1957). The protein content of protoplast extracts was less and was measured by the modified biuret method of Bürgi, Richterich & Briner (1967).

Bacterial density determination. Bacterial densities were determined by measuring extinction at 600 nm. in cuvettes with a 1 cm. light path.

Bacterial mass determination. Dry weights of washed bacteria were determined as described by Coleman & Elliott (1962).

Casein hydrolysate. 'Oxoid' casein hydrolysate (acid) was used as a nitrogen supplement ('Oxoid' Division of Oxo Ltd, London E.C.4).

RESULTS

Comparison of galactokinase levels in Bacillus megaterium 216 and Escherichia coli K12

It was of interest to make an initial comparison between the levels of enzyme in *Bacillus megaterium* 216 and *Escherichia coli* K12 before and after induction with D-galactose. Whilst a low level of enzyme was detected in both the uninduced bacteria, in *B. megaterium* the level was less than one-tenth of that in *E. coli*. After induction with D-galactose (20 mM) comparable levels of enzyme were found in both organisms, the level in *B. megaterium* being 30 % higher than in *E. coli*. However, due to the lower basal level in *B. megaterium* a 208-fold increase in enzyme was observed on induction compared with a 13-fold increase in *E. coli*.

Stability of sucrose-stabilized protoplasts

It was important to determine the period during which the protoplasts remained stable without evidence of spontaneous lysis. A sucrose-stabilized protoplast suspension in basal medium was induced by the addition of 20 mM D-galactose, and the time course of increase in galactokinase was followed together with the change in intracellular protein. There was a rapid increase in both protein and galactokinase during the first 60 min. of incubation and thereafter levels of both fell, suggesting that after this period the protoplasts become unstable and start to lyse.

It was possible that lysis also occurred to a limited extent during the first 60 min. but was not detected because of the masking effect of net increases in enzyme and protein. By expressing results in terms of specific activity, this possible source of error or experimental variation may be eliminated.

Effect of casein hydrolysate concentration

The effect of casein hydrolysate on the induction of galactokinase in protoplasts was compared with its effect on induction in whole bacteria.

The addition of 0·005% casein hydrolysate to protoplasts induced with 5 mM D-galactose caused a doubling in specific activity of the enzyme (Table 1). Increase in the casein hydrolysate concentration to 0·05% produced only a further 20% increase in the specific activity of galactokinase. In contrast, similar additions of casein hydrolysate (0·005 to 0·05%) had no effect on the specific activity of galactokinase in protoplasts induced with 5 mM D-fucose (6-deoxy-D-galactose), a gratuitous inducer of galactokinase (Buttin, 1963).

Table 1. *Effect of casein hydrolysate as a nitrogen supplement on galactokinase formation*

Protoplasts and washed bacteria were incubated in basal medium containing increasing concentrations of casein hydrolysate together with inducer as indicated. Extracts of the protoplasts were prepared for assay after 60 min. incubation and extracts of whole bacteria were prepared after 3 h. incubation.

Casein hydrolysate	Specific activity of galactokinase (units/mg. protein) in		
	Protoplasts induced by		Whole bacteria induced by
%	5 mM D-fucose	5 mM D-galactose	20 mM D-galactose
0·000	1·92	1·48	16·2
0·005	1·96	3·06	23·8
0·010	1·92	3·73	22·0
0·050	1·85	3·57	21·1

Galactokinase was induced in whole bacteria by both 5 mM and 20 mM D-galactose in the absence of casein hydrolysate to give a specific activity three times greater than that achieved in protoplasts after the same incubation period of 1 h. The specific activity in whole bacteria was increased a further threefold after 3 h. incubation with 20 mM D-galactose. The effect of adding 0·005% casein hydrolysate under these latter conditions was to produce a further 37% increase in specific activity. Increase in the concentration of casein hydrolysate to 0·05% did not stimulate the formation of any more galactokinase.

A concentration of D-galactose of 20 mM was employed as inducer with whole bacteria in preference to 5 mM during a 3 h. incubation since at the lower concentration all the D-galactose was metabolized, whilst at the higher concentration D-galactose was detectable in the medium at the end of the incubation.

Effect of inducer concentration

Studies of the effect of different concentrations of D-galactose and D-fucose on galactokinase induction in protoplasts showed that with both inducers the specific activity of the galactokinase increased with increase in inducer concentration, reaching a maximum at 3 mM D-galactose and 5 mM D-fucose (Fig. 1 *a*). Greater concentrations of inducer produced little change in specific activity of the enzyme. The maximum

specific activity in the presence of D-galactose was twice that achieved in the presence of D-fucose. In whole bacteria induced with D-galactose similar characteristics were observed (Fig. 1*b*), maximum activity being achieved at a concentration of 5 mM; increasing the inducer concentration above this had no effect. However, in the presence of D-fucose the pattern was rather different; with 0·5 mM D-fucose the level of galactokinase activity was similar to the maximum amount induced by D-galactose. Further increase in D-fucose concentration from 0·5 to 20 mM resulted in a doubling of the specific activity of galactokinase.

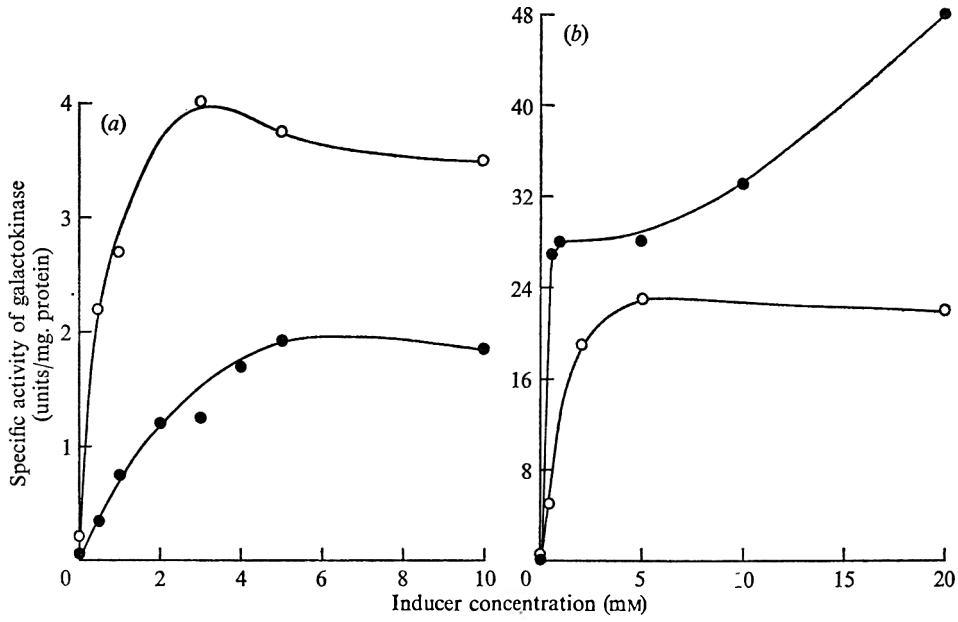

Fig. 1. Effect of inducer concentration on the induction of galactokinase in (*a*) protoplasts and (*b*) whole bacteria. Protoplasts were incubated for 60 min. and whole bacteria for 3 h. in basal medium containing 0·01 % casein hydrolysate and different concentrations of D-galactose (○) or D-fucose (●).

Progress of galactokinase formation

The progress of galactokinase induction was studied in the presence of basal medium containing 0·01 % casein hydrolysate and D-galactose or D-fucose at a concentration of 5 mM for protoplasts and 20 mM for whole bacteria.

In the presence of D-galactose the galactokinase and protein content of the protoplasts increased and reached a maximum at 60 min., at which time maximum specific activity was also achieved (Fig. 2*a*). On further incubation both protein and galactokinase activity fell, indicating that the protoplasts were becoming unstable and lysing.

In the presence of the gratuitous inducer D-fucose total galactokinase and specific activity again reached a maximum after 60 min. (Fig. 2*b*), and as expected protein content changed relatively little over the period of the experiment. In this experiment the development of protoplast instability was less pronounced than in the presence of D-galactose, and specific activity remained relatively constant at about half the maximum level achieved with the nongratuitous inducer.

The progress of galactokinase induction in whole bacteria (Fig. 3*a*, *b*) produced the expected patterns of increase in bacterial density over 3 h. in the presence of D-galactose and little change in bacterial density in the presence of D-fucose. This resulted in specific activities being at least twofold greater throughout the 3 h. incubation for the D-fucose-induced bacteria.

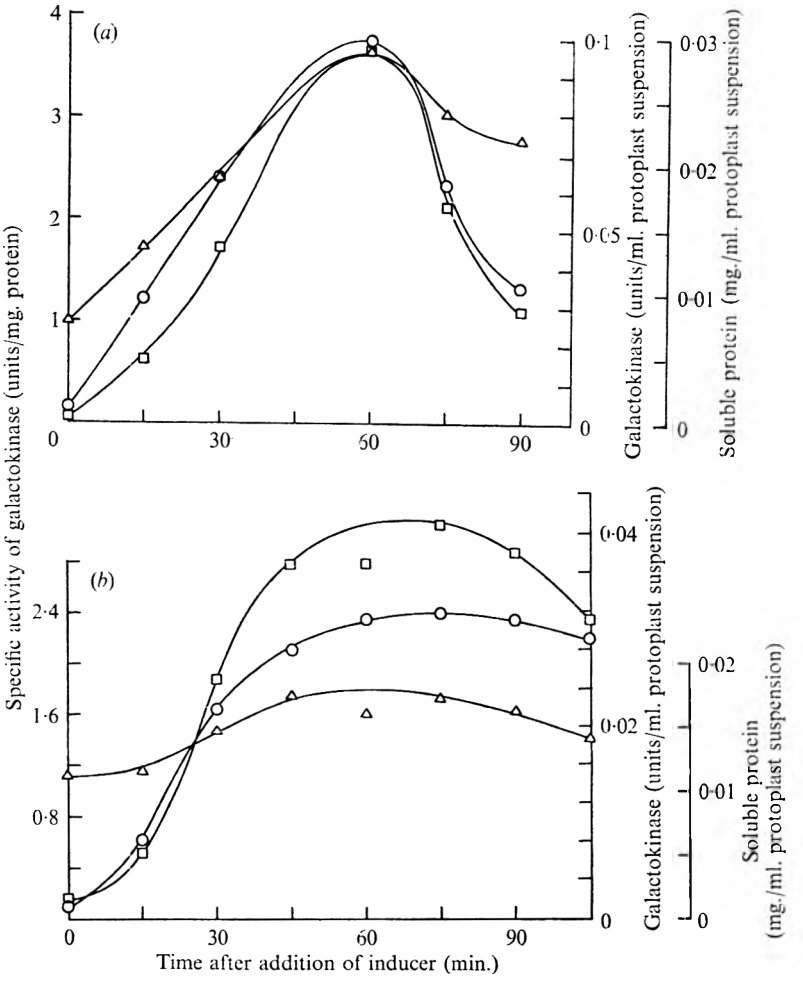

Fig. 2. Progress of galactokinase induction in protoplasts by (*a*) D-galactose and (*b*) D-fucose. Protoplasts were incubated in basal medium containing 0·01 % casein hydrolysate, 20 % (w/v) sucrose and inducer at a concentration of 5 mM. Samples were taken at intervals for galactokinase (□) and soluble protein (△) assay. Specific activities of galactokinase (○) were calculated from the data obtained.

The progress curves for galactokinase formation induced by D-galactose in protoplasts (Fig. 2*a*) and in whole bacteria (Fig. 3*a*) show that the specific activity of the enzyme increased by the same amount during the first 15 min. after induction. Thereafter the specific activity in whole bacteria increased more rapidly, being 50 % higher after 30 min. and three times higher after 1 h. It was of interest to establish whether

this difference was due to more favourable external conditions imposed on whole bacteria compared with protoplasts or whether it was related to a more highly developed enzyme-forming machinery in whole bacteria.

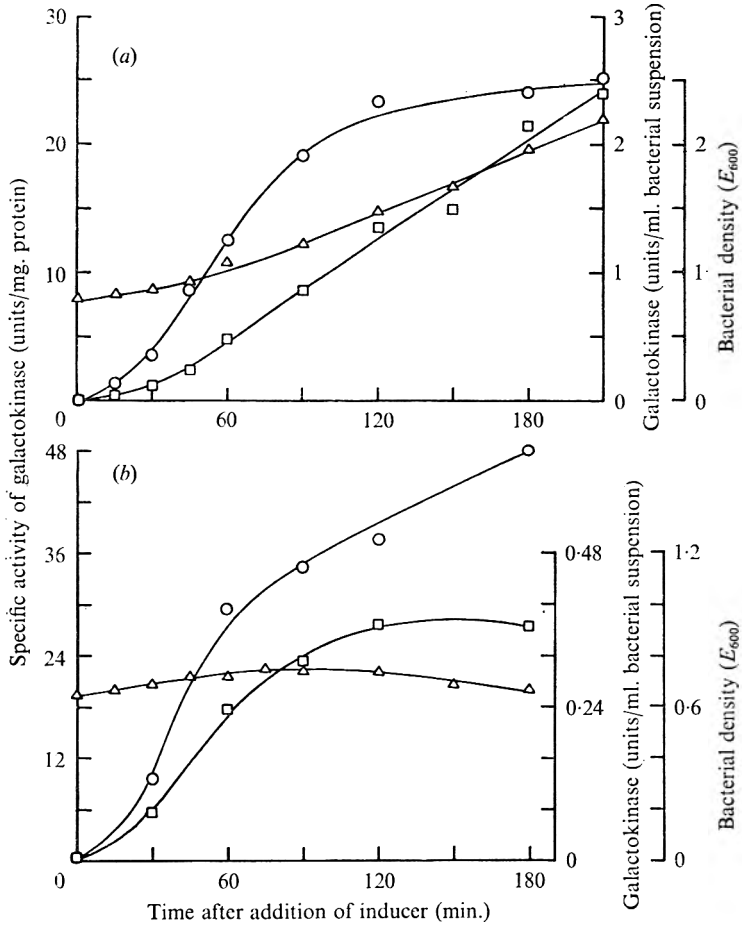

Fig. 3. Progress of galactokinase induction and change in bacterial density in the presence of (*a*) D-galactose and (*b*) D-fucose. Intact bacteria were incubated in basal medium containing 0·01 % casein hydrolysate and inducer at a concentration of 20 mM. At different times during the incubation bacterial density was determined in terms of extinction at 600 nm. (△) and samples were taken for determination of total galactokinase (□) and its specific activity (○).

The addition of 20 % (w/v) sucrose to a suspension of whole bacteria, in the presence of 5mM D-galactose caused a 50 % reduction in specific activity of the enzyme over a 60 min. incubation (Fig. 4). Reduction of the shaking rate to that which was optimal for maintaining protoplast stability further reduced the specific activity to 66 % of that achieved in protoplasts under similar conditions.

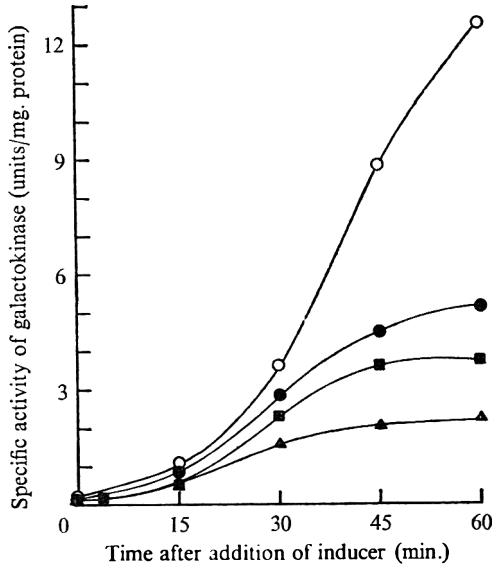

Fig. 4. Effect of incubating washed bacteria under the conditions employed for the induction of galactokinase in protoplasts. The increase in specific activity of galactokinase in whole bacteria shaken at 240 oscillations/min. in the presence (●) and absence (○) of 20 % (w/v) sucrose was compared with whole bacteria in the presence of 20 % (w/v) sucrose (▲) and sucrose-stabilized protoplasts (■) shaken at 120 oscillations/min. All the preparations were suspended in basal medium containing 0·01 % casein hydrolysate together with 5 mM D-galactose as inducer.

DISCUSSION

The results of this investigation show that the general characteristics of induction of galactokinase were similar in whole bacteria and protoplasts of *Bacillus megaterium* although differences were observed in the levels of enzyme and specific activities achieved in the two systems.

The effect of casein hydrolysate supplementation was consistent with the supply of amino acids being a limiting factor during the induction of galactokinase in protoplasts by the nongratuitous inducer D-galactose. On induction of protoplasts with D-fucose, where there was little opportunity for increase in total cellular material due to the unavailability of a metabolizable carbon source, no such limitation was demonstrated.

The higher levels of enzyme and higher specific activities observed in whole bacteria compared with protoplasts was due largely to the greater stability of whole bacteria during the experimental period. This was reflected by their ability to produce enzyme over longer periods. Over shorter time periods, during which protoplasts remain stable, the differences in specific activity appeared to be related to differences in environmental conditions. Evidence in favour of this idea was obtained by incubating whole bacteria in sucrose at the reduced shaking rate necessary to maintain protoplast stability since in these conditions the production of enzyme was reduced to a level similar to that achieved in protoplasts.

Thus, whilst whole bacteria will form greater amounts of galactokinase for longer

periods of time, the advantages which osmotic fragility confer on protoplasts as a source of a cell-free enzyme-forming system still make them an attractive proposition worthy of further study.

P. J. A. wishes to express his thanks to the Science Research Council for the award of a Research Studentship.

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Serological Properties of the Wall and Membrane Teichoic Acids from *Lactobacillus helveticus* NCIB 8025

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SUMMARY

Both wall and membrane teichoic acids from *Lactobacillus helveticus* NCIB 8025 are glycerol phosphate polymers partially substituted with α-D-glucosyl residues. The membrane teichoic acid, isolated as a complex with lipid (lipoteichoic acid), was antigenic when injected into rabbits with Freund's adjuvant. The α-D-glucosyl substituents are primarily responsible for the serological specificity of the membrane antigen, and account for the reaction of wall teichoic acid with antisera to the membrane teichoic acid. Glycerol teichoic acids either differing in or lacking sugar substitution may cross-react and the significance of these observations is discussed.

INTRODUCTION

The serological classification of micro-organisms may be based on the agglutination of whole organisms or the reactivity of solubilized cell components with specific antisera. Both procedures have been utilized in the serological classification of the lactobacilli. The work of Sharpe (1955) showed that most strains of lactobacilli can be divided into one of six serological groups on the basis of the reaction of Lancefield acid extracts with antisera prepared against whole organisms. Subsequently, Sharpe, Davison & Baddiley (1964) concluded that the group A antigen (*Lactobacillus helveticus-jugurti* group) was an intracellular (membrane) glycerol teichoic acid. Mills (1969) has recently studied the agglutinating antigens of *L. jugurti* and concluded that the cell-wall teichoic acid is the major antigen in the cell wall of group A lactobacilli, while the intracellular teichoic acid was also serologically active.

This study reports more definitive investigations into the chemical structure and serological properties of the wall and membrane (intracellular) teichoic acids. The reaction of these teichoic acids with antisera against other lactobacilli and group A streptococci has also been investigated.

METHODS

Organisms. Strains of lactobacilli were obtained originally from the National Collection of Type Cultures, Colindale, London, or from Dr M. E. Sharpe, National Institute for Research in Dairying, Reading, Berkshire, and were representative of

each of the serological groups: *Lactobacillus helveticus* NCIB8025 (group A), *L. casei* NIRDH831 (group B), *L. casei* NIRDR094 (group C), *L. plantarum* NCIB7220 (group D), *L. lactis* NCIB7228 (group E), and *L. fermenti* NCTC6991 (group F). A strain of group A streptococcus was isolated and identified by Mr H. C. Spies of this Institute. Organisms were grown for 18 h. at 37°, lactobacilli in the medium described by Sharpe *et al.* (1964), and streptococci in Todd–Hewitt broth.

Preparation of teichoic acid. Suspensions of washed *Lactobacillus helveticus* were disrupted with Ballotini beads (no. 13) and cell walls isolated and washed by the method described previously (Knox & Hall, 1965). Teichoic acid was extracted from these cell walls by two procedures: (i) Walls were extracted conventionally with cold 10% trichloracetic acid (TCA) for 48 h., crude teichoic acid being recovered from the clarified supernatant of centrifuged extracts by precipitation with 5 vol. of 96% ethanol. (ii) Suspensions of cell walls in 0·5N-NaOH were stirred at room temperature for 4 h. (Archibald, Coapes & Stafford, 1969), and after centrifugation the clear supernatant fluid was neutralized with HCl and then dialysed. Crude teichoic acid was recovered by freeze-drying. Teichoic acid preparations from both (i) and (ii) were purified further by chromatography in 0·2M-ammonium acetate, pH6·9, on columns of Sephadex G 75 (Wicken & Knox, 1970).

The nonsedimentable material obtained after centrifuging disintegrated organisms (12,000 *g*, 20 min., Serval RC 2 centrifuge) was used as the source of membrane teichoic acid. Teichoic acid was extracted with cold aqueous phenol and purified by chromatography in 0·2M-ammonium acetate on columns of 6% agarose (Litex, Denmark) as described previously (Wicken & Knox, 1970).

Dr M. McCarty kindly provided a sample of polyglycerophosphate (glycerol teichoic acid) prepared from a group A streptococcus strain D58 (type 3) (McCarty, 1959).

Preparation of antisera. Group-specific antisera were prepared by the intravenous injection of suspensions of bacteria (Sharpe, 1955; Knox, Hewett & Wicken, 1970). The antigenicity of the membrane teichoic acid from *Lactobacillus helveticus* was examined by injecting subcutaneously an emulsion containing equal volumes of teichoic acid solution and Freund's complete adjuvant (Knox, Hewett & Wicken, 1970).

Serological methods. The specificity of group antisera was examined initially by the qualitative ring precipitin method (Sharpe, 1955). Quantitative precipitin reactions and haemagglutination titres were determined by the methods previously used with *Lactobacillus fermenti* (Hewett, Knox & Wicken, 1970; Knox *et al.* 1970).

Analytical methods. Conditions for the acidic and alkaline hydrolysis of teichoic acids, enzymic dephosphorylation, characterization of glycosides and identification of products by paper chromatography, unless stated in the text, are essentially as described previously (Forrester & Wicken, 1966; Wicken, 1966). Procedures for quantitative analyses have been detailed by Wicken & Knox (1970).

Paper chromatography. The following solvent systems were used for paper chromatography: (*a*) propan-1-ol + aq. ammonia (sp.gr. 0·88) + water (6 + 3 + 1, by vol.; Hanes & Isherwood, 1949), Whatman paper no. 4, ascending; (*b*) butan-1-ol + pyridine + water (6 + 4 + 3, by vol.; Jeanes, Wise & Dimler, 1951), Whatman paper no. 1 or 3MM, descending; (*c*) ethyl acetate + pyridine − water (5 + 2 + 5, by vol., upper layer; Sastry & Kates, 1964), Whatman paper no. 1 or 3MM, descending. Chromatographic spray reagents were as detailed previously (Wicken & Knox, 1970).

RESULTS

Teichoic acids of Lactobacillus helveticus

Wall teichoic acid was obtained in yields of 13·6% and 9·1% of the dry weight of cell walls by TCA and sodium hydroxide extraction respectively, after chromatography on Sephadex G 75 (K_d *ca.* 0·5). Paper chromatography of acid hydrolysates of both preparations showed products typical of glycerol teichoic acids substituted with glucose and, in the case of TCA-extracted teichoic acid, D-alanyl esters. Quantitative estimation of D-glucose and phosphorus in acid hydrolysates gave mole ratios of 0·64:1·00 and 0·56:1·00 respectively for TCA- and sodium hydroxide-extracted teichoic acids. These values compared favourably with the glucose:phosphorus mole ratio of 0·61:1·00 for whole cell walls. Isolated cell walls contained 1·37 μmoles phosphorus/mg. dry weight which is indicative of a total teichoic acid content of about 35% by weight, assuming that all of the phosphorus in the cell wall is accounted for by teichoic acid; this would represent approximately 7% of the total cell mass.

Phenol-extracted membrane teichoic acid was eluted, free of nucleic acid material, close to the void volume from 6% agarose columns, and appeared to be of the same order of molecular size as the P- or lipoteichoic acid isolated from *Lactobacillus fermenti* NCTC6991 (Wicken & Knox, 1970); the yield from 21 g. dry weight of organisms was 96 mg., or approximately 0·5% of the total cell mass. Acid hydrolysates of this material showed, in addition to the components found in wall teichoic acid, fatty acid(s) and traces of galactose as well as associated protein material. Quantitative analyses gave 4·4% fatty acid esters (as palmitic acid) and the mole ratio of glucose:galactose:phosphorus was 0·45:0·025:1·00.

Alkaline phosphomonoesterase treatment of alkali-hydrolysed wall teichoic acid gave glycerol, traces of diglycerol monophosphate, a small amount of a glycoside, traces of an organic phosphate ester, and appreciable quantities of a material containing organic phosphate. The latter migrated as a broad band ($R_{glycerol}$ = 0·0 to 0·18) in solvent A and had a glucose:phosphorus ratio of 1·09:1. Diglycerol monophosphate was identified by its mobility in solvent A and rapid reaction with the periodate+Schiff's reagent on paper chromatograms. The glycoside gave a slow reaction with the periodate+Schiff's reagent, characteristic of a 2-substituted glycerol, and after acid hydrolysis showed D-glucose and glycerol as the only constitutents in the mole ratio of 0·93:1·00. It was not hydrolysed by β-glucosidase and was chromatographically indistinguishable from authentic 2-O-α-D-glucopyranosyl-glycerol, having $R_{glucose}$ values of 1·10 and 1·03 in solvents B and C respectively. Hydrolysis of wall teichoic acid in 60% HF at 0° (Glaser & Burger, 1964) followed by treatment with phosphomonoesterase gave a good yield of this glycoside as well as glycerol, and no evidence of higher glycosidic substitution of glycerol was obtained. These results are consistent with 2-O-α-D-glucosyl-substitution of many of the glycerol moieties in these teichoic acid preparations. The phosphate ester (R_F = 0·43 in solvent A) obtained from alkaline+enzymic hydrolysis was unchanged by further treatment with these agents, and gave a slow reaction with the periodate+Schiff's reagent analogous to that given by the glycoside above. Acid hydrolysis gave glycerol, glucose and glycerol monophosphates. The ester is therefore likely to be diglucosyldiglycerol monophosphate which has an identical reported R_F in solvent A (Shaw & Baddiley, 1964).

Degradation of membrane teichoic acid by alkali and phosphomonoesterase or by

60% HF gave products essentially similar to those from wall teichoic acid. In addition, hydrolysis by alkali yielded a small quantity of a glycoside containing glucose and galactose ($R_{glucose}$ = 0·31 in solvent B) and reacting rapidly with the periodate+ Schiff's reagent. The monoglucosylglycoside was chromatographically identical to that obtained from wall teichoic acid and similarly was not hydrolysed by β-glucosidase.

Reaction of teichoic acids from Lactobacillus helveticus with homologous antisera

Reactivity of antisera to Lactobacillus helveticus cells

Antisera obtained from three rabbits injected with a suspension of *Lactobacillus helveticus* gave a positive reaction in the qualitative ring precipitin test with an acid extract of the organisms. When examined by the quantitative precipitin method the most active serum (rabbit 159) contained, per ml., only 0·23 mg. of antibody reacting with membrane teichoic acid and < 0·1 mg. reacting with wall teichoic acid.

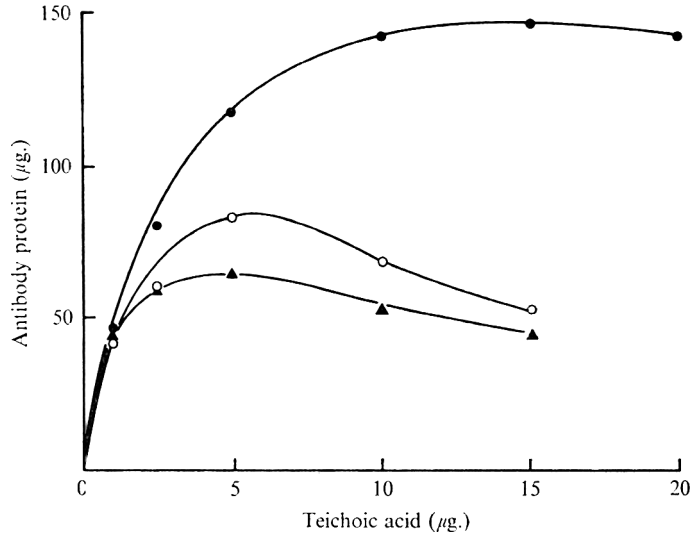

Fig. 1. Precipitation of teichoic acids from *Lactobacillus helveticus* by antiserum (0·1 ml.) against membrane teichoic acid (rabbit 176); ●, Membrane teichoic acid; ○, ammonia-degraded membrane teichoic acid; ▲, wall teichoic acid.

Reactivity of antisera to membrane teichoic acid

Antisera prepared against the isolated membrane teichoic acid reacted with both the wall and membrane teichoic acids; with membrane teichoic acid the amount of antibody in sera from rabbits 175, 176 and 177 was estimated to be 1·2, 1·5 and 0·8 mg./ml. respectively. The reactions of the wall and membrane teichoic acids with antiserum 176 (0·1 ml.) are compared in Fig. 1. The weaker reaction of wall teichoic acid could be related to its molecular weight being considerably less than that for the membrane teichoic acid. It was shown previously (Knox *et al.* 1970) that aqueous ammonia degraded high molecular weight membrane teichoic acid from *Lactobacillus fermenti*, removing fatty acids and yielding a product with serological properties similar to those of low molecular weight teichoic acid. Treatment of a solution of *L. helveticus* mem-

brane teichoic acid with an equal volume of aqueous ammonia (sp.gr. 0·88) at room temperature for 16 h. decreased its serological reactivity, the quantitative precipitin curve now being similar to that for the wall teichoic acid (Fig. 2).

In earlier studies on teichoic acid preparations from *Lactobacillus fermenti* (Hewett *et al.* 1970), the application of the haemagglutination procedure indicated that lipoteichoic acids adsorbed to sheep erythrocytes whereas lipid-free teichoic acid did not. Similar studies with *L. helveticus* preparations have shown that the membrane lipoteichoic acid but not the wall teichoic acid sensitizes sheep erythrocytes. The titre of antiserum 176 using these sensitized erythrocytes was 800; prior absorption of antiserum with whole organisms (Hewett *et al.* 1970) decreased the titre to 200.

Table 1. *Inhibition of precipitin reaction between teichoic acids and antisera prepared against membrane teichoic acid from Lactobacillus helveticus*

The antisera were examined for serological specificity using membrane teichoic acid, ammonia-degraded membrane teichoic acid and wall teichoic acid prepared from *L. helveticus*. Results are recorded in the form of percentage inhibition of the precipitin reaction when glucose, methyl-α-D-glucoside or methyl-β-D-glucoside was present.

Antiserum from rabbit	Teichoic acid	Inhibition (%) produced by sugar (20 μmoles)		
		Glucose	Methyl-α-D glucosice	Methyl-β-D glucoside
175	Membrane	38	52	18
177	Membrane	25	51	13
176	Membrane	35	47	20
	Degraded membrane	44	59	22
	Wall	42	61	25

Specificity of antisera to membrane teichoic acid

D-Glucose, methyl-α-D-glucoside and methyl-β-D-glucoside were examined for their ability to inhibit the precipitin reaction between membrane teichoic acid (10 μg.) and each of the antisera 175 (0·1 ml.), 176 (0·1 ml.) and 177 (0·2 ml.). The detailed results for antiserum 176 are given in Fig. 2 while Table 1 compares the degree of inhibition by 20 μmoles of carbohydrate for each of the sera. The results indicate that α-D-glucosyl substituents were primarily responsible for the reaction between membrane teichoic acid and the antisera.

D-Glucose, methyl-α-D-glucoside and methyl-β-D-glucoside were also compared as inhibitors of the precipitin reactions between antiserum 176 (0·2 ml.) and both wall teichoic acid (5 μg.) and ammonia-degraded membrane teichoic acid (10 μg.). The results for 20 μmoles of carbohydrate are included in Table 1 and the detailed results for wall teichoic acid are given in Fig. 3. The results with degraded membrane teichoic acid indicate that it retained its serological specificity. The results with wall teichoic acid indicate that this preparation cross-reacted with antiserum to membrane teichoic acid because of the presence of α-D-glucosyl substituents. Figure 3 also shows that galactose partially inhibited this precipitin reaction although galactose is not a substituent of either the wall teichoic acid or the polyglycerophosphate component of the membrane teichoic acid.

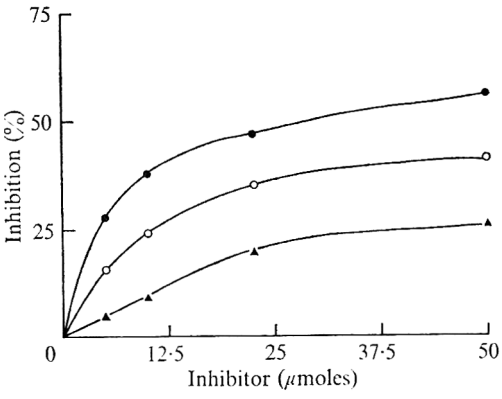

Fig. 2

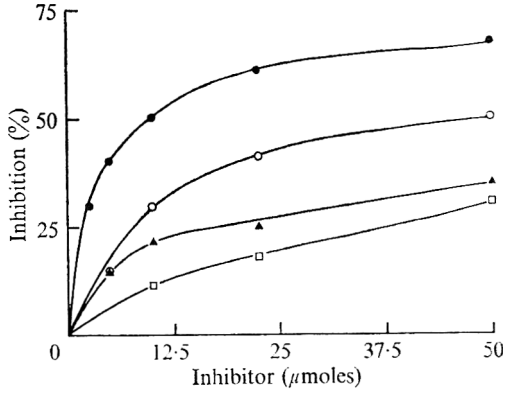

Fig. 3

Fig. 2. Inhibition of the precipitin reaction between membrane teichoic acid (10 μg.) and homologous antiserum (rabbit 176, 0·1 ml.) by D-glucose (○), methyl-α-D-glucoside (●) and methyl-β-D-glucoside (▲).

Fig. 3. Inhibition of precipitin reaction between wall teichoic acid (5 μg.) and antiserum 176 (0·2 ml.) by D-glucose (○), methyl-α-D-glucoside (●), methyl-β-D-glucoside (▲) and galactose (□).

Serological cross-reactions between teichoic acids from different bacterial species

Reaction of antisera to Lactobacillus helveticus teichoic acid with other teichoic acids

The results of the chemical and serological experiments described above indicate that both the cell wall and membrane teichoic acids of *Lactobacillus helveticus* are glycerophosphate polymers partially substituted with α-D-glucosyl residues. Antiserum against membrane teichoic acid (rabbit 176) was next tested for its reaction with polyglycerophosphate from group A streptococci (McCarty, 1959), and membrane teichoic acid from *L. fermenti* NCTC6991; the latter preparation, designated P-teichoic acid (Wicken & Knox, 1970), contained glucose and galactose substituents. The cross-reactions were determined by the quantitative precipitin method, the maximum amount of antibody precipitated being expressed as a percentage of the maximum precipitated in the homologous reaction. The results (Table 2) showed that there was a moderate degree of cross-reaction between *L. fermenti* teichoic acid and antiserum 176 whereas streptococcal polyglycerophosphate reacted very weakly.

The cross-reaction with *Lactobacillus fermenti* teichoic acid was also examined by the haemagglutination procedure using sheep erythrocytes sensitized with this teichoic acid preparation; the titre with antiserum 176 was 200 compared with 800 for the homologous reaction using sheep erythrocytes sensitized with *L. helveticus* membrane teichoic acid.

Reaction of teichoic acids with antisera to group A streptococci

Each of the three rabbits (170, 171, 172) injected with group A streptococci gave antisera which reacted strongly in the qualitative ring precipitin test with streptococcal polyglycerophosphate (100 μg./ml.). All quantitative tests were carried out with antiserum 170. This antiserum reacted strongly with *Lactobacillus fermenti* and *L. helveticus* membrane teichoic acids (Fig. 4) and moderately with *L. helveticus* wall teichoic acid

(Table 2). Using sheep erythrocytes sensitized with *L. helveticus* membrane teichoic acid, the haemagglutination titre of antiserum 170 was 1600. These results showed that whereas streptococcal polyglycerophosphate reacted very weakly with antiserum specific for α-D-glucosyl substituents (rabbit 176), teichoic acids from *L. helveticus* reacted well with antisera to group A streptococci (rabbit 170). The reaction of *L. helveticus* membrane teichoic acid with antiserum 170 was not inhibited by 100 μmoles of D-glucose, suggesting that the cross-reaction was dependent on the polyglycerophosphate component of the membrane teichoic acid rather than due to the presence of an unknown α-glucosyl-specific antigen in the streptococci.

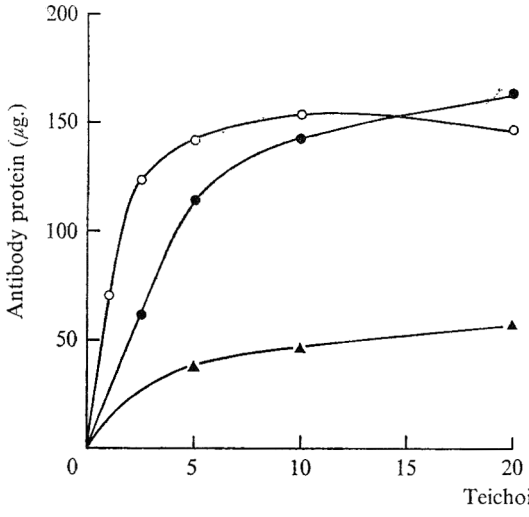

Fig. 4

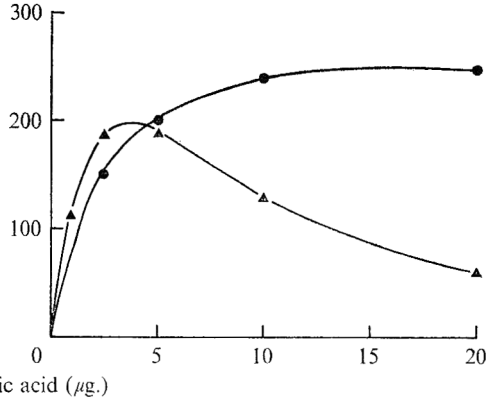

Fig. 5

Fig. 4. Precipitation of teichoic acids by antiserum (0·3 ml.) against group A streptococci (rabbit 170). ○, Streptococcal polyglycerophosphate; ●, membrane teichoic acid and, ▲, wall teichoic acid from *Lactobacillus helveticus*.

Fig. 5. Precipitation of teichoic acids from *Lactobacillus helveticus* by antiserum (0·1 ml.) against *L. plantarum* (rabbit 164). ●, Membrane teichoic acid; ▲, wall teichoic acid.

Table 2. *Cross-reactions of antisera with preparations of teichoic acid*

Values are expressed as a percentage of the amount of antibody precipitated in the homologous reaction.

		Source of teichoic acid			
Preparation injected	Antiserum from rabbit	Group A strepto-coccus	*L. fermenti* membrane	*L. helveticus* membrane	*L. helveticus* wall
Lactobacillus helveticus membrane teichoic acid	176	5	30	100	38
Group A streptococcus	170	100	102	105	28
L. fermenti membrane teichoic acid	146	32*	100	72	32
	149	32*	100	73	21
	147	15*	100	38	17
	148	25*	100	40	16

* From Knox, Hewett & Wicken, 1970.

To confirm that the cross-reaction depended on antibodies against the streptococcal glycerol teichoic acid, antiserum 170 (0·75 ml.) was mixed with streptococcal polyglycerophosphate (25 ug./0·25 ml.), the resultant precipitate removed, and the supernatant solution (0·4 ml.) tested for its reaction with *Lactobacillus helveticus* membrane teichoic acid (20 μg.). The amount of precipitate that formed on standing was $< 10\%$ of that formed in the control experiment omitting polyglycerophosphate.

In a similar experiment absorption of antiserum 170 (10 ml.) with *Lactobacillus fermenti* P-teichoic acid (1 mg.) decreased the reactivity of the antiserum with streptococcal polyglycerophosphate by 80%, and the reaction with *L. helveticus* membrane teichoic acid by 90%. The precipitate that had formed on the addition of the *L. fermenti* teichoic acid was analysed to confirm the presence of glucose and galactose: relative to the amount of teichoic acid added, the recoveries of phosphorus, glucose and galactose were 75%, 63% and 67% respectively.

Reaction of teichoic acids with antisera to Lactobacillus fermenti

Antisera prepared against *Lactobacillus fermenti* membrane teichoic acid were examined for their reaction with *L. helveticus* teichoic acids; their reaction with the streptococcal polyglycerophosphate has already been described (Knox *et al.* 1970). The sera chosen illustrated the differences in specificity which were observed, and the values in brackets indicate the percentage inhibition of the homologous reaction by 100 μmoles of glucose and galactose respectively (Knox *et al.* 1970): antiserum 146 (6%, 10%), 149 (4%, 11%), 147 (7%, 36%) and 148 (22%, 44%). The cross-reactions with the *L. helveticus* teichoic acids (Table 2) reflect the specificities of the antisera, with 147 and 148 cross-reacting less than 146 and 149.

The cross-reactions were also detected by the haemagglutination procedure: the titre of antiserum 146 was 1600 when tested with erythrocytes sensitized with *Lactobacillus fermenti* P-teichoic acid and 800 when tested with *L. helveticus* membrane teichoic acid, whilst for antiserum 148 the respective titres were 3200 and 1600.

Reaction of Lactobacillus helveticus teichoic acids with antisera to other lactobacilli

By using the qualitative ring precipitin test *Lactobacillus helveticus* membrane teichoic acid (100 μg./ml.) was shown to react well with antisera against groups D and E lactobacilli, but not with antisera against groups B and C lactobacilli. The quantitative reactions of *L. helveticus* membrane and wall teichoic acids with group D antiserum (0·1 ml. from rabbit 164) are compared in Fig. 5. The amount of antibody precipitated by the membrane teichoic acid was approximately the same as that precipitated by an impure preparation of membrane teichoic acid from the group D strain, *L. plantarum* NCIB 7220 (Wicken & Knox, unpublished observations).

A comparison of the results for *Lactobacillus helveticus* wall and membrane teichoic acids in Table 2 and Fig. 1, 4 and 5 shows that the wall teichoic acid reacted strongly with only group D antisera, where it precipitated 84% of the amount of antibody precipitated by membrane teichoic acid. Ammonia-degraded membrane teichoic acid also reacted better with group D antiserum, giving 75% of the reaction of undegraded material compared with 54% for the reaction with antiserum 176 (Fig. 1).

DISCUSSION

Sharpe *et al.* (1964) indicated that the wall and membrane (intracellular) teichoic acids of *Lactobacillus helveticus* NCIB 8025 were of the glycerol type but only the membrane teichoic acid was appreciably substituted with glucose, a glucosylglycerol being isolated after alkaline hydrolysis of the latter.

The teichoic acid which we isolated from *Lactobacillus helveticus* NCIB 8025 cell walls by either TCA- or sodium hydroxide-extraction contained appreciable quantities of glucose. The glucose:phosphorus mole ratios of these preparations reflected that of unextracted cell walls and suggested that no enrichment of a glucosylated teichoic acid had taken place during extraction. The products of alkali and hydrofluoric acid degradation of wall teichoic acid were consistent with a structure having the normal arrangement of phosphodiester groups between positions 1 and 3 of each glycerol moiety, and with many of the latter residues being substituted on the secondary hydroxyl group with α-D-glucose. The enrichment of glucose in teichoic acid fragments apparently stable to alkali hydrolysis leads us to suggest either regions of full and partial glycosidic substitution within the same polymer chain or a mixture of fully and partially substituted chains: no glucosylglycerol or diglucosyldiglycerol monophosphate could be expected from alkaline and enzymic degradation of a mixture of fully glucosylated and unsubstituted polymers. In this connection it is interesting to note that the wall teichoic acid from *L. buchneri* has been shown to be partially substituted with glucose and has regions of the polymer where adjacent glycerol residues bear glucose substituents (Archibald, Baddiley & Heptinstall, 1969).

The membrane teichoic acid from *Lactobacillus helveticus* has a lower degree of α-glucosyl substitution than the wall polymer. The product isolated by phenol extraction resembled the lipo- or P-teichoic acid of *L. fermenti* NCTC 6991, being of high apparent molecular weight and containing a glycolipid component. Evidence for a glycolipid being present was provided by the detection of fatty-acid ester residues and of a glycoside containing glucose and galactose and assumed to be a 1-substituted glycerol from its rapid reaction with the periodate + Schiff's reagent. The small amount of the glycoside in alkaline hydrolysates probably reflects the low galactose content of the polymer.

The studies by Sharpe *et al.* (1964) on the teichoic acids of group A lactobacilli (including *Lactobacillus helveticus* NCIB 8025) led them to conclude that the membrane teichoic acid was the group antigen, the preparations of wall teichoic acid being serologically inactive when examined by the precipitin reaction. More recently, Mills (1969) studied the agglutinating antigens of a strain of *L. jugurti* of serological group A and concluded that glycerol teichoic acid, containing 'variable traces of glucose', was the major wall component responsible for the agglutination of whole organisms by homologous antisera. Preparations of wall and membrane teichoic acids appeared to be immunologically identical, as determined by the Ouchterlony method, but it was considered unlikely that the membrane teichoic acid component of intact organisms would react with antibodies. Studies on the group F antigen of lactobacilli have indicated that antibody can react with the membrane teichoic acid *in situ* (Hewett *et al.* 1970).

The present studies have shown that antibodies prepared against membrane teichoic acid from *Lactobacillus helveticus* NCIB 8025 reacted with wall teichoic acid because of

the presence of the α-D-glucosyl substituents on the polyglycerophosphate component of both wall and membrane teichoic acid. In terms of the reactivity of Lancefield acid extracts of *L. helveticus* (Sharpe, 1955) or the agglutinability of this organism (Mills, 1969), it is to be expected that the wall teichoic acid would be the major contributor to the serological reaction because of the greater amount of teichoic acid in the wall. However, none of the studies on the group A lactobacilli has resolved whether the antibodies formed on the injection of whole cells are elicited by the wall teichoic acid, the membrane teichoic acid or by both of these substances. Thus the classification of a lactobacillus as belonging to serological group A may depend on the production of antibodies to the membrane teichoic acid and their reaction primarily with the wall component. The definition of the group antigen in this case would therefore depend on whether it is defined as the component eliciting antibody production or the component detected in a particular serological procedure.

Quantitative precipitin tests have shown that teichoic acid from *Lactobacillus helveticus* cross-reacted with antisera to groups D, E and F lactobacilli and group A streptococci. In the case of the group D reaction, the *L. helveticus* membrane teichoic acid was probably combining with antibodies against the *L. plantarum* membrane glycerol teichoic acid rather than the wall ribitol teichoic acid; studies on the membrane component from one strain of *L. plantarum* (= *L. arabinosus* 17–5) indicated a low degree of α-glucosyl substitution (Critchley, Archibald & Baddiley, 1962). The reaction with group E antiserum, however, could depend on antibodies against the wall glycerol teichoic acid. This is the group antigen which has been shown in four species to contain glucose substituents (Sharpe *et al.* 1964) and in one of them (*L. buchneri*) to be partially substituted with α-D-glucosyl residues (Shaw & Baddiley, 1964).

The results with antisera against group F lactobacilli and group A streptococci indicated that glycerol teichoic acids differing in structure may nevertheless cross-react. The teichoic acid from group A streptococci is unsubstituted with sugar (McCarty, 1959) whereas the corresponding product from group F lactobacilli (*Lactobacillus fermenti*) contains galactose and a disaccharide of glucose and galactose (Wicken & Knox, 1970).

The reaction of certain antisera against group A streptococci with extracts from various Gram-positive bacteria, including streptococci, staphylococci and sporulating bacilli, was observed by McCarty (1959); polyglycerophosphate was isolated from a group A streptococcus, and it was concluded that this component is widespread amongst Gram-positive bacteria. More recently, Sharpe & Brock (personal communication) showed that antisera against a strain of *Lactobacillus acidophilus* reacted with 'almost all of 98 strains of lactobacilli' as well as some other Gram-positive bacteria; glucosyl-glycerol teichoic acid was isolated from one of the reacting strains of *L. casei*, and it was suggested that this component was responsible for the serological reactivity of the other strains. Both of these studies implied that a common serological reaction was due to a teichoic acid, the structure of which was similar to that isolated from one particular strain. However, the results of the present study indicate that such a conclusion is not necessarily valid because of the cross-reaction of teichoic acids differing in structure.

With regard to the observations on serological cross-reactions, the situation may be analogous to that discussed by Kabat (1966). McCarty (1964) had noted that certain group A streptococcal antisera reacted equally well with streptococcal glycerol

teichoic acid whether or not alanine was substituted on carbon 2, and Kabat suggested that such antisera 'would have a specificity complementary to the —CH— aspect of carbon 2 of the glycerol teichoic acid and not involving the more hydrophilic side of the molecule'. A molecular model of part of an α-D-glucosyl-substituted glycerol teichoic acid can be constructed in which all the glucosyl residues project in the same direction, thus fulfilling the requirements for Kabat's proposition. Different antibodies reacting with the teichoic acid could then have specificities depending primarily on the glucosyl substituents (e.g. antiserum 176) or independent of the glucosyl substituents but dependent on the glycerol phosphate 'backbone' (e.g. antiserum 170). An analogous situation has been proposed by Lüderitz, Staub & Westphal (1966) to account for the serological specificity of the branched chain O-specific polysaccharides of *Salmonella*.

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Division of Mycoplasmas into Subgroups

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SUMMARY

The deoxyribonucleic acid (DNA) base compositions of 12 recently isolated or new species of mycoplasmas fell within the range known for the Mycoplasma group. By DNA–DNA hybridization a new sterol-nonrequiring strain, S-743, was unrelated to *Mycoplasma laidlawii* or *M. granularum*, the latter two strains showing moderate cross-reactivity; swine strain B3 appeared to be related to *M. mycoides* var. *capri*. From these results and information in the literature, the mycoplasmas have been divided into six provisional subgroups on the basis of physiological characters, DNA base composition and nucleic acid hybridization. Some speculations are made on the significance of heterogeneity among mycoplasmas.

INTRODUCTION

Earlier work has emphasized the heterogeneity of mycoplasmas (Neimark & Pickett, 1960). Studies showing that the guanine+cytosine (GC) contents of Mycoplasma DNA range widely from approximately 23 to 40 moles % established that this heterogeneity was fundamental (Neimark & Pene, 1965). Studies also revealed that a cluster of mycoplasmas exists with GC contents lower than those of any known aerobic bacteria (Jones & Walker, 1963; Neimark & Pene, 1965). Data now available on the DNA base compositions of most known mycoplasmas (McGee, Rogul & Wittler, 1967; Neimark, 1967; Bak & Black, 1968; Kelton & Mandel, 1969; Williams, Wittler & Burris, 1969) indicate that heterogeneity in base composition extends through both fermentative and nonfermentative strains and divides the mycoplasmas into subgroups that are incompatible in a single genus (Neimark, 1967). Nucleic acid hybridization studies have been carried out between several mycoplasmas (Somerson, Reich, Chanock & Weissman, 1967; McGee *et al.* 1967; Peterson & Pollock, 1969). The present paper reports DNA base compositions of some recently isolated mycoplasmas (some representing new species) and results of DNA–DNA hybridization between selected mycoplasmas. Six subgroups are proposed and speculations made on the implications of divisions within the mycoplasmas. These results were presented in preliminary form (Neimark, 1969).

METHODS

Organisms, media and growth conditions. The organisms used in this study were kindly supplied by several colleagues and, whenever possible, were obtained from the laboratories which originally isolated or identified them. Strains S-410 and S-743 (Tully & Razin, 1969) and B3 (Dinter, Danielsson & Bakos, 1965) were provided by

Dr J. Tully; goat 145 by Dr J. Tully and Dr R. Del Giudice; UM30847 and *Mycoplasma arginini* strain G230 (Barile *et al.* 1968) by Dr M. Barile; strains 747v and S3–7 (Davidson & Thomas, 1968) by Dr M. Davidson; *M. agalactiae* var. *bovis* (Hale, Helmboldt, Plastridge & Stula, 1962) by Dr A. Mosher. The latter strain does not produce acid from glucose in amounts that can be detected by change in pH indicator, even after prolonged serial passage on media containing glucose. *Mycoplasma gallisepticum* strain S6, obtained from Dr H. Adler, and *M. granularum*, from Dr W. Switzer, were used in hybridization studies. The identity of the *M. granularum* strain used in this study was kindly confirmed by Dr J. Tully. Each strain was cloned three times. DNA from Connecticut goat, designated *M. mycoides* var. *capri* (Jonas & Barber, 1969), VOM strain, and Mexican strain was prepared through the courtesy of Dr J. J. Callis and Dr T. Barber, United States Department of Agriculture, Plum Island Animal Disease Laboratory.

Media and growth conditions were essentially as described previously (Neimark & Pickett, 1960; Neimark, 1967). Neither inhibitory agents nor antibiotics were employed.

Labelled DNA was produced by growing mycoplasmas in liquid media containing 0·5 μCi/ml. of thymidine-methyl-^{3}H.

Enzymes and isotopes. Ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) was heated at 85° for 10 min to inactivate any contaminant deoxyribonuclease. Pronase (Calbiochem, Los Angeles, California) was self-digested for 2 h. at 37° (Saito & Miura, 1963; Gillespie & Spiegelman, 1965). Thymidine-methyl-^{3}H (specific activity approximately 15 Ci/mmole) was purchased from Schwartz BioResearch, Orangeburg, New York.

Isolation of DNA. Cells were collected from broth by centrifugation at approximately 20,000 *g*, washed once with cold saline-EDTA (0·15 M-NaCl + 0·1 M-Na_2 ethylenediamine tetra-acetate, pH 8·0), and their DNA isolated by the method of Marmur (1961).

Cells from strains 747v and S3–7 could be obtained only in small amounts, and their DNA was partially purified. After suspensions had been lysed with 1% sodium dodecyl sulphate, they were diluted with an equal volume of saline-EDTA and digested with Pronase (50 μg./ml.) for 12 to 16 h. at 37°. The lysate was then deproteinized by shaking with chloroform-isoamyl alcohol, the aqueous phase precipitated with 2 vol. ethanol, and the DNA dissolved in a small volume of SSC (0·15 M-NaCl + 0·015 M-sodium citrate).

Determination of DNA base composition. The average GC content of DNA was estimated from buoyant density in a CsCl density gradient as described by Schildkraut, Marmur & Doty (1962) using *Escherichia coli* DNA as reference (taken to have a buoyant density of 1·710 g./cm.^{3}). All samples were analysed at least twice.

Immobilization of DNA on filters. DNA was bound to membrane filters by the procedures of Gillespie & Spiegelman (1965) and Denhardt (1966), with minor modifications. Native DNA (50 μg./ml.) was denatured by boiling for 8 min. and then quickly cooled by immersion in an ice bath and dilution with 9 vol. cold sixfold concentrated SSC. Denatured DNA was filtered through 25 mm. membrane filters (HA, 0·45 μm; Millipore Corporation, Bedford, Massachusetts) prewashed in sixfold concentrated SSC. Filters with retained DNA were washed with sixfold concentrated SSC, dried overnight in a desiccator at 22°, and baked in a vacuum oven for 2 h. at 80°.

Labelled DNA and competitor DNA were sheared by sonic oscillation (Biosonic II, microtip probe; Will Scientific, New York) and then denatured.

DNA–DNA duplex formation. Filters with immobilized DNA were preincubated in the medium of Denhardt (1966) for 2 to 6 h. at the temperature to be used for hybridization, drained and blotted, and then placed in vials which contained 1 μg, homologous or heterologous labelled DNA fragments in twofold concentrated SSC. For competition experiments (Hoyer, McCarthy & Bolton, 1964), a large excess of unlabelled DNA fragments was added to the reaction mixture (final volume of 0·5 ml.) to compete with labelled DNA fragments for binding sites on the immobilized DNA. The ratio of membrane-bound to labelled DNA was 10:1 or, in some cases, 40:1. Hybridization was carried out for 12 to 16 h. at 25° below the optical melting temperature (T_m) of the DNA (Marmur & Doty, 1961, 1962); where DNA samples differed in T_m, the higher temperature, which provided more stringent test conditions, was used. Filters were then washed on each side with twofold concentrated SSC at the temperature of hybridization, dried at 60° and counted in a Picker Liquimat or Beckman liquid scintillation counter.

RESULTS

DNA base compositions. For several recently isolated mycoplasmas the GC contents of carbohydrate-fermenting strains ranged from 26 to 31 moles % and those for nonfermentative strains ranged from 26 % to 32 % (Table 1). In addition, GC contents of 35·2 % and 32·4 % were found for *Mycoplasma gallisepticum* S6 and *M. granularum*. No satellite bands were observed for any of these strains.

Table 1. *DNA base compositions of some recent isolates and new Mycoplasma species*

Species or strain	Origin	Density* (g./cm³)	GC content (mole %)
	A. Fermentive strains		
S-410	Cell culture	1·6907	31·3
S-743	Cell culture	1·6907	31·3
Connecticut goat	Goat	1·6892	29·8
VOM goat	Goat	1·687	27·6
Mexican goat	Goat	1·6868	27·4
UM 30847	Goat	1·686	26·5
B3	Swine	1·6857	26·3
	B. Nonfermentative strains		
M. agalactiae. var *bovis*†	Cow	1·692	32·7
Goat 145	Goat	1·689	29·6
M. arginini G230	Several animals	1·688	28·6
747V	Primate	1·687	27·6
S3–7	Primate	1·686	26·5

* Average of two determinations.

† This strain does not produce acid from glucose in amounts that can be detected by pH change.

DNA–DNA hybridization. The sterol-nonrequiring strains S-410 and S-743 have identical average base compositions of 31·3 moles % GC (Table 1). Strain S-743 was tested against the other known sterol-nonrequiring mycoplasmas, *Mycoplasma laidlawii* and *M. granularum*, to determine its relationship to them. Reciprocal hybridiza-

tion tests between S-743 and *M. granularum* showed only very low levels of DNA binding (Table 2). In competition reactions between S-743 and *M. laidlawii* A (Table 3), the addition of homologous unlabelled DNA fragments reduced binding of labelled DNA by approximately two-thirds in each case, whereas addition of heterologous fragments produced no significant decrease in binding. Thus there is little or no genetic relatedness between S-743 and *M. laidlawii* A or *M. granularum*. One-way crosses between DNAs from S-743, *M. pneumoniae* and *M. gallisepticum* also showed only a very low level of duplex formation (Table 2).

Table 2. *DNA–DNA hybridization tests between Mycoplasma strains*

Relations among strains are expressed as % relative to the homologous reaction which is designated 100%. Averages of duplicate trials are reported.

Source of DNA on filter	Source of ^{3}H-labelled DNA fragments	DNA bound (%)
S-743	S-743	100
M. granularum	S-743	3
M. gallisepticum S6	S-743	5
M. pneumoniae FH	S-743	3
M. granularum	*M. granularum*	100
S-743	*M. granularum*	6
M. laidlawii A	*M. granularum*	34
M. laidlawii A	*M. laidlawii* A	100
M. granularum	*M. laidlawii* A	32

Table 3. *DNA competition reactions between selected Mycoplasma strains*

Relations among strains are expressed as % relative to the homologous reaction which is designated 100%. Averages of duplicate trials are reported.

Source of DNA on filter	Source of ^{3}H-labelled DNA fragments	Competitor DNA fragments added (50 μg.)	DNA bound (%)
A. S-743 against *M. laidlawii* A			
S-743	S-743	None	100
S-743	S-743	S-743	33
S-743	S-743	*M. laidlawii*	98
M. laidlawii A	*M. laidlawii* A	None	100
M. laidlawii A	*M. laidlawii* A	*M. laidlawii* A	30
M. laidlawii A	*M. laidlawii* A	S-743	105
B. Strain B3 against VOM strain of *M. mycoides* var. *capri*			
B3	B3	None	100
B3	B3	B3	34
B3	B3	VOM	33
VOM	B3	None	73
VOM	B3	B3	25
VOM	B3	VOM	22

Duplex formation between *Mycoplasma laidlawii* and *M. granularum* DNA was observed at levels of about one-third that of the homologous reactions (Table 2). For comparisons between strain B3 and the VOM strain of *M. mycoides* var. *capri*, the DNA of strain VOM was prepared under quarantine at the United States Department of Agriculture Plum Island Animal Disease Laboratory by the author and was sterility

tested by Plum Island Laboratory staff. Under these conditions it was not possible to prepare radio-labelled DNA, and therefore reciprocal hybridization tests could not be done. However, DNA fragments of B3 and VOM were approximately equivalent in their competition with labelled B3 fragments for sites on membrane-bound DNA of B3 and VOM (Table 3). This clearly demonstrates that strain B3 and *M. mycoides* var. *capri* are genetically related.

DISCUSSION

The DNA base compositions reported here fall within the known range of 22·8 to 41·0 moles % for the mycoplasmas. A GC content of 23 % would appear to be near a lower limit, considering that GC contents of some mycoplasmas are amongst the lowest known for prokaryotic or any other organisms (Jones & Walker, 1963; Neimark & Pene, 1965) and because of limits to the degeneracy of the genetic code.

Sterol-nonrequiring mycoplasmas. The sterol-nonrequiring organisms *Mycoplasma laidlawii* and *M. granularum* are physiologically distinct from all other mycoplasmas (Edward, 1967; Tully & Razin, 1968). Gel-electrophoresis patterns revealed some similarity in their proteins, and serological tests showed some sharing of antigens (Tully & Razin, 1968). My finding of a moderate amount of DNA duplex formation between *M. laidlawii* A and *M. granularum* agrees partially with the results of Pollock & Bonner (1969), who reported that DNA from *M. granularum* strain BTS-39 bound DNA from *M. laidlawii* A to somewhat less than one-fourth that of the homologous DNA. *M. laidlawii* and *M. granularum* are thus related.

Tully & Razin (1969) showed that two new sterol-nonrequiring isolates, S-410 and S-743, differed from *Mycoplasma laidlawii* and *M. granularum* by serological tests, their inability to synthesize pigmented carotinoids, and gel-electrophoresis patterns of cellular proteins; they also differed serologically from the known sterol-requiring mycoplasmas. My DNA hybridization experiments showed only very low levels of homology between S-743 and *M. laidawii* or *M. granularum*. Isolate S-743 is distinct from *M. laidlawii* and *M. granularum* and is more distant from these two organisms than they are from one another. Tully and Razin (personal communication) propose to nominate this organism as a new species. The occurrence of additional sterol-nonrequiring mycoplasmas distinct from *M. laidlawii* gives further support for the establishment of a new genus in the order Mycoplasmatales (Edward *et al.* 1967; Tully & Razin, 1969). Recently, Edward & Freundt (1969) formally proposed a second family and genus within the Mycoplasmatales to recognize the distinction between the sterol-nonrequiring organisms and the rest of the group.

Fermentative mycoplasmas of low GC content. Strain B3 has been distinguished from mycoplasmas isolated from pigs (Dinter *et al.* 1965), and although a number of random samples of porcine and avian sera contained antibody to B3, no serological relationship with any strain was demonstrated (Taylor-Robinson & Dinter, 1968). Strain B3 is closely related to *Mycoplasma mycoides* var. *capri*, as indicated by the proximity of their GC contents and the high level of duplex formation between B3 and VOM DNA; Razin (1968) has also noted the similarity. Strain B3 and the strains of *M. mycoides* var. *capri* examined here are all rapidly growing, fermentative organisms and, with *M. mycoides* var. *mycoides*, form a cluster of organisms with GC contents within a 4 % span. Strain UM30847 is related to B3 and VOM and appears to be a member of this cluster (Neimark, 1969) even though growth inhibition tests by the author of UM30847

and B3 with *M. mycoides* var. *mycoides* and var. *capri* antisera were negative (sera kindly provided by Dr J. J. Callis, Plum Island Animal Disease Laboratory).

Although these organisms form a cluster they are distinguishable by DNA homology and serological procedures (Jonas & Barber, 1969). These differences may be similar to the species heterogeneity found in *Mycoplasma laidlawii* (Peterson & Pollock, 1969), *M. hominis* and *M. salivarium*, but not in *M. pneumoniae* (Reich *et al.* 1966; Somerson *et al.* 1966) or *M. hyorhinis* (Somerson *et al.* 1966).

Mycoplasma agalactiae and *M. agalactiae* var. *bovis*. Although the biological properties of these organisms are not inconsistent (Leach, 1967), no relationship has been demonstrated (Jain, Jasper & Dellinger, 1967); the similarities of gel-electrophoresis patterns (Razin, 1968) and GC content, shown here, are evidence of a relationship. Their classification as nonfermentative also must be re-examined, since *M. agalactiae* consistently produces slight acidity in glucose broth (Cottew *et al.* 1968) and strains of *M. agalactiae* var. *bovis* are known to produce an acid pH change in media containing glucose.

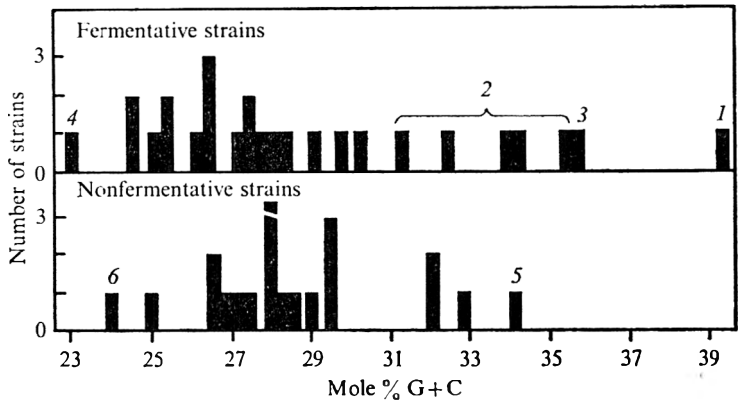

Fig. 1. Distribution of mole % GC contents among carbohydrate fermenting and nonfermenting mycoplasmas. The numbers over the bars refer to the subgroups discussed in the text. Bar No. *1* is *Mycoplasma pneumoniae*. The bracket, *2*, indicates the span where sterol-nonrequiring organisms occur. Bars *3*, *4*, *5* and *6* are the organisms that mark the upper or lower ends of the other provisional subgroups.

Division of the mycoplasmas into subgroups. Values for the DNA base compositions of most of the recognized fermentative and nonfermentative mycoplasmas are in the literature (Table 4). The GC contents of the mycoplasmas form an almost continuous span from 23 to 35 %, followed by a gap with *Mycoplasma pneumoniae* set apart. The range of GC values for the fermentative and nonfermentative strains is shown graphically in Fig. 1. The arrangement of organisms by ability to ferment carbohydrates may reflect an evolutionary separation within the mycoplasmas (Neimark & Pickett, 1960). However, it should be recognized that the division is based only on results of fermentation tests and there may exist mycoplasmas that are phenotypically nonfermentative but genotypically fermentative; a possible example is the sterol-nonrequiring strain which cannot ferment glucose but which was identified as *M. laidlawii* (Leach, 1967; Tully & Razin, 1968), a carbohydrate-fermenting species.

Table 4. *DNA base compositions of Mycoplasma strains*

Species or designation	Strain	GC (mole %) by Density	T_m	Chromatography	References
		A. Fermentative Mycoplasma			
M. pneumoniae FH		40·8	39·0	—	Neimark & Pene, 1965; Neimark, 1967
	FH:15531[a]	—	39·9	—	Williams *et al.* 1969
	Bru:15377	—	39·3 38·6	—	McGee *et al.* 1965; McGee *et al.* 1967
M. gallisepticum	801, PG31, 1010	35·7	—	—	Kelton & Mandel, 1969
	A5969	34·0	35·0	—	Marmur & Doty, 1962; Schildkraut *et al.* 1962
	S6	35·2 35·2 34·6[b]	—	—	Kelton & Mandel, 1969; Neimark, this paper; Reich *et al.* 1966
	S6:15302	—	32·5, 32·0	—	Rogul *et al.* 1965; McGee *et al.* 1967
	1150	34·7	—	—	Kelton & Mandel, 1969
	A5969	34·7[b] 33·7	 33·4	—	Morowitz *et al.* 1962; Morowitz *et al.* 1967
	PG31:19610	—	31·8	—	Williams *et al.* 1969
M. laidlawii A	—	35·7	31·7	—	Neimark, 1967
	—	35·7	30·9	—	Neimark & Pene, 1965
	—	—	34·1	—	Morowitz *et al.* 1967
	PG8:23206	—	33·0	—	Williams *et al.* 1969
	14089	35	32·5	—	Peterson & Pollack, 1969; McGee *et al.* 1967
	PG10	34·2	—	—	Kelton & Mandel, 1969
M. laidlawii B	PG9	33·6[b]	—	33·3	Reich *et al.* 1966; Chelton *et al.* 1968
	PG9:23217	—	32·4	—	Williams *et al.* 1969
	14192	34	32·3	—	Peterson & Pollock, 1969; McGee *et al.* 1967
	—	33·7	33·2	—	Morowitz *et al.* 1967
M. synoviae	1853	34·2	—	—	Kelton & Mandel, 1969
M. granularum	—	32·4	—	—	Neimark, this paper
	—	32·1	—	—	Kelton & Mandel, 1969
	BTS-39:19168	—	30·4	—	McGee *et al.* 1967
S-410/S-743	—	31·3	—	—	Niemark, table 1[c]
Avian serotype C	TU	30·5	—	—	Kelton & Mandel, 1969
	C	29·1	—	—	Kelton & Mandel, 1969
M. edwardii	PG24 (C21): 23462	—	29·2	—	Williams *et al.* 1969
Connecticut goat	—	29·8	—	—	Neimark, table 1
Mycoplasma sp. (goat)[d]	C30KS-1: 15718	—	28·9	—	Williams *et al.* 1969
M. canis	PG14	29·1	28·4	—	Kelton & Mandel, 1969; McGee *et al.* 1967

Table 4 (*cont.*)

Species or designation	Strain	GC (mole %) by Density	T_m	Chromatography	References
M. pulmonis	Sabin type C: 23115[a]	—	29·2	—	Williams *et al.* 1969
	Negroni	—	28·3	—	McGee *et al.* 1967
	ASH (PG 34): 19612	—	27·9	—	Williams *et al.* 1969
	KON: 14267	—	27·5	—	McGee *et al.* 1967
Avian serotype D	PSU-4	28·6	—	—	Kelton & Mandel, 1969
	NY	28·1	—	—	Kelton & Mandel, 1969
M. bovigenitalium	PG 11	28·1	—	—	Kelton & Mandel, 1969
	PG 11: 19852	—	30·4	—	Williams *et al.* 1969
	PG 11: 14173	—	29·6	—	McGee *et al.* 1967
M. fermentans	PG 18(G): 19989	—	28·7	—	Williams *et al.* 1969
	G2	27·6	—	—	Neimark, 1967
	G	27·5[b]	—	—	Reich *et al.* 1966
	GII: 15474	—	27·8	—	McGee *et al.* 1967
M. hyorhinis	GDL	—	27·8	—	McGee *et al.* 1967
	BTS-7: 17981	—	27·3	—	McGee *et al.* 1967
Mycoplasma sp. (sheep)	67-166: 23243	—	27·2	—	Williams *et al.* 1969
M. mycoides var. *capri*	VOM	27·6	—	—	Neimark, table 1
	PG 3	—	—	26–24 24·8 25·0	Jones & Walker, 1963; Jones *et al.* 1965; Walker, 1967
Mexican goat	—	27·4	—	—	Neimark, table 1
Goat UM 30847	—	26·5	—	—	Neimark, table 1
M. mycoides var. *mycoides*	T 3	—	—	30·0	Chelton *et al.* 1968
	GLADYSDALE	26·5	26·8	—	Neimark, 1967
	V 5	—	26·1	—	McGee *et al.* 1967
California calf	—	26·5 26·5	23·6 23·5	—	Neimark & Pene, 1965; Neimark, 1967
Avian serotype F	SA	26·5	—	—	Kelton & Mandel, 1969
B 3 (Dinter)	—	26·3	—	—	Neimark, table 1
M. felis	CO: 23391	—	25·2	—	Williams *et al.* 1969
California goat	Kid	25·5	24·1	—	Neimark & Pene, 1965
Avian serotypes	(J) 693	25·5	—	—	Kelton & Mandel, 1969
group I, J, K, N,	(I) 695	24·5	—	—	Kelton & Mandel, 1969
Q, R	(K) 1805	24·5	—	—	Kelton & Mandel, 1969
M. bovirhinis	PG 43	24·5	—	—	Kelton & Mandel, 1969
	PG 43 (5M 331): 19884	—	25·4	—	Williams *et al.* 1969
M. neurolyticum	Type A: 19988	—	26·2	—	Williams *et al.* 1969
	Type A	22·8	—	—	Neimark, 1967
	KSA: 15049	—	23·0	—	McGee *et al.* 1967

Table 4 (*cont.*)

Species or designation	Strain	GC (mole %) by Density	T_m	Chromatography	References
		B. Nonfermentative strains			
M. agalactiae[f]	—	34·2	33·6	—	Neimark, 1967
M. agalactiae var. *bovis*[f]	DONETTA	32·7	32·9	—	Neimark, table 1; Morowitz *et al.* 1967
Avian serotype L	694	32·1	—	—	Kelton & Mandel, 1969
M. arthritidis	PG 27[g]	32·6[b]	—	—	Reich *et al.* 1966
	—	32·6	30·0	—	Morowitz *et al.* 1967
	07	32·1	—	—	Kelton & Mandel, 1969
	39	32·1	—	—	Kelton & Mandel, 1969
	H 606: 13988	—	31·9	—	McGee *et al.* 1967
	CAMPO: 14152[g]	—	31·7	—	McGee *et al.* 1967
	PG 6: 19611	—	31·3	—	Williams *et al.* 1969
Goat 145	—	29·6	—	—	Neimark, table 1
M. maculosum	PG 15	29·6	—	—	Kelton & Mandel, 1969
	PG 15: 19327	—	26·7	—	McGee *et al.* 1967
M. iners	O	29·6	—	—	Kelton & Mandel, 1969
	PG 30	29·1	—	—	Kelton & Mandel, 1969
	PG 30 (M): 19705	—	29·1	—	Williams *et al.* 1969
M. spumans	PG 13	29·1	—	—	Kelton & Mandel, 1969
	PG 13 (C 48): 19526	—	28·6	—	Williams *et al.* 1969
	PG 13: 15147	—	28·4	—	McGee *et al.* 1967
M. arginini	G 230	28·6	—	—	Neimark, table 1
M. meleagridis	529	28·6	—	—	Kelton & Mandel, 1969
	N	28·1	—	—	Kelton & Mandel, 1969
M. salivarium	PG 20 (H 110): 23064	—	31·4	—	Williams *et al.* 1969
	PG 20	28·5[b]	—	—	Reich *et al.* 1966
	MANIRE	—	27·3	—	McGee *et al.* 1967
Primate 747 V	—	27·6	—	—	Neimark, table 1
M. hominis	H 39	31·6	33·7	—	Morowitz *et al.* 1967
M. hominis type 1	PG 21 (H 50): 23114	—	29·2	—	Williams *et al.* 1969
	H 34: 15056	—	29·2	—	McGee *et al.* 1967
	4387: 14027	—	28·7	—	McGee *et al.* 1967
	4330	27·3	—	—	Kelton & Mandel, 1969
M. gateae	CS: 23392	—	28·5	—	Williams *et al.* 1969
T strains (seven human serotypes)	—	—	28·5–27·7	—	Bak & Black, 1968

Table 4 (*cont.*)

Species or designation	Strain	GC (mole %) by Density	T_m	Chromatography	References
M. gallinarum	54-537	27·0	—	—	Kelton & Mandel, 1969
	PG 16: 15319	—	28·0 27·5	—	Rogul *et al.* 1965; McGee *et al.* 1967
	PG 16: 19708	—	27·0	—	Williams *et al.* 1969
	PG 16	26·5	—	—	Kelton & Mandel, 1969
M. orale type 1[h]	CH 19299: 23714	27·5[j]	27·8	—	Reich *et al.* 1966; Williams *et al.* 1969
	823 B: 15539	—	26·6	—	McGee *et al.* 1967
M. pharyngis[h]	LGM: 19524	—	27·0	—	McGee *et al.* 1967
	PATT: 15544	—	23·9	—	Williams *et al.* 1969
M. orale type 2	DC 1600	26·5[j]	26·4	—	Reich *et al.* 1966; Williams *et al.* 1969
	CH 20247: 23636	—	26·1	—	Williams *et al.* 1969
Mycoplasma sp. (human)	NAVEL: 15497	—	26·8	—	Williams *et al.* 1969
Primate S3–7	—	26·5	—	—	Neimark, table 1
Avian serotype M[i]	R49	25·0	—	—	Kelton & Mandel, 1969
Avian serotype E	HPR–15	24·0	—	—	Kelton & Mandel, 1969

[a] Numbers after colons in strain designations are American Type Culture Collection numbers.

[b] Calculated from data in reference cited, taking *Escherichia coli* density as 1·710 g./cm^3.

[c] Table 1 refers to results reported in this paper.

[d] This strain was believed to be a subculture of Cordy & Adler's goat pathogen (Williams *et al.* 1969); another subculture appears in the table as California goat, kid strain.

[e] Sabin type C, *Mycoplasma histotropicum*, has been shown to be identical with *M. pulmonis* (Lemcke, Forshaw & Fallon, 1969).

[f] These strains have been reported to produce slight acidity in glucose broth (see text).

[g] Formerly *M. hominis* type 2.

[h] The names *M. pharyngis* and *M. orale* type 1 are synonyms; the epithet *orale* is favoured (D. G. ff. Edward & E. A. Freundt, personal communication).

[i] Avian strain R 49, from which all serotype M cultures are derived, is a mixed culture that contains *M. granularum* and an unknown fermenter (J. Fabricant, personal communication). However, density gradient centrifugation of DNA from R 49 did not reveal significant contamination.

Mycoplasma neurolyticum and *M. pneumoniae*, at the extremes of the span of base compositions of fermentative organisms, are approximately 17 % apart in GC content, and both differ by more than 10 % from several other fermentative mycoplasmas. Since bacteria with GC contents differing by more than 10 % would be expected to have little genetic relatedness (Sueoka, 1961), the fermentative mycoplasmas may contain at least two unrelated subgroups.

Mycoplasma pneumoniae is not related physiologically or by nucleic acid homology to any of the nearby sterol-nonrequiring mycoplasmas (McGee *et al.* 1967; Table 2). Only rather low-level DNA reactions were found by the DNA-agar method between *M. pneumoniae* and *M. gallisepticum* (separated by *ca.* 5 % in GC content) and

M. fermentans (differing by *ca.* 12%) (McGee *et al.* 1967); a DNA–RNA method did not detect any reaction between *M. pneumoniae* and *M. fermentans* (Reich *et al.* 1966). Consequently, *M. pneumoniae* is distinct from the middle and low-range GC mycoplasmas. Even excluding *M. pneumoniae* and the sterol-nonrequiring subgroup, the fermentative mycoplasmas still are heterogeneous with a span of 12%; probably *M. gallisepticum* (GC *ca.* 35%) at the upper extreme is unrelated to *M. neurolyticum* at the lower extreme.

The nonfermentative mycoplasmas span a range of 10% in GC content, and here, too, the organisms at opposite ends of the span would fall into separate subgroups; the range of GC content will be contracted if *M. agalactiae* (and possibly *M. agalactiae* var. *bovis*) is proved to ferment carbohydrates. This division contains the human T strain mycoplasmas (Shepard, 1967; Taylor-Robinson, Williams & Haig, 1968), of which seven have the same base composition but are serologically distinct (Bak & Black, 1968).

Of those nonfermentative organisms examined for DNA–RNA homology, *Mycoplasma salivarium*, *M. orale* types 1 and 2, *M. hominis* and *M. arthritidis* were readily separated, although low-level cross-reactions were demonstrated, in agreement with serological findings (Reich *et al.* 1966). These organisms fall within a span of 26 to 32% in GC content and utilize arginine (Barile, Schimke & Riggs, 1966); they also show low-level DNA homology with *M. fermentans*, an arginine-utilizing, carbohydrate-fermenting Mycoplasma (Reich *et al.* 1966). DNA homology studies (McGee *et al.* 1967; Pollock & Bonner, 1969; Peterson & Pollock, 1969) reveal no reaction between fermentative and nonfermentative organisms except for a low-level cross-reaction between *M. fermentans* and *M. gallinarum* (McGee *et al.* 1967).

To provide 'working' units for studying relationships, the mycoplasmas can be divided tentatively into six subgroups:

(1) *Mycoplasma pneumoniae.* GC content *ca.* 40%. Set apart from all other mycoplasmas.

(2) Sterol-nonrequiring mycoplasmas. Contains *M. laidlawii*, *M. granularum*, and strains S-743/S-410. GC *ca.* 31 to 35%.

(3) High GC fermentative organisms. GC *ca.* 35% and less. Contains *M. gallisepticum.*

(4) Low GC fermentative organisms. GC *ca.* 23% and greater. Contains *M. neurolyticum.*

(5) High GC nonfermentative organisms. GC *ca.* 34% and less. Contains *M. agalactiae.*

(6) Low GC nonfermentative organisms. GC *ca.* 24% and greater. Contains strain HPR-15, avian serotype E. If *M. agalactiae* and *M. agalactiae* var. *bovis* prove to be fermentative, the distinction between subgroups 5 and 6 will depend on there being no DNA homology between strain HPR-15 and *M. arthritidis.*

The demonstration of homology between fermentative and nonfermentative mycoplasmas would be less surprising than the finding of a relationship between organisms at extremes of the GC range, for the latter would contradict accepted principles of molecular genetics.

Serology and homology studies generally agree in instances where comparable data are available (Reich *et al.* 1966; Somerson *et al.* 1967). *Mycoplasma gallisepticum* and *M. synoviae* might be compared further, since they are serologically related (Olsen,

Yamamoto & Ortmayer, 1965) and their GC contents are not far apart; *M. hominis* and *M. gateae* also share antigens (Cole, Golightly & Ward, 1967) and differ by about 1% in GC content. Serological cross-reactions are known for mycoplasmas that otherwise appear unrelated: for example, between *M. pneumoniae* and *M. mycoides* (Lemcke, Marmion & Plackett, 1967) and between *M. iners* and avian serotype E (Kelton & Mandel, 1969). Kenny (1969) reported that five fermentative organisms with GC contents of 23 to 28% were serologically related, while *M. pneumoniae* and *M. gallisepticum* were distinct; this supports the proposed subgroup divisions.

Significance of Mycoplasma subgroups. Genetic heterogeneity is a major feature of the mycoplasmas, but its origins are obscure. This diversity may indicate that mycoplasmas are derived from a variety of sources or, alternatively, may merely reflect breadth developed through evolution from a single source. Whether mycoplasmas share ancestors with or are ancestral to bacteria, or whether they developed from bacteria is uncertain; at present there is no evidence that they arose by simple discontinuous events from bacteria. The hypothesis that mycoplasmas are L-forms of bacteria is not supported by homology studies (McGee, Rogul, Falkow & Wittler, 1965; Somerson *et al.* 1967; McGee *et al.* 1967; Neimark, 1967). Also, the mycoplasmas with uniquely low GC contents do not appear to be stable L-forms of known bacteria (Jones & Walker, 1963; Neimark & Pene, 1965).

Theoretically the mycoplasmas could have developed through mutants with alterations in GC content; reports of such mutants in bacteria have not been confirmed (see Neimark, 1967; and Mandel, 1969). One stable L-form of *Streptococcus faecalis* lacks some 4 to 6% of DNA sequences present in the parent (Hoyer & King, 1969); such deletion mutants could conceivably have been factors in the development of mycoplasmas.

On the other hand, if the Mycoplasma are a true biological group, it should be possible to demonstrate some common fundamental properties in addition to the absence of a cell wall. One property that has been examined is genome size. Values for seven strains ranged from $4{\cdot}4 \times 10^8$ to $1{\cdot}2 \times 10^9$ daltons (Bode & Morowitz, 1967; Morowitz, Bode & Kirk, 1967; Ryan & Morowitz, 1969). Bak, Black, Christiansen & Freundt (1969) determined genome sizes in 12 mycoplasmas, and their results were partly in accord with those of Morowitz *et al.* (1967); however, the mycoplasmas fell into only two groups, each composed of strains with identical genome size. One group containing the sterol-nonrequiring organisms had genome sizes of about $1{\cdot}0 \times 10^9$ daltons (cf. *Haemophilus influenzae*, $7{\cdot}0 \times 10^8$ to $1{\cdot}0 \times 10^9$ daltons). All the serum-requiring strains examined were in the second group, with genome sizes of about $4{\cdot}6 \times 10^8$ daltons, a value half that for the sterol-nonrequiring strains and smaller than any known for bacteria. The finding of identical genome size may reflect common phylogenetic origin (Bak *et al.* 1969; Peterson & Pollock, 1969; Mandel, 1969); the discontinuity between the two groups implies that organisms in each may have arisen or developed independently. These findings, if confirmed and shown to be representative of the entire Mycoplasma group, would indicate that the extensive genetic heterogeneity observed in GC content does not extend to genome size and would provide a fundamental trait with which homogeneous divisions among the mycoplasmas could be established. If the mycoplasmas still to be examined should fall into additional groups, then the possibility would have to be considered that the Mycoplasma group is composed of clusters of organisms that arose and evolved independently.

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SHORT COMMUNICATION

Decreased Uptake of Cadmium by a Resistant Strain of *Staphylococcus aureus*

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(*Accepted for publication 14 July 1970*)

Penicillinase plasmids, and some related extrachromosomal elements in *Staphylococcus aureus*, can specify resistance to inorganic ions, including Hg^{2+} and Cd^{2+} (Richmond & John, 1964; Novick & Roth, 1968; Peyru, Wexler & Novick, 1969). There is some tentative evidence that resistance to Hg^{2+} ions is due to the impermeability of the cells to the ions and not to a higher concentration of free –SH groups in resistant cells (Vaczi, Fodor, Milch & Rethy, 1962), but the biochemical basis of resistance to cadmium ions is unknown. The experiments described here show that there is a markedly decreased rate of uptake of Cd^{2+} ions by resistant cells when compared with strains that lack the *cad-r* gene.

The uptake of Cd^{2+} ions has been studied in the cadmium resistant *Staphylococcus aureus* strain 8325 (*α.i*$^+$*p*$^+$*.cad-r.mer-r*) and in its cadmium-sensitive derivative, strain 8325(N) (for this nomenclature, see Richmond, 1968). Strain 8325(N) was obtained from 8325 (*α.i*$^+$*.cad-r.mer-r*) by isolating a variant which had spontaneously lost the *α* penicillinase plasmid specifying resistance to Cd^{2+} ions (Novick, 1963; Novick & Roth, 1968). Cadmium uptake was estimated by adding $^{115m}CdCl_2$ (final concentration: 10^{-4}M) to exponentially growing cultures of the sensitive and resistant strains and following the uptake of radioactivity. The initial specific activity of the $^{115m}CdCl_2$ solution was 80 μCi/μmole, and the bacterial culture density at the point of addition of the tracer was approximately 10^8 bacteria/ml. (about 0·08 mg. dry wt/ml.). The experiments were carried out in nutrient broth. After addition of the tracer, the cultures were incubated with shaking at 37° and samples removed at intervals for estimation of $^{115m}Cd^{2+}$ ion uptake. The samples were filtered rapidly through Whatman GF/C glass fibre filters, washed with two batches of prewarmed growth medium lacking added $CdCl_2$ (5 ml. each), and the radioactive content of the cells was measured by placing the dried filters in vials containing scintillant followed by estimation in a liquid scintillation spectrometer. The quantity of cadmium taken up was calculated on the basis of a counting efficiency of 90% and a specific activity of the Cd^{2+} of 2·5 μCi/μmole.

The kinetics of Cd^{2+} uptake by the sensitive and resistant cultures is shown in Fig. 1. The values quoted have been corrected for nonspecific binding of Cd^{2+} ions to components of the growth medium. This was determined by the same procedure but with the medium alone and no added bacteria. The nonspecific binding of Cd^{2+} amounted to 170×10^{12} ions/ml. medium, which is equivalent to about 15% of the total

Cd^{2+} taken up by 1 ml. of culture of the sensitive strain. The total uptake of cadmium by cadmium-sensitive cells was about 15 times that found with the resistant organisms (Fig. 1). In this experiment the total uptake was approximately 106×10^{14} Cd^{2+} ions/mg. dry wt of sensitive organisms.

To decide whether the Cd^{2+} ions taken up by the bacteria were inside the cells or adsorbed to the cell surface, attempts were made to release the isotopic tracer from the sensitive bacteria. Sensitive organisms were exposed to $^{115m}Cd^{2+}$ as above, and samples were washed with two batches of ice-cold 5% (w/v) trichloroacetic acid (5 ml. each) followed by three batches of 1% (v/v) acetic acid (5 ml. each). Dried filters were placed in scintillant and radioactivity estimated as before. This treatment with cold trichloroacetic acid completely removed all tracer from the bacteria. Control experiments showed that labelled organisms retained radioactivity after washing with prewarmed broth (two portions of 5 ml. each) followed by 1% (v/v) acetic acid (three portions of 5 ml. each).

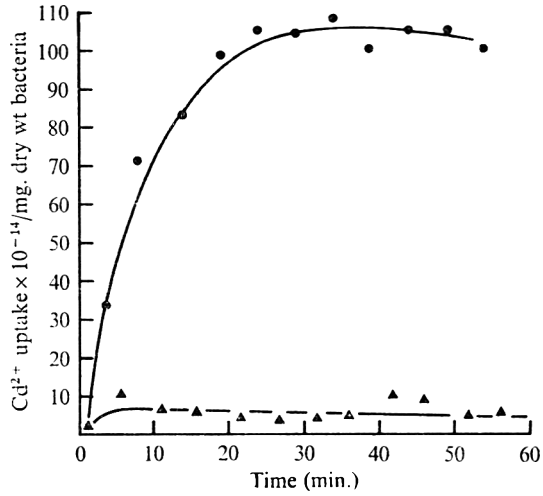

Fig. 1. The uptake of Cd^{2+} ions, on a dry weight basis, by cadmium-sensitive (●) and by cadmium-resistant (▲) staphylococci. Initial specific activity of $CdCl_2$: 2·5 μCi/μmole. $CdCl_2$ concentration: 10^{-4}M. Samples were treated as described in the text. All values have been corrected for nonspecific binding of tracer by the growth medium.

Exchange experiments were also performed in which sensitive organisms were labelled for 40 min. and then resuspended in non-radioactive medium. When prewarmed medium containing 10^{-4}M-$CdCl_2$ was used for this purpose, about 60% of the radioactive cadmium ions were displaced in 60 min., further loss being much slower. Therefore, about 40% of the radioactive Cd^{2+} ions taken up by cadmium-sensitive staphylococci reaches a location in the cell that is immediately accessible to trichloroacetic acid but not to further Cd^{2+} ions. It seems, therefore, that the non-exchangeable Cd^{2+} ions are likely to be bound to some structure within the cell rather than adsorbed adventitiously to the surface.

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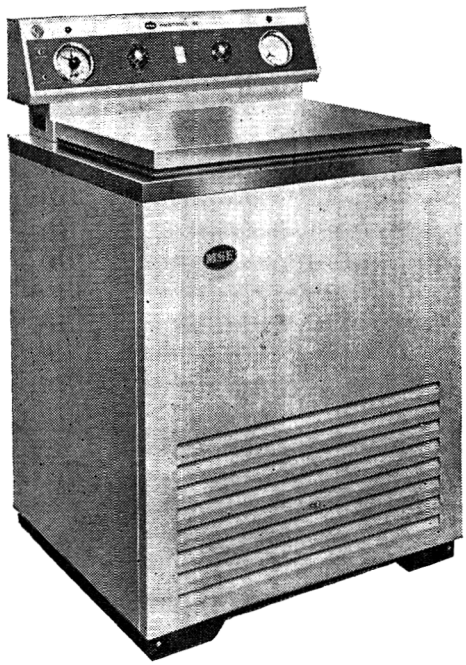

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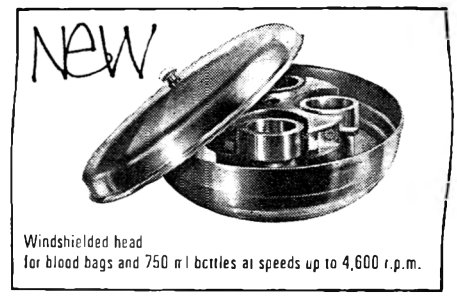

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