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PHOSPHATE DEPRIVATION OF Saccharomyces cerevisiae

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Submitted for the Degree of Doctor of Philosophy
of The University of Glasgow

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Declaration

I hereby declare that the work reported in this thesis is a true record of the research which I carried out, as a member of the Department of Microbiology, Glasgow University, and has not been submitted elsewhere for any other degree.

Signed

Date

16th April 1977

Andrew M. Ramsay

UNIVERSITY OF GLASGOW

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Phosphate Deprivation of Saccharomyces cerevisiae

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SUMMARY

Substantial changes have been reported in both the cell-wall and lipid composition of a variety of bacteria when these organisms were grown under conditions of phosphate limitation. Similar detailed studies with yeasts have not yet been described, however. The major objective of the present work was therefore an analysis of changes in the content and composition of these components resulting from growth of Saccharomyces cerevisiae NCYC 366 under phosphate-limiting conditions.

Phosphate-limited cells obtained by either batch or continuous culture of the yeast contained less total phosphorus than did cells grown in medium with an optimum concentration of phosphate. Detailed analysis of these cells revealed complex changes in both cell-wall and lipid composition although the changes observed were less dramatic than those described for bacteria.

Walls prepared from phosphate-limited cells had a lower phosphate content than did those from control cells. No evidence was obtained for the presence in these walls of uronic acid or succinyl residues although they did appear to contain an increased proportion of one or more unidentified anionic components. Walls prepared from chemostat-grown, phosphate-limited cells contained less carbohydrate than did those from control cells and this was reflected in a considerably decreased glucan content. The glucosamine content of the walls was only slightly affected however, while that of protein increased.

The most striking change observed in the lipid composition of phosphate-limited cells was a decrease of both sterol esters and

triacylglycerols. Phospholipid synthesis, overall, appeared to be stimulated, leading to an increased proportion of phosphatidylethanolamine and phosphatidylcholine. The phosphatidylserine content on the other hand was decreased.

These changes in both cell-wall and lipid composition were interpreted in terms of a diminished population of small or large vesicles in the phosphate-limited yeast.

A secondary objective of this study was to investigate the reported occurrence of teichoic acid in Candida intermedia and then to extend the survey for these polymers to other yeast species.

Trichloroacetic acid extracts of C. intermedia, C. utilis and Sacch. cerevisiae were subjected to acid hydrolysis and the hydrolysates examined for products characteristic of a glycerol teichoic acid. Traces of glycerol were found in almost all of the hydrolysates, but glycerol phosphate was only identified in the hydrolysate of one extract from C. intermedia. Glycerol diphosphate was not detected. Some evidence was obtained for the synthesis of a polymeric product from CDP-glycerol by subcellular fractions of C. intermedia. These results were considered too equivocal to support a claim for the presence of teichoic acids in yeasts.

LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
CDP	Cytidine 5'diphosphate
DEAE-cellulose	Diethylaminoethylcellulose
DNA	Deoxyribonucleic acid
E	Extinction coefficient
$E_{\substack{650 \\ 1\text{cm}}}$	Extinction value of a solution in a cell of 1 cm light path at a wavelength of 650 nm
EDTA	Ethylenediaminetetra-acetate
NAD	Nicotinamide-adenine dinucleotide
NADP	Nicotinamide-adenine dinucleotide phosphate
psi	Pounds per square inch
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
rpm	Revolutions per minute
Tris	2-Amino-2-hydroxymethylpropane-1,3-diol

To My Mother, Father and Wife

"It is better to light one small candle
than to curse the darkness."

Confucius.

INTRODUCTION

2

A.

GENERAL REMARKS

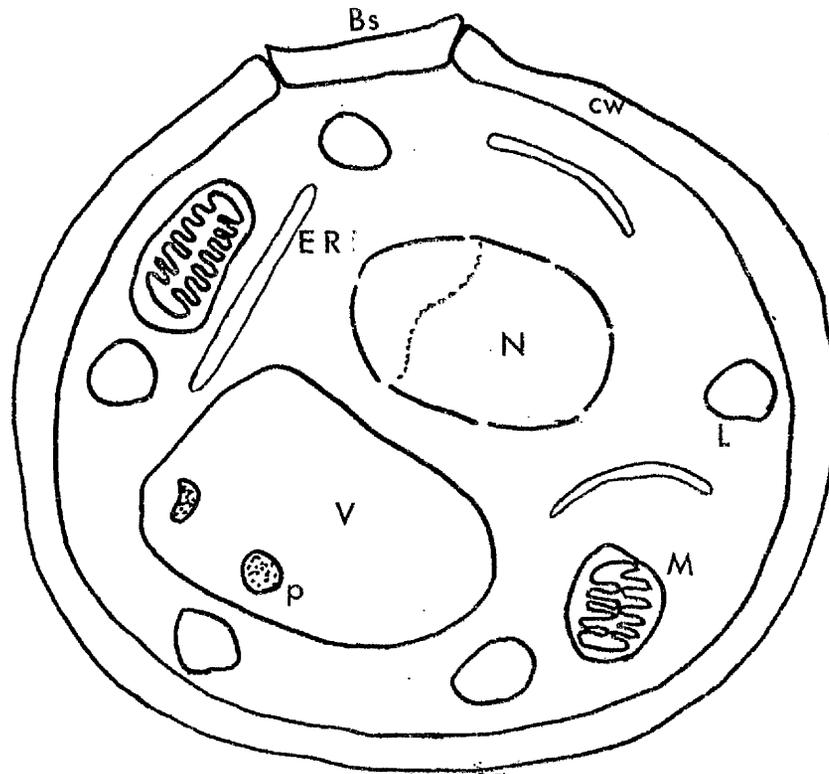
The group of micro-organisms known as "yeasts" is by traditional agreement limited to fungi in which the unicellular form is predominant. They are uninucleate cells which reproduce asexually by budding or by fission. The diversity of the yeasts is illustrated by the fact that 39 genera and some 350 species are now recognised (Kreger - Van Rij, 1969).

The Dutchman, Antonie van Leeuwenhoek, is credited with being the first man to have observed yeasts microscopically. In 1680 he sent descriptions and drawings to the Royal Society in London of "globular bodies", oval or spherical in shape, which were present in fermenting beer. In recent times, the study of yeast morphology and cytology has advanced considerably with the introduction of the electron microscope as a research tool and the development of freeze-etching techniques. The fine structure of yeast cells, as revealed by freeze etching, was described by Moor & Muhlethaller (1963). More recently, the whole subject of yeast cytology has been comprehensively reviewed by Matile, Moor & Robinow (1969).

The principal features of a cell of Saccharomyces cerevisiae are shown in Fig 1. The outermost layer is the cell wall which appears to be composed of strata. Streiblova (1968) has recognised three layers in replicas of yeast cells obtained by freeze-etching. Improved visualisation of cell wall ultrastructure following prefixation treatment with tris-I aziridinylphosphine oxide and acrolein has been reported (Cassone, 1973). Using this fixation protocol, it is claimed that four layers are visible. Many attempts have been made to assign the cell-wall components to specific layers but without much success.

Fig 1.

Schematic diagram of a typical yeast cell in
cross section



Bs	Bud scar
cw	Cell wall
L	Lipid granule
M	Mitochondrion
p	Polyphosphate granule
V	Vacuole
N	Nucleus
ER	Endoplasmic reticulum

The plasma membrane is a triple layered structure about 8 nm wide (Vitols, North & Linnane, 1961; Kopp, 1972). The outer surface of the membrane, as revealed by freeze-etching, is sculptured with hexagonal arrays of particles 15 nm in diameter (Moor & Muhlethaller, 1963). This feature has been used as a morphological marker for plasma membrane (Matile, Moor & Muhlethaller, 1967).

A double membrane system located in the cytoplasm of the yeast cell is thought to resemble very closely the endoplasmic reticulum of higher plants (Moor & Muhlethaller, 1963). The organelles which appear to be derived from the endoplasmic reticulum include the nuclear envelope, lipid granules (sphaerosomes or small vesicles), microbodies and the Golgi apparatus.

In thin sections of yeast cells, the vacuole (or large vesicle) appears as an electron-transparent area delimited by a single membrane (Vitols et al., 1961). The diameter of this structure has been shown to vary from 0.3 μm to 3.0 μm (Moor & Muhlethaller, 1963). In the last decade, many properties and functions have been attributed to the vacuole including the storage of polyphosphate (Brachet, 1957; Indge, 1968) and a variety of lytic enzymes (Matile & Wiemken, 1967). Evidence has also been presented for the attachment of ribosomes to the surface of the vacuole (Matile, Moor & Robinow, 1969).

The remaining organelles - the nucleus and the mitochondria - are very similar in morphology to those of higher plants. The yeast nucleus is globular, delimited by a nuclear membrane (probably containing about 200 pores) and with a constant diameter of 2.3 μm (Moor & Muhlethaller, 1963).

The matrix of the cytoplasm contains 80S ribosomes and yeast glycogen which is very similar in architecture to animal glycogen (Silbereisen, 1960).

B. CELL WALLS OF YEASTS

Current knowledge of the yeast cell wall is based upon the accumulation and integration of data obtained by chemists, biochemists, enzymologists and cytologists. Early methods of extraction of specific components were severe, with the consequent degradation of the polymer under study. With time, the methods of extraction became less drastic and consequently views on structure and morphology have changed.

In Saccharomyces cerevisiae, the yeast species that has been most extensively studied, the wall contains approximately equal amounts of glucan and mannan which, together, account for about 85 per cent of the dry weight of the wall. The remainder of the wall is made up of protein, lipid and glucosamine. When walls of Sacch. cerevisiae are treated with hot, dilute sodium hydroxide solution, the mannan and some of the glucan are extracted, leaving a shell which retains the shape of the intact yeast (Phaff, 1971). It is presumed, therefore, that the glucan which remains after alkaline extraction, constitutes the structural component of the wall in this yeast. Fibrils of glucan have indeed been observed on the surfaces of protoplasts of Sacch. cerevisiae incubated under conditions that allow a partial regeneration of the cell wall (Darling, Thielade & Birch-Anderson, 1969).

1. Glucans

a) Structure

Early studies revealed little about the chemical nature of

baker's yeast glucan except that it contained β -(1 \rightarrow 3) bonds. There was also a vague implication that it was a branched structure (Phaff, 1971).

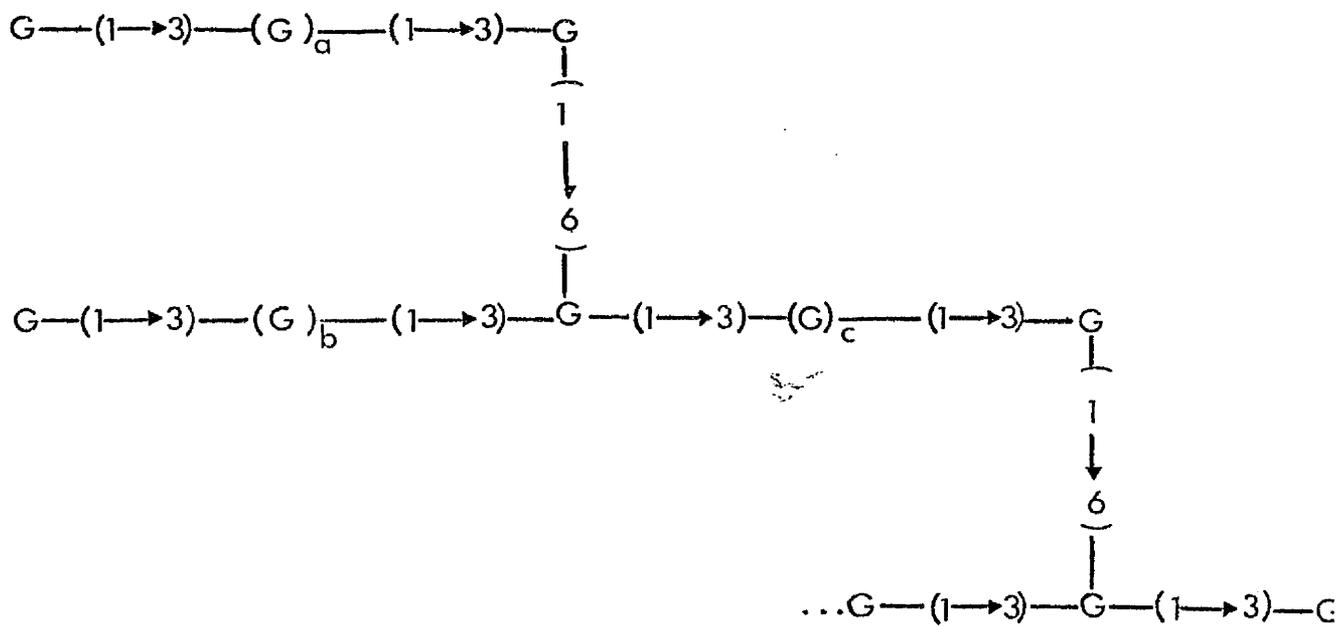
Enzymic analysis of whole cell walls or of purified wall fractions with various glucanases has contributed significantly to chemical studies. Tanaka, Phaff & Higgins (1965) observed that if a strain of Bacillus circulans was grown on baker's yeast cell walls it produced a mixture of endo- β -(1 \rightarrow 3) and endo- β -(1 \rightarrow 6) glucanases which were easily separable by column chromatography on DEAE cellulose. Treatment of baker's yeast with either of these enzymes caused lysis, although the β -(1 \rightarrow 3) glucanase was much the more efficient. Previously, Tanaka (1963) had treated purified cell walls with each enzyme separately and had concluded that there were twice as many linkages susceptible to the β -(1 \rightarrow 3) glucanase as were susceptible to the β -(1 \rightarrow 6) enzyme. Buecher (1968) repeated the work of Tanaka using slightly modified conditions and found that the ratio of susceptible linkages was 1:1. He concluded that long blocks of β -(1 \rightarrow 6) glucan must be present since the endo- β -(1 \rightarrow 6) glucanase could not hydrolyse gentiobiose and hydrolysed gentiotriose only very slowly. Recently a new model for the structure of baker's yeast glucan has been proposed (Manners, Masson & Patterson, 1973) which takes into account the heterogeneous nature of glucan preparations. The major component (about 85%) is a branched β -(1 \rightarrow 3) glucan of high molecular weight (about 240,000 daltons) containing 3% β -(1 \rightarrow 6) glycosidic interchain linkages (Fig 2). The minor component is a highly branched β -(1 \rightarrow 6) glucan containing a smaller proportion of β -(1 \rightarrow 3) glycosidic bonds that serve mainly as interchain linkages although some may be interresidue links.

Roelofsen (1953) and Kessler & Nickerson (1959) reported that an alkali-soluble glucan accounted for 20-25% of the cell wall of baker's yeast.

Fig 2.

Partial Structure of a Segment of Sacch. cerevisiae

β -(1 \rightarrow 3) Glucan (Manners et al., 1973)



a + b + c comprise about 60 glucose residues, although the exact lengths are unknown.

Recently Fleet & Manners (1976) have isolated and characterised this polymer from the walls of Sacch. cerevisiae NCYC 1109. They showed that it has a molecular weight of approximately 250,000 daltons and contains 80 - 85% β -(1 \rightarrow 3) linkages, 8 - 12% β -(1 \rightarrow 6) linkages and 3% to 4% branched residues. They were also able to demonstrate that an amorphous layer is lost from the cell surface during alkaline extraction.

b) Biosynthesis

Little is known about the biosynthesis of the glucan component of the yeast cell wall. Sentandreu, Elorza & Villaneuva (1975) demonstrated the incorporation of a small amount of $[^{14}\text{C}]$ -glucose from UDP- $[^{14}\text{C}]$ -glucose into the cell wall glucan of toluene-treated Sacch. cerevisiae. Very recent work (Balint, Farkas & Bauer, 1976) suggests that GDP-glucose may also be required as a glucose donor. No evidence has as yet been obtained for involvement of a lipid-linked intermediate in glucan biosynthesis.

2. Mannans

a) Isolation

Mannan is a major polysaccharide of many but not all yeasts (Phaff, 1971). Although it was discovered in 1894 by Salkowski it was not studied in any detail until the middle of this century. Early methods for the extraction of mannan from the yeast cell involved drastic chemical treatments. Haworth, Hirst & Isherwood (1937), for example, boiled whole cells in 6% sodium hydroxide solution, a treatment which dissolved the mannan and left most of the glucan and the chitin as insoluble residues. The mannan was subsequently freed from other soluble polysaccharides by precipitation as an insoluble copper complex. The precipitate was then

washed in water and redissolved in hydrochloric acid. A milder extraction procedure involves autoclaving intact cells in neutral citrate buffer (Peat, Whelan & Edwards, 1961). Mannan prepared in this way is an amorphous, water-soluble mixture of macromolecules and has been subjected to a variety of fractionation procedures including ion-exchange chromatography (Thieme & Ballou, 1971) and gel filtration (Thieme & Ballou, 1972). The fractionated preparations contained covalently-linked protein or polypeptide material indicating that most or all of the cell-wall mannan is present as glycoprotein. Simple cell breakage gives the least altered mannan-protein preparations, yielding active external invertase and other mannan-linked enzymes that can be purified to maximum specific activity (Neuman & Lampen, 1967). Any mannan adhering to the glucan component of the cell wall matrix may be freed by treatment with glucanases (Phaff, 1971).

b) Techniques used in structural analyses

Much of the information on mannan structure has come from studies on baker's yeast. Characteristically, this mannan is composed of long chains of D-mannose residues linked by α -(1 \rightarrow 6) glycosidic bonds, with short side chains in α -(1 \rightarrow 2) and α -(1 \rightarrow 3) linkage. Phosphodiester-linked mannose residues are also present. These macromolecules are attached to asparagine in the protein. In addition, there are short α -(1 \rightarrow 2) and α -(1 \rightarrow 3)-linked oligomannosides attached to serine or threonine in the protein.

i) Partial acid hydrolysis. The phosphodiester bonds are the most acid-labile and can be broken by gentle conditions (0.01M HCl at 100°C for 30 min) with quantitative release of free mannose (Rosenfeld & Ballou, 1974). Recovery of the residue and subsequent rehydrolysis releases very little additional mannose, indicating the specificity of the reaction.

ii) Alkaline hydrolysis. Mild alkaline hydrolysis (0.05M at 50°C for 18 hours) liberates mannose and mannose oligosaccharides attached to serine and threonine moieties in the protein (Nakajima & Ballou, 1974a). The oligosaccharides thus released may be recovered, separated into classes and analysed. In this way, the carbohydrate attached to serine and threonine has been characterised. Stronger hydrolysis (M NaOH at 100°C for 5 h) releases the larger polymannose chains which are attached to asparagine in the protein (Nakajima & Ballou, 1974a).

iii) Selective acetolysis. A reaction mixture containing concentrated sulphuric acid and acetic anhydride splits α -(1 \rightarrow 6)-linked mannose residues with great specificity (Gorin & Perlin, 1956; Kocourek & Ballou, 1969). This reaction has been one of the most important in the determination of the structure of yeast mannan. The side chains liberated by this reaction have been characterised structurally (Nakajima & Ballou, 1974b) and have been used to prepare haptens with which the structural specificity of the immunochemical reactions of yeasts has been analysed (Ballou, 1970).

iv) Enzymic degradation. The most useful enzyme has been a bacterial exo- α -mannanase from a species of Arthrobacter which hydrolyses preferentially α -(1 \rightarrow 2) and α -(1 \rightarrow 3)-linked mannose residues. It also hydrolyses α -(1 \rightarrow 6)-linked units, but only very slowly (Jones & Ballou, 1968, 1969a). Consequently, most of the side chains of the mannan can be removed leaving the backbone essentially intact (Jones & Ballou, 1969b). Side chains substituted with phosphate (Thieme & Ballou, 1971) or other sugars such as N-acetylglucosamine (Raschke & Ballou, 1972) are not attacked.

Other enzymes which have been used in mannan structural analyses include an endo- α -(1 \rightarrow 6) mannanase which is only active against the

unsubstituted mannan backbone and a β -N acetyl-D-glucosaminidase which releases the mannan from its protein component (Ballou, 1976).

v) Immunochemistry. Immunochemistry, too, has been important in the elucidation of the structure of mannan. It is possible to obtain antisera which are specific for different features of the mannan molecule (Ballou, 1970), the most common determinant being the terminal α -(1 \rightarrow 3)-linked mannose unit.

Antiserum directed against wild-type mannan has been used to select for mutants with altered mannan structure in a mutagenized culture of yeast (Raschke, Kern, Antalis & Ballou, 1973). Ballou (1976) suggests that this may lead to a series of mutants related to each other by single alterations in mannan structure arising from a stepwise loss of biosynthetic activity.

c) Structure

The first structures for yeast mannan were postulated over thirty years ago, based on the results of methylation analyses (Haworth, Hirst & Isherwood, 1937; Haworth, Heath & Peat, 1941). The mannan molecule appeared to be highly branched and to contain a degree of polymerisation of 200-400 units. Its high dextrorotation indicated the presence of α -linkages although the presence of some β -linkages could not be excluded. Periodate oxidation demonstrated (1 \rightarrow 6), (1 \rightarrow 2) and (1 \rightarrow 3) linkages in the ratio 2:3:1 (Lindstedt, 1945). Peat, Whelan & Edwards (1961) found that the backbone of the highly branched mannan contained α -(1 \rightarrow 6) and not α -(1 \rightarrow 2) linkages as had been previously suggested.

An exhaustive research programme using the techniques described

in the previous section has enabled Ballou's group to postulate a structure for the mannan molecule of Sacch. cerevisiae X2180(Ballou, 1976). This structure is shown in Fig 3. The molecule consists of three parts: an outer chain, an inner core and oligosaccharides which are linked to protein through serine or threonine residues. Some of the mannose residues in the outer chain are joined in phosphodiester linkage while the inner core is attached to asparagine in the protein through a di-N-acetylchitobiose grouping.

d) Biosynthesis

The mechanism of mannan biosynthesis is only poorly understood, partly because the structure of the molecule has not been fully elucidated and partly because of the inherent difficulties of working with such a complex macromolecule.

In a very recent review, Ballou (1976) proposes a scheme for mannan biosynthesis. He envisages that following the synthesis of the protein moiety, the inner core di-N-acetylchitobiose-mannosyl unit may be synthesised on a lipid carrier and then transferred to the polypeptide chain (Hsu, Baynes & Heath, 1974). The lipid carrier is thought to be a dolichol phosphate similar to that isolated by Tanner (1969), Sentandreu & Lampen (1971, 1972) and Jung & Tanner (1973). Similarly, the first mannose attached to serine and threonine is derived from a dolichol phosphate intermediate (Babczinski & Tanner, 1973; Sharma, Babczinski, Lehle & Tanner, 1974) although the subsequent additions of mannose units are probably mediated by the sugar nucleotide GDP-mannose. The outer chain portion of the mannose polysaccharide is probably built up on the inner core. Preliminary evidence indicates that GDP-mannose is the mannose donor

during this part of the biosynthetic process because a lipid requirement is not apparent with the α -(1 \rightarrow 6) transferase-activity (Behrens & Cabib, 1968). Side chain modification consists of the transfer of mannosyl-phosphate groups from GDP-mannose to certain portions of the outer chain.

3. Chitin

Early investigators generally inferred the presence of chitin by testing for glucosamine or chitosan (diacetylated chitin) after drastic treatment with hot acid or alkali. Eddy (1958), however, observed the release of N-acetylglucosamine following treatment of baker's yeast walls with crude chitinase preparations. Based on the amounts of enzyme-liberated and residual N-acetylglucosamine, he estimated the apparent glucosamine content of the wall to be about 0.8% to 0.9%. Korn & Northcote (1960) fractionated cell walls into three components using an ethylene-diamine extraction procedure. They concluded that only 9% of the apparent glucosamine content was similar to crustacean chitin which is completely insoluble in this solvent.

Bacon, Davidson, Jones & Taylor (1966) using a mild extraction procedure on pressed baker's yeast obtained a preparation consisting almost entirely of bud scars. Its infrared spectrum and chemical analysis showed that it consisted of chitin (50%) and glucan (30 - 35%). Although the bud scar region is very rich in chitin, Bacon et al. (1966) could only account for 20% of the original glucosamine content.

Cabib & Bowers (1971) prepared cell wall ghosts by acid and alkaline treatment of wall hulls of Sacch. carlsbergensis. Electron microscopy showed that the ghosts were rich in bud scars. Treatment with a crude preparation of chitinase led to the destruction of the bud scars

while glucanase treatment destroyed the integrity of the ghosts but left the bud scars intact.

Chitin biosynthesis has been extensively studied by Cabib and his group who have shown that the chitin synthetase is present in the cell as a zymogen (Cabib & Farkas, 1971); the activating factor is a yeast protease (Cabib & Ulane, 1973); the chitin synthetase zymogen is sedimentable with the plasma membrane (Duran, Bowers & Cabib, 1975). The activating factor is located in vesicles (Cabib & Ulane, 1973).

Chitin is the major component of the primary septum in yeast (Cabib & Farkas, 1971). Cabib & Bowers (1975) examined the timing of chitin synthesis with respect to septum formation and bud emergence. They showed that chitin synthesis is induced at a stage in the cell cycle prior to septum formation. Chitin synthetase activity is not required for bud emergence although in the wild type it begins at or shortly after bud emergence.

4. Lipids

There is considerable variation in the reported lipid content of isolated yeast cell walls. That for cell walls of Saccharomyces species, for example, ranges from 2% to 13.5% (Eddy, 1958; Miller & Phaff, 1958). There is also great disparity in the values for phospholipid based on the nitrogen and phosphorus levels in the isolated lipid. These apparent discrepancies are probably due to variable contamination of wall preparations with plasma membrane fragments.

Suomalainen and his colleagues have examined the neutral lipid and glycolipid components of purified cell walls (Nurminen, Kottinen &

Suomalainen, 1975) and cell envelopes (Tyorinoja, Nurminen & Suomalainen, 1974) of baker's yeast. They claim that 1.6% of the cell wall is lipid, with neutral lipids (mainly acylglycerols and sterol ester) accounting for 74% of the total, while glycolipids (mostly in the form of sphingolipids) account for 5% of the lipid from isolated cell envelopes.

5. Protein and carbohydrate - protein complexes

Before techniques were developed to prepare relatively pure cell walls, it was not possible to study the protein components. Early data by Northcote & Horne (1952) on the protein content (13%) of washed cell walls of mechanically ruptured baker's yeast may be considered too high due to contamination with small particles entrapped in the cell envelope. A number of later studies showed that the protein content of baker's yeast walls is approximately 5 - 7% (Roelofsen, 1953; Falcone & Nickerson, 1956; Miller & Phaff, 1958).

It is well established that the protein of yeast walls is not extractable by repeated washing of the walls with water or buffers. Northcote & Horne (1952) first demonstrated the association of protein with the mannan component. Falcone & Nickerson (1956) treated walls with alkali and solubilised 75% of the wall material. Part of the extract was water-soluble and appeared to consist of a tightly bound mannan-protein complex, whereas the water-insoluble component was a glucan protein. Other workers have subsequently isolated mannan-protein complexes and glucan-mannan-protein complexes by a variety of different extraction procedures (Phaff, 1971).

A number of hydrolytic enzymes in yeast are found external to the cytoplasmic membrane and bound tightly or loosely to certain wall

components. Such enzymes are frequently excreted into the medium during growth and presumably enable the yeast to utilise substrates to which the plasma membrane is impermeable. The whole subject of external enzymes is the topic of a review by Lampen (1968).

The external enzymes of Saccharomyces species include invertase, acid phosphatase, melibiase, catalase, glucanases (β -(1 \rightarrow 3) and β -(1 \rightarrow 6)), aryl β -glucosidase, glucamylase (β) and phospholipase. Invertase and acid phosphatase have been purified and shown to be mannan proteins (Lampen, 1968). During the process of protoplasting baker's yeast with snail gut juice, invertase is released as a "large" molecule. Mechanical rupture of untreated cells, however, releases a mixture of "large" and "small" invertase molecules, the latter being a cytoplasmic constituent of much lower molecular weight.

Although the kinetic properties of the "large" and "small" invertases are very similar, fundamental differences between the two proteins, such as amino acid composition, have been found (Lampen, 1968). Hence, there is no clear evidence that the "small" internal invertase is converted into the large form after excretion through the plasma membrane, followed by complexing with mannan.

Lampen envisages that invertase lies between the glucan or mannan layers, bound to both polymers by phosphodiester bonds. However, the existence of any covalent linkage between the glycoprotein and either glucan or mannan is a matter of dispute (Kidby & Davies, 1970). Other workers have suggested that invertase, acid phosphatase (Arnold, 1972) and also an esterase (Wheeler & Rose, 1973) are located in the periplasmic space. Recent studies using fluorescent antibody labelling techniques

indicate that newly-formed invertase molecules are located at the cell surface surrounding the bud (Tkacz & Lampen, 1972).

6. Molecular architecture

The cell wall of baker's yeast is approximately 70 ± 10 nm thick (Moor & Muhlethaller, 1963) and accounts for 15 - 16% of the dry weight of the cell (Bacon et al., 1969). Many attempts have been made to demonstrate particular polysaccharides in particular layers by examination of ultra-thin sections under the electron microscope, but the results have been rather inconclusive. Recently, however, four layers have been recognised in thin sections of Sacch. cerevisiae (Cassone, 1973). It is claimed that layers 1 and 2 contain mannan-protein (layer 1 being furthest from the plasma membrane). Layer 3 is predominantly mannan-glucan-protein. Layer 4 is closely appressed to the plasma membrane and may be the alkali-insoluble glucan. This interpretation agrees fairly well with the cell-wall model proposed in 1970 by Kidby & Davies which is shown diagrammatically in Fig 4.

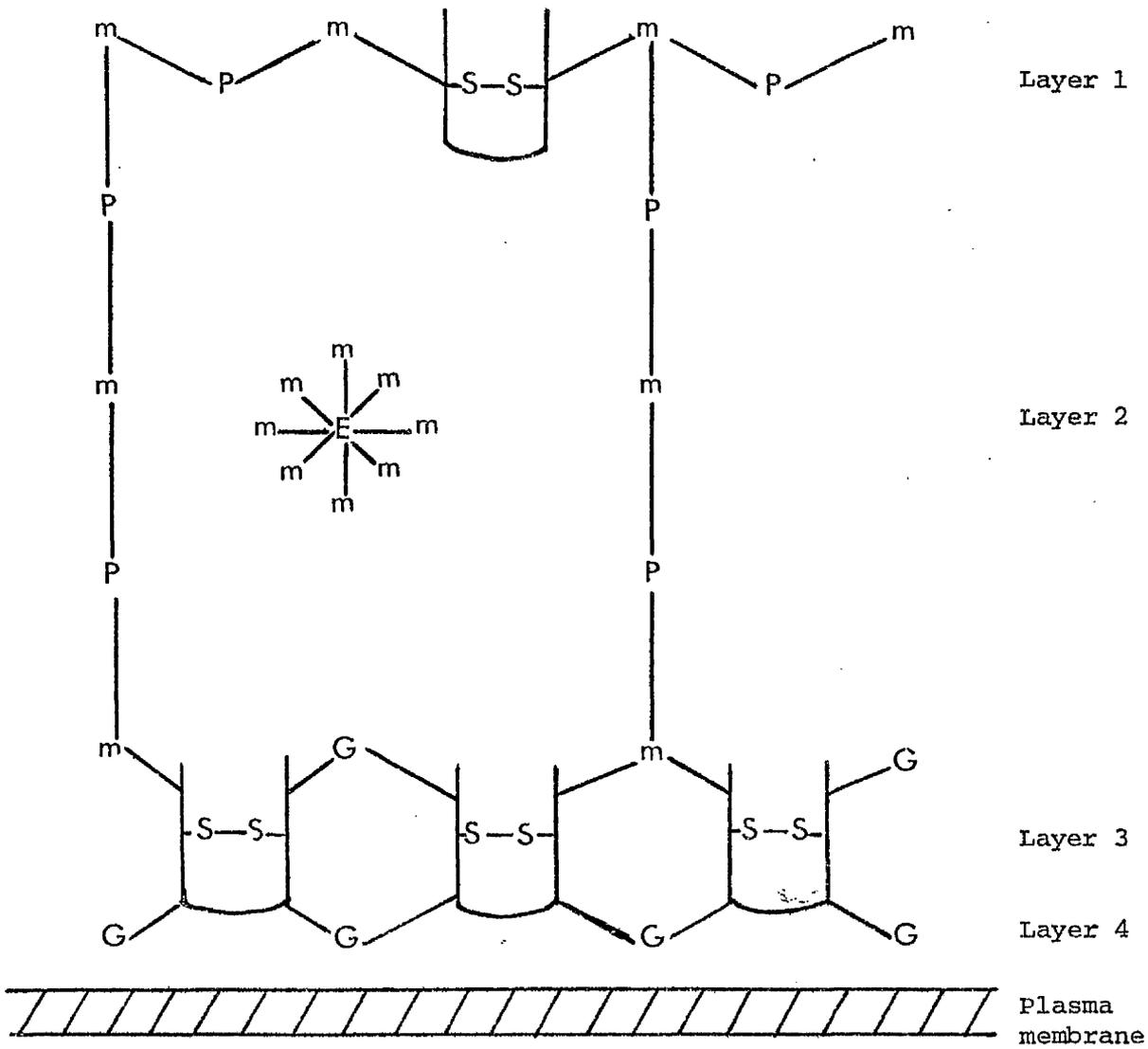
C. LIPIDS OF YEASTS

1. Total Lipid Content

Any survey of the literature relating to yeast lipids quickly reveals that the total lipid content varies widely from species to species. Nevertheless it is possible to distinguish at least two groups of yeasts by their lipid content. The majority contain between 7% and 15% of their dry weight as lipid. However, a second, smaller class have a lipid content of between 30% and 60% (Hunter & Rose, 1971); these are the so-called "fat" yeasts.

Fig 4.

Model of yeast cell wall (Kidby & Davies, 1970)

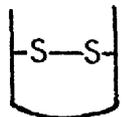


KEY

m = mannan

G = glucan

E = enzyme

 protein molecule

P phosphate group

Table 1 Comparative efficiency of methods of lipid extraction

<u>Extraction method 1</u> ¹	Most Efficient	<u>Extraction method 2</u> ²
(Nyns, Chiang & Wiaux, 1968)		(Suzuki, Takigawa & Hasegawa, 1973)
1. Cold chloroform : methanol (1:1, v/v)	1. Enzymic disruptions followed by extraction with chloroform : methanol (2:1, v/v)	
2. Hot ethanol : butanol (1:4, v/v)	2. Cold chloroform : methanol (1:1, v/v)	
3. Hot ethanol : petroleum ether (1:1, v/v)	3. Treatment with conc. HCl followed by extraction with diethyl ether	
4. Cold ethanol : diethyl ether (1:1, v/v)	4. Disruption with glass beads followed by extraction with chloroform : methanol (2:1, v/v)	
5. Cold chloroform : methanol (2:1, v/v)	5. Cold chloroform : methanol (2:1, v/v) method of Folch	
6. Cold ethanol : diethyl ether (3:1, v/v)	6. Cold chloroform : methanol (2:1, v/v) method of Bligh & Dyer	
1. Freeze-dried cells were used in extractions 1 - 2; a wet paste in 3 - 6.	Least Efficient	
2. Either freeze-dried or heat-dried cells were used.		

The lipid content of Sacch. cerevisiae has been variously reported as 8% (Letters, 1966) and 14.4% (Hunter & Rose, 1972). Such discrepancies presumably reflect the growth conditions used (see Section C-5) and the efficiency of the extraction method employed.

2. Methods of extraction

Although a wide variety of organic solvents have been used to extract yeast lipids, it is unfortunate that very few studies of comparative efficacy have been made. The results of two such studies are shown in Table 1. It has been suggested that non-polar solvents are efficient only when the cells have been previously disrupted (Harrison & Trevelyan, 1963), whereas Letters (1966) has claimed that neutral solvents must be acidified with hydrochloric acid if the full complement of phospholipids is to be extracted.

Great care must be taken to avoid possible enzymic decomposition of the phospholipid components during the extraction procedure. Letters & Snell (1963) found that treatment with organic solvents, particularly at room temperature, is sufficient to activate membrane-bound phospholipases. The major enzyme involved is thought to be a phospholipase C (Harrison & Trevelyan, 1963). However the problem can be overcome by heating the yeast cells in 80% (v/v) ethanol for fifteen minutes prior to extraction with chloroform and methanol. These conditions are sufficient to inactivate the phospholipases (Letters, 1968).

3. Lipid components

a) Fatty acids

Significant amounts of free fatty acids (approximately 5% of the total lipid content) have been found in Sacch. cerevisiae (Castelli,

Barbaresi & Bertoli, 1969; Hunter & Rose, 1972). However, this may not be a true figure since fatty acids can also arise from the enzymic hydrolysis of phospholipids, as already mentioned.

A typical lipid composition for yeast would be anticipated to have an overall fatty acid content of 70% to 90% (Rattray, Schibeci & Kidby, 1975), with the C₁₆ and C₁₈ acids predominating. Baraud, Maurice & Napias (1970) have identified thirty-three different fatty acids in extracts from Sacch. cerevisiae ranging in chain length from C₈ to C₂₆. However acids of chain length C₂₀ - C₃₀ have been found to account for only 1% to 2% of the total complement (Welch & Burlinghame, 1973).

Yeasts in general abound in unsaturated fatty acids and Hunter & Rose (1972) have shown that in Sacch. cerevisiae NCYC 366 the C_{16:1} and C_{18:1} components account for almost 70% of the fatty acid content.

b) Acylglycerols

The acylglycerols (Fig 5a) are esters of long chain fatty acids and glycerol. Triacylglycerols, together with phospholipids and sterols, account for the bulk of cellular lipids in yeasts (Hunter & Rose, 1971). These three classes of compounds are easily separated by thin layer, paper and column chromatography.

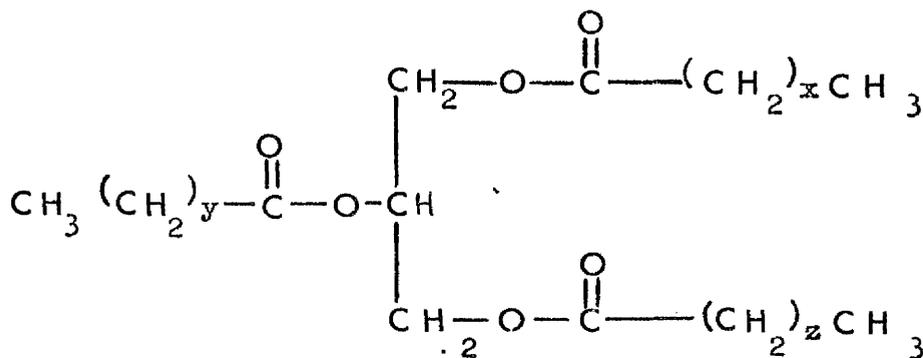
As with triacylglycerols of mammalian origin, the unsaturated fatty acid component in yeasts is preferentially esterified to the C₂ atom of the glycerol backbone (Meyer & Bloch, 1963).

Diacylglycerols and monoacylglycerols have also been detected in lipid extracts from yeasts (Kates & Baxter, 1962). These were mostly 1,2 diacyl-sn glycerols but a small amount of 1,3 diacyl-sn

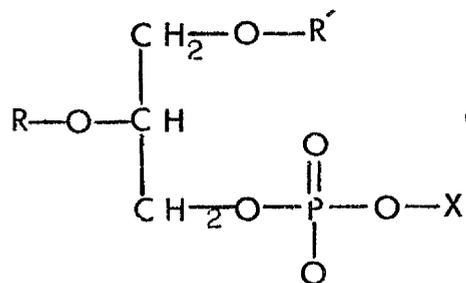
Figure 3

Structure of different lipids found in yeasts

A Triacylglycerols



B Phospholipids



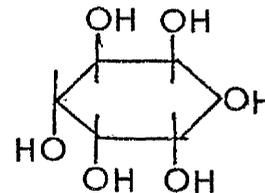
R, R' = fatty acids

X =

choline $\text{HOCH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$

ethanolamine $\text{HOCH}_2\text{CH}_2\text{N}^+\text{H}_3$

inositol



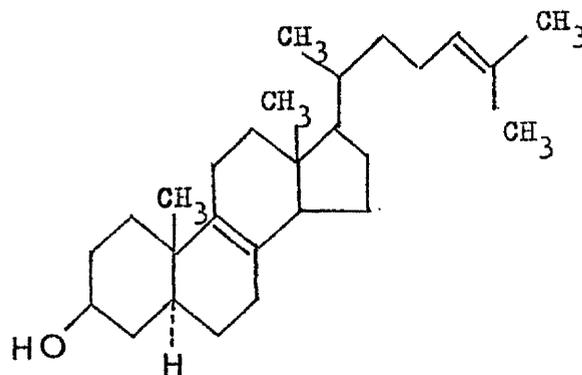
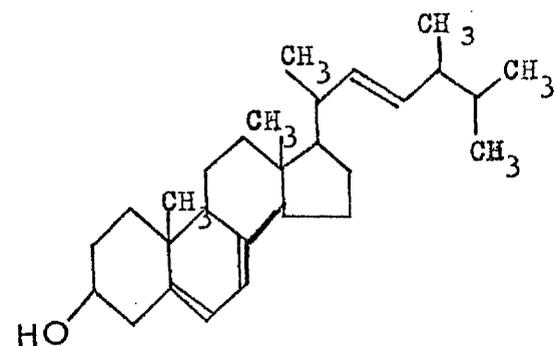
serine

$\text{HOCH}_2\text{CHNH}_2\text{COOH}$

C Sterols

Ergosterol

zymosterol



glycerols was detected. The monoacylglycerol was shown to be 1, acyl-sn glycerol.

c) Phospholipids

Most of the yeast species have a phospholipid content of between 3% and 7% of the cell dry weight. Structures for the most common phospholipids are given in Fig 5b. A representative distribution (as the percentage of total phospholipid) of 35% to 55% phosphatidyl choline, 20% to 32% phosphatidyl ethanolamine, 9% to 22% phosphatidyl inositol, 4% to 18% phosphatidyl serine and less than 10% of minor component, has been described by Letters (1968). The minor phospholipids include phosphatidic acid, cardiolipin, phosphatidyl glycerol, N'monomethyl phosphatidyl ethanolamine and N'N' dimethyl phosphatidyl ethanolamine (Rattray, Schibeci & Kidby, 1975). The amino acid esters of phosphatidyl glycerol (the lipoamino acids), common in bacteria, have not as yet been isolated from yeasts (Hunter & Rose, 1972). Similarly, plasmalogens appear to be absent or present in only trace amounts (Letters & Snell, 1963).

Lester and his colleagues have investigated the nature of the inositol-containing yeast phospholipids. So far seven have been isolated and characterised. Phosphatidyl inositol is predominant. The others are di- and tri-phosphatidyl inositol and four inositol-containing sphingolipids (Steiner, Smith, Waechter & Lester, 1969; Lester & Steiner, 1972; Steiner & Lester, 1972a,b).

Lysophospholipids have been recognised in lipid extracts of yeasts, although they generally account for only a small proportion of the total lipid phosphorus (Hunter & Rose, 1972). Contrary to this, Letters (1968) has found that anaerobically grown Sacch. carlsbergensis contains

large amounts of lysophosphatidyl choline accounting for some 27% of the total lipid phosphorus.

Recently a novel phospholipid - pyrophosphatidic acid - has been isolated and characterised from Cryptococcus neoformans. It may also be present in some other species of yeast (Itoh & Kaneko, 1974).

d) Hydrocarbons

Due to the lack of a reliable assay very little work has been done on this class of lipid. However, Russian workers reported that yeasts contain between 0.1% to 0.7% of their dry weight as hydrocarbon (Maksimova, Vorboreva, Grigoreva & Botnikova, 1967). A more detailed study of the hydrocarbon fraction from Sacch. oviformis and Sacch. ludwigii suggested that this class of lipid could account for up to 20% of the lipids in the cell (Baraud, Cassagne, Genevois & Joneau, 1967). More than forty different straight- and branched-chain alkanes were detected varying in chain length from C₁₀ to C₃₁. The same group confirmed the presence of hydrocarbons in Sacch. cerevisiae (Baraud, Maurice & Napias, 1970).

e) Polyprenols

Polyprenols have been demonstrated in yeasts only relatively recently (Dunphy, Kerr, Pennock, Whittle & Feeny, 1967). A total of 5 mg of dolichols was isolated from 1 kg of pressed baker's yeast. The chain length of these compounds varied from C₇₀ to C₁₀₅. Subsequently, Jung & Tanner (1973) fractionated the naturally occurring dolichols in Sacch. cerevisiae into five homologous polyprenols saturated with α -isoprene units. Phosphorylated forms accounted for 10% to 20% of the free dolichols.

f) Sphingolipids

Sphingolipids are hydroxy fatty acid esters of long chain amino alcohols or sphingosines and comprise three main types known as sphingomyelins, cerebrosides and gangliosides respectively. There have been many reports of sphingolipids in yeasts. All those examined in detail have been cerebrosides and derivatives of phytosphingosine (Reindel, Wieckmann, Picard, Luber & Turula, 1940; Stanacev & Kates, 1963; Wagner & Zofcsik, 1966a,b; Trevelyan, 1968; Tanner, 1968; Steiner et al., 1969). Wagner & Zofcsik (1966a,b) isolated and characterised two sphingolipids from Sacch. cerevisiae and Candida utilis. From one of these lipids three sphingosine bases, α -hydroxy stearic acid and galactose were released upon hydrolysis. The products of hydrolysis of the other lipid included C_{18} and C_{20} phytosphingosines; C_{18} dihydro-sphingosine; C_{20} , C_{22} and C_{24} fatty acids; inorganic phosphate, inositol and mannose. This latter sphingolipid was called a "mycoglycolipid". Later evidence (Tanner, 1968) precludes the possibility that it is an inositol mannoside-containing glycolipid of the type found in mycobacteria.

Recently, Suomalainen and his associates have demonstrated several complex sphingolipids containing phosphate and inositol in the cell envelope of baker's yeast (Tyorinoja, Nurminen & Suomalainen, 1974).

g) Glycolipids

In addition to the complex glycosphingolipids, small quantities of other glycolipids have been found in Sacch. cerevisiae. Monogalactosyl-diacyl glucose for example has been recognised in lipid extracts from stationary phase cells (Baraud, Maurice & Napias, 1970). Such glycolipids are characterised by an abundance of long chain (greater than C_{18}) and odd-chain fatty acids.

The presence of sterol glycosides, monogalactosyl diacyl glycerols and acyl sugars has been demonstrated in cell envelopes of baker's yeast (Työrinoja, Nurminen & Suomalainen, 1974).

h) Sterols

The total sterol content of yeasts can range from 0.03% to 4.6% of the cell dry weight, accounting for up to 10% of the total cell lipid. Yeasts of the genus Saccharomyces are particularly rich in sterols. The most common yeast sterols are ergosterol and zymosterol (Fig 5c; Hunter & Rose, 1971) and ergosterol may account for over 90% of the total sterol in some species. Some of the minor sterols present in Sacch. cerevisiae are listed in Table 2.

Sterols can occur free or esterified to fatty acids characterised by a high content of C_{16:1}, C_{18:1} and in certain genera C_{18:2} acids. Sterol esters were first reported in yeasts as long ago as 1940 (Maguigan & Walker, 1940). The bulk of the sterol component in Sacch. cerevisiae is esterified (Hunter & Rose, 1971). It would appear that sterols can also be esterified to a polysaccharide (Adams & Parks, 1967, 1968) which may be identical to the cell-wall mannan (Thompson, Knights & Parks, 1973).

4. Cellular Distribution

Up to the beginning of this decade it was generally held that in non-fat yeasts, the cellular lipids were located in a membrane system (Hunter & Rose, 1971). Recently there have been a number of reports about the presence and/or composition of lipid droplets, granules, particles or vesicles in Sacch. cerevisiae (Dawson & Craig, 1966; Baraud,

Table 2

Minor Sterols in Lipid Extracts of Sacch. cerevisiae

STEROLS	REFERENCE
Dimethyl-4-4'zymosterol	Ponsinet & Ourisson (1965)
Methyl-4 α -zymosterol	"
$\Delta^{5,7,22,24(28)}$ ergostetraen-3 β -ol*	Breivik, Oivades & Light (1954)
Episterol	Longley, Rose & Knights (1968)
	Wieland, Rath & Hess (1941)
Ascosterol	"
Cerevisterol	Fieser & Fieser (1959)
14-dehydrocholesterol	"
5-dihydrocholesterol	Callow (1931)
4 α -methylene-24-25-dihydrozymosterol	Barton, Harrison & Widdowson (1968)
4,14 dimethylcholesta-8,24-dien-3 β -ol	Trocha, Jasne & Sprinson (1974)
4,14 dimethylergosta-8,24-dien-3 β -ol	"
4,14 dimethylergosta-8,24(28)-dien-3 β -ol	"

*This sterol has been shown to be a major component of the sterol fraction of Sacch. cerevisiae NCYC 366 (Longley, Rose & Knights, 1968; Hunter & Rose, 1972).

Maurice & Napias, 1970; Hossack, Wheeler & Rose, 1973; Cartledge & Rose, 1973; Clausen, Christiansen, Jennsen & Behnke, 1974).

Longley, Rose & Knights (1968) found very few differences in lipid composition between isolated plasma membranes and whole cells, although they were able to demonstrate that the protoplast membrane contained a greater proportion of phosphatidyl ethanoline and phosphatidyl inositol plus phosphatidyl serine than whole cells, but less phosphatidyl choline. Minor changes in the distribution of fatty acids were also reported. In contrast to this earlier study, Hossack, Wheeler & Rose (1973) using plasma membranes isolated by a sucrose density gradient fractionation of sphaeroplast lysates found that they were deficient in sterol esters and concluded that Sacch. cerevisiae may have a plasma membrane lipid composition similar to that of animal cell-surface membranes.

A rather broad range of values have been reported for the amount of lipid that is genuinely part of the cell wall. Thus, Kessler & Nickerson (1958) reported a lipid content of 8% to 10% while Eddy (1958) quoted a figure of 2%. A range of lipid contents from 2% to 9% has been shown to exist in cell walls prepared from Sacch. cerevisiae grown under different conditions of substrate limitation (McMurrough & Rose, 1967). Suomalainen's group have examined the lipids from the cell walls of brewer's and baker's yeast in great detail and have reported yields of 1.4% and 1.6% lipid respectively. They have further shown that the lipid of the cell walls prepared from baker's yeast comprises 74% neutral lipid with acylglycerols and sterol esters accounting for 83% of this (Nurminen, Konttinen & Suomalainen, 1975).

Much attention has been paid recently to the lipid composition of a low density vesicle fraction which can be isolated relatively easily

from sphaeroplast lysates of Sacch. cerevisiae. Hossack, Wheeler & Rose (1973) reported that the lipid composition was 32% phospholipid, 38% sterol ester and 26% triacylglycerol. In contrast, Cartledge & Rose (1973), using the same strain of yeast but another fractionation procedure, reported a quite different lipid composition for these vesicles. The most notable changes were a decrease in the sterol ester and phospholipid levels to 10% and 17% respectively and a large increase in the content of free fatty acids (from 0 to 30% of the total lipid). They also showed that the distribution of phospholipids in the vesicle fraction is different from that in whole cells.

A Danish group has also isolated lipid particles from Sacch. cerevisiae and has reported that these structures contain 94% lipid which is mainly composed of triacylglycerol and sterol ester (Clausen, Christiansen, Jensen & Behnke, 1974).

5. Effects of growth conditions on lipid content

A variety of conditions influence the growth of yeast and must be taken into account in any meaningful assessment of the cellular lipid composition. The effects of the environmental factors on the cellular lipid composition of Sacch. cerevisiae have been summarised in Table 3. Those attributable to phosphate deprivation are discussed in more detail in Section E. below.

6. Biological significance

a) Fatty acids

Fatty acid composition, it would appear, is an important variable in membrane morphology (Henry & Keith, 1971; James, Branton, Wisnieski &

<u>Growth conditions</u>	<u>Effect on lipid composition</u>
Growth during early exponential phase	Decrease in total lipid mainly at expense of triacylglycerols.
Growth during late exponential phase	Increase in total lipid production.
Entry into stationary phase	Rapid decrease in lipid content as a result of increased lipid peroxidase activity.
Decreased growth rate	Increased synthesis of phosphatidyl choline and sterol ester.
Increasing the concentration of glucose in the culture medium from 0.2% (w/v) to 1.0% (w/v)	Decreased total lipid mainly at expense of phospholipid and sterol ester. Increased proportion of unsaturated fatty acids.
Phosphate deficiency	Increased total lipid mainly as triacylglycerol.
Inositol deficiency	Increased total lipid, mostly as triacylglycerol. Overall level of phospholipid unaltered. Fall in level of phosphatidyl inositol. Accumulated lipid stored as globules. Affects composition of cell wall lipids.
Biotin deficiency	Lowered level of C ₁₆ and C ₁₈ fatty acids
Anaerobiosis	Lowered amounts of total lipid, phospholipid and sterol. Variable acyl-glycerol content. Increased quantities of lysophospholipids. High levels of saturated fatty acids. Induction of fatty acid and sterol auxotrophy.
Decrease in growth temperature	Increased synthesis of triacylglycerols and phospholipids. Decreased content of total and esterified sterols. Little or no effect on unsaturated or composition of fatty acids.
Fall in pH from 6.0 to 5.5	Affects general composition rather than quantity of cellular lipid.

Keith, 1972). Considerable information has been obtained from studying the growth requirements of fatty acid desaturase mutants (Keith, Wisnieski, Henry & Williams, 1973). Marked growth-promoting ability has been observed with fatty acids containing a cis- Δ^9 bond (Wisnieski, Keith & Resnick, 1970; Wisnieski & Kiyomoto, 1972). Barber & Lands (1973) have demonstrated that ^{de}increasing the saturation of the fatty acid supplement increases the cell yield, with the C_{22:6}, C_{20:5} and C_{20:4} acids producing greatest activity. The biological significance of this finding is not clear since these acids are not normally found in Sacch. cerevisiae. When the level of unsaturation of the supplement was decreased it was found that respiratory incompetent cytoplasmic petite mutants were induced (Marzuki, Hall & Linnane, 1974). Proudlock, Haslam & Linnane (1969, 1971) have demonstrated that unsaturated fatty acids are necessary components of mitochondrial lipid if electron transport is to be coupled to oxidative phosphorylation. A similar relationship has been shown for active cation transport (Haslam, Spithill, Linnane & Chappell, 1973).

By exploiting the anaerobically-induced requirement of Sacch. cerevisiae for an unsaturated fatty acid and a sterol, Altherthum & Rose (1973) were able to study the effect of lipid unsaturation on the ability of the yeast plasma membrane to resist stretching. They grew cells enriched in either oleic, linoleic or linolenic residues and found that the susceptibility to osmotic lysis of sphaeroplasts prepared from such cells increased as the proportion of fatty-acyl unsaturation was raised.

b) Sterols and sterol esters

Sterols have been isolated from a variety of membrane systems (Rattray, Schibeci & Kidby, 1972) and from vesicle preparations (Hossack, Wheeler & Rose, 1973). Although the cell wall of Sacch. cerevisiae has

been found to be deficient in sterol (Baraud et al., 1970; Nurminen & Suomalainen, 1971) its mannan component possesses a general capacity to bind sterol in vitro (Thompson, Knights & Parks, 1973).

The major function of sterols in yeast has been regarded by Proudlock, Wheeldon, Jollow & Linnane (1968) to be one of structural influence on the dynamic state of membranes. Recently, Hossack & Rose (1976) have examined the effect of different sterol enrichments on the ability of the plasma membrane to remain intact when sphaeroplasts are exposed to hypotonic solutions of sorbitol. The capacity to resist stretching was greatest when the membrane was enriched in a sterol with an unsaturated side chain at C₁₇ (e.g. ergosterol and stigmasterol).

c) Phospholipids

The relative constancy of the total phospholipid component in yeast is suggestive of some primary role (Rattray, Schibeci & Kidby, 1975). It has been proposed by Jollow, Kellerman & Linnane (1968) that phospholipids are the major structural components of yeast membranes.

Several isolated functions have been ascribed to individual phospholipids. Talwalker & Lester (1973) have observed that a cyclical turnover of polyphosphoinositides is accompanied by changes in adenylate energy charge which may possibly facilitate diffusion of ions across membranes in Sacch. cerevisiae. Hydrolysis of phosphatidyl glycerol phosphate to phosphatidyl glycerol may be important in active transport of D-galactose and amino acids (Deierkauf & Booiij, 1968). Phospholipid has been shown to protect mitochondrial ATPase against the action of oligomycin (Somlo & Krupa, 1974).

D. TEICHOIC ACIDS IN MICRO-ORGANISMS

The term "teichoic acid" (Gr. teichos = wall) was originally used to describe polymers containing phosphate which were isolated from the cell walls of Gram-positive bacteria. The first of these to be examined in detail were all polymers of either glycerol phosphate or ribitol phosphate in which repeating units were joined together through phosphodiester linkages. Glycosyl and D-alanine ester substituents were also usually present. Subsequently, polymers possessing considerable variation on this basic structure were isolated. In addition, glycerol phosphate polymers were found to be almost ubiquitous components of the membranes of Gram-positive bacteria; these compounds appear to be covalently bound to lipid and are now generally referred to as lipoteichoic acids. Consequently the definition of teichoic acids has now been broadened to include all wall, membrane or capsular polymers containing glycerol phosphate or ribitol phosphate residues (Baddiley, 1972).

1. Occurrence and location

Teichoic acids were first extracted from the walls of Gram-positive bacteria by treatment with cold trichloroacetic acid (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). They are major cell wall components, often accounting for between 20% and 50% of the dry weight of the cell wall and are covalently linked to peptidoglycan (Baddiley, 1972). Their distribution is widespread and their occurrence, structure and antigenicity can be of taxonomic importance (Knox & Wicken, 1973). All wild strains of Staphylococcus aureus for example contain ribitol teichoic acid in their walls.

Some confusion still exists about the precise location of membrane-associated teichoic acids. They were first isolated from whole cell extracts of bacteria which lacked a wall teichoic acid and at that time were given the name "intracellular teichoic acids". Later studies on polymer release during protoplast formation suggested a periplasmic location (Hay, Wicken & Baddiley, 1963). However, subsequent isolation of teichoic acid covalently bound to lipid was demonstrated (lipoteichoic acid; Toon, Brown & Baddiley, 1972). Recent work (Huff, Cole & Theodore, 1974) now suggests that lipoteichoic acid may be associated with mesosomal vesicles, at least in some bacteria, although this has been disputed (Joseph & Shockman, 1975). Lipoteichoic acids have been found in all Gram-positive bacteria examined so far with the exception of Micrococcus lysodeikticus (Powell, Duckworth & Baddiley, 1974) and Sarcina lutea (L.J. Douglas & J.H. Freer, personal communication). These organisms do, however, possess related acidic polysaccharides which also appear to be located in the mesosomes (Owen & Salton, 1975).

Although for many years it was supposed that teichoic acids were confined to Gram-positive bacteria, recent reports indicate their distribution may be more widespread. It has been shown that a simple unsubstituted poly (glycerol phosphate) teichoic acid is present in a yeast - Candida intermedia (Naumova & Streshinskaya, 1973). These workers have tentatively suggested an intracellular location for this polymer. Recently, a lipoteichoic acid has been detected in hot phenol extracts of the Gram-negative rumen anaerobe Butyrivibrio fibriosolvens (Sharpe, Brock & Phillips, 1975). This material was subsequently extracted, isolated and analysed and the presence of a lipoteichoic acid confirmed (Hewett, Wicken, Knox & Sharpe, 1976). Ribitol and ribitol phosphate have been released upon alkaline and acid hydrolyses respectively from a lipopolysaccharide

preparation of Proteus mirabilis (Gmeiner, 1975). The significance of this observation is not yet clear, although it may indicate the presence of ribitol teichoic acid in a Gram-negative organism.

2. Structure

a) Poly (ribitol phosphate) teichoic acids

Ribitol teichoic acids of this type are confined exclusively to bacterial walls and the most extensively studied are those from strains of Staph. aureus (Baddiley, Buchanan, Martin & RajBhandry, 1962; Baddiley, Buchanan, RajBhandry & Sanderson, 1962). In all of those examined, the phosphodiester linkages are between positions 1 and 5 on ribitol and an N-acetylglucosaminyl residue is present at the D-4 position on each ribitol residue (Fig 6A). The glycosidic linkages may be α or β but in the majority of strains both types of linkage are present (Davidson & Baddiley, 1963). Most of the ribitol residues also possess a D-alanine ester residue and the extracted polymer has an average chain length of 5 - 9 repeating units.

Similar ribitol teichoic acids are found in the walls of bacilli and lactobacilli. In a strain of Bacillus subtilis the sugar substituents are β -glucopyranosyl (Armstrong, Baddiley & Buchanan, 1961) whereas in Lactobacillus arabinosus 17 - 5 they are α -glucopyranosyl (Archibald, Baddiley & Buchanan, 1961). Ribitol teichoic acids of rather more complex structure also comprise the capsular material of some pneumococci (Chittenden, Roberts, Buchanan & Baddiley, 1968; Rao, Buchanan & Baddiley, 1966; Brundish & Baddiley, 1968).

b) Poly (glycerol phosphate) teichoic acids

Teichoic acids containing glycerol phosphate are more widespread than are those containing ribitol phosphate. The first examples to be subjected to structural examination were polymers of glycerol phosphate in which phosphodiester linkages join the 1- and 3-positions of adjacent glycerol residues (Kelemen & Baddiley, 1961). These were lipoteichoic acids and subsequent work supports the conclusion that all lipoteichoic acids are of this type (Baddiley & Davidson, 1961; Davidson & Baddiley, 1963; Archibald, Baddiley & Button, 1968). Many of them lack glycosyl substituents completely, but a few have glycosyl substituents on 2-hydroxyl groups of occasional glycerol residues (Critchley, Archibald & Baddiley, 1962). Alanine ester residues occupy hydroxyl groups on most of those glycerol moieties that are not glycosylated. An example of this type of structure is shown in Fig 6B. Glycerol teichoic acids also exist in which the phosphodiester linkage is between C1 and C2 of adjacent glycerol residues. These have been found in actinomycetes (Naumova & Zaretskaya, 1964) and in Bacillus stearothermophilus (Wicken, 1966).

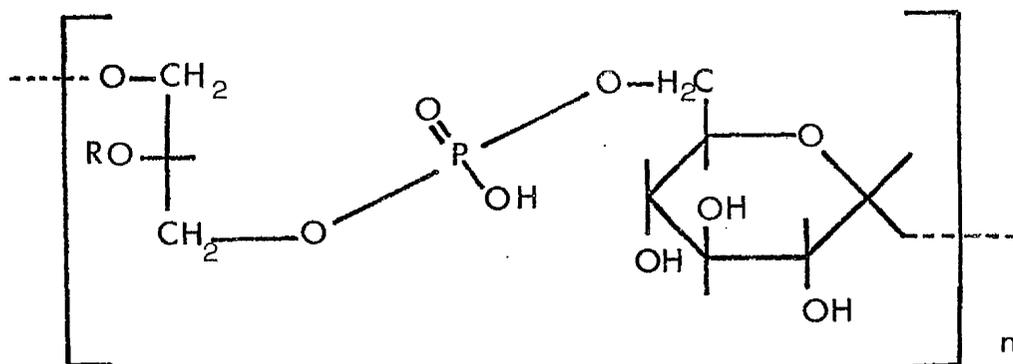
c) Complex glycerol teichoic acids

Glycerol teichoic acids have been recognised in which the sugar residue forms part of the main polymer chain. An example of this type of polymer is the glucosylglycerol phosphate teichoic acid from Bacillus licheniformis ATCC 9945 (Fig 7A). A galactosylglycerol phosphate teichoic acid of similar structure also occurs separately in the wall of this strain of B. licheniformis.

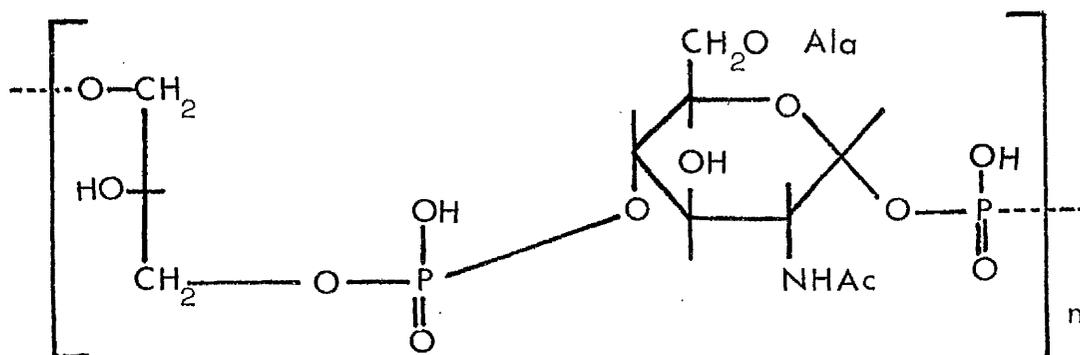
A related polymer has been identified in the walls of Staph. lactis I3. It contains a repeating unit in which glycerol phosphate is

Fig. 7 STRUCTURES OF OTHER GLYCEROL TEICHOIC ACIDS

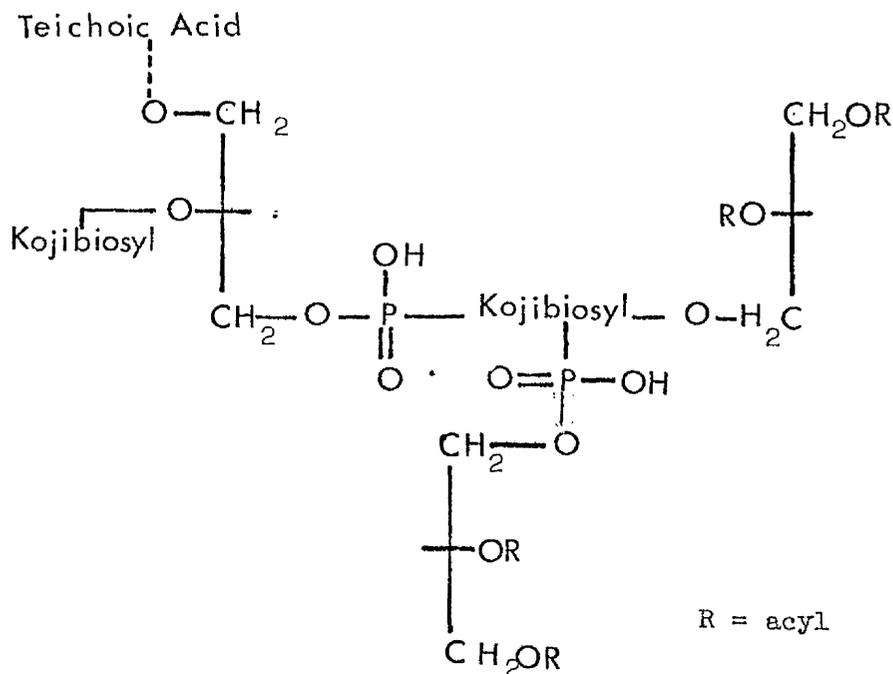
- A. Glucosylglycerol Phosphate Teichoic Acid from B. licheniformis
A.T.C.C. 9945 (Burger & Glaser, 1966)



- B. Teichoic Acid containing Sugar 1-phosphate Linkages from the Walls of Staph. lactis I3



- C. Structure of the Lipoteichoic Acid from Strep. faecalis N.C.I.B. 8191



attached to the hydroxyl at C4 on N-acetylglucosamine 1-phosphate; D-alanine ester residues are present on the C6 hydroxyl of the N-acetylglucosamine residue (Fig 7B).

d) Lipoteichoic acids

A mixture of phenol and water can be used to extract membrane teichoic acids from disrupted cells (Burger & Glaser, 1964). The suggestion (Archibald, Baddiley & Blumson, 1968) that in such preparations the linkage to membrane components remains intact has been verified in a study of teichoic acids extracted from lactobacilli (Knox & Wicken, 1970; Wicken & Knox, 1970). Such preparations of teichoic acid and membrane lipid are now termed "lipoteichoic acids".

Detailed analysis of the lipoteichoic acid from Strep. faecalis showed that the glycerol phosphate chain of the teichoic acid comprises 28 - 35 repeating units and is covalently attached through its terminal phosphate to a phosphatidyl glycolipid that is a normal component of the membrane. The phosphodiester linkage is attached to an unidentified hydroxyl on one of the two glucose residues in the glycolipid (Toon, Brown & Baddiley, 1972) and the phosphatidyl residue is attached to another unidentified hydroxyl on a glucose residue (Fig 7C).

More recently lipoteichoic acids have been extracted by the cold-phenol procedure from a variety of lactobacilli, streptococci, bacilli and staphylococci (Coley, Duckworth & Baddiley, 1972; Knox & Wicken, 1973).

3. Biosynthesis

The incorporation of glycerol and ribitol into the corresponding

polyol polymer has been accomplished using CDP-glycerol and CDP-ribitol as precursors (Burger & Glaser, 1964; Glaser, 1964; Ishimoto & Strominger, 1966) and washed cytoplasmic membrane or particulate preparations corresponding to fragmented membrane as the enzyme source. The particulate enzyme system from B. subtilis NCTC 3610 can also carry out a transglycosylation reaction in which α -D-glycopyranosyl residues are transferred from UDP-glucose to poly (glycerol phosphate). For the cell-free synthesis of the complex wall teichoic acid from Staph. lactis I3 (Fig 7B) both CDP-glycerol and UDP-N-acetylglucosamine are required simultaneously (Baddiley, Blumsom & Douglas, 1968).

Lipid intermediates, similar to those involved in peptidoglycan synthesis, have been shown to be important in the biosynthesis of some teichoic acids (Douglas & Baddiley, 1968; Hussey & Baddiley, 1972). Repeating units of the teichoic acids of B. licheniformis ATCC 9945 and Staph. lactis I3 are assembled stepwise on the lipid then transferred to the nascent polymer chain (Hancock & Baddiley, 1972; Anderson, Hussey & Baddiley, 1972; Hussey & Baddiley, 1972). A common pool of lipid, which has been identified as undecaprenol phosphate, has been shown to exist for peptidoglycan and teichoic and biosyntheses (Watkinson, Hussey & Baddiley, 1971; Anderson, Hussey & Baddiley, 1972).

Poly (ribitol phosphate) synthetase from Staph. aureus H has been solubilised and shown to use lipoteichoic acid as an acceptor for the newly synthesised polymer (Fiedler & Glaser, 1974a). Evidence has also been presented that lipoteichoic acids from a wide variety of sources can act as acceptor in this system (Fiedler & Glaser, 1974b).

Recent studies have shown that the poly (ribitol phosphate) teichoic acid of Staph. aureus H is attached to peptidoglycan through a

oligomer of glycerol phosphate (Coley, Archibald & Baddiley, 1976).

This linkage unit appears to be synthesised from CDP-glycerol (Bracha & Glaser, 1976; Hancock & Baddiley, 1976).

4. Function

A considerable proportion of the metabolic activity in many Gram-positive bacteria is directed towards synthesis of teichoic acid and so it seems reasonable to assume that these polymers have some role or function which is of value to the cell. Thus far evidence has been obtained for three possible functions. Several observations support the idea that one important function is in the binding of cations and the provision of the correct cationic environment at the plasma membrane. Secondly, the lysis and autolysis of certain bacteria is influenced by teichoic acid, and it appears that teichoic acids may participate in the activation and regulation of autolytic enzymes during cell growth. Finally, a role for lipoteichoic acid has been proposed in the synthesis of wall ribitol teichoic acid in some bacteria (see Section D.3).

In addition, teichoic acids are frequently antigenic (Knox & Wicken, 1973) and often form part of the specific receptor site for various bacteriophages (Glaser, Ionesco & Schaeffer, 1966; Young, 1967). However important though these properties are, they are not usually regarded as true biological functions since they are unlikely to confer any advantage on the cell.

The cation-binding properties of walls of certain staphylococci (Cutineui & Galdiero, 1967; Bradley & Parker, 1968) have been ascribed to the teichoic acid component (Hepinstall, Archibald & Baddiley, 1970). The ability of teichoic acid to act as a cation buffer (that is to maintain, at

the membrane, an optimal concentration of cation which is almost independent of the cation concentration of the suspending medium) has been demonstrated with a cell-free system from B. licheniformis ATCC 9945 (Hughes, Hancock & Baddiley, 1973). An inverse relationship between the amount of ester-bound alanine on the teichoic acid and the amount of Mg^{++} bound by walls of staphylococci has been established (Archibald, Baddiley & Hepinstall, 1973). It was also suggested that the alanyl-ester content may regulate the surface charge and cation binding properties of the wall. The importance of a negatively-charged polymer in the wall of many Gram-positive bacteria has been demonstrated by the finding that under conditions of phosphate limitation, teichoic acid is replaced by teichuronic acid (see Section E.1).

Bacterial autolysins have been implicated in the processes of surface growth and cell division (Rogers, 1970; Shockman, Daneo-Moore & Higgins, 1974). Since one result of autolytic activity is cell lysis, regulatory mechanisms at the cellular level have been postulated (Shockman, Daneo-Moore & Higgins, 1974). It has been proposed that lipoteichoic acid may act as an inhibitor of autolytic systems (Holtje & Tomasz, 1975; Cleveland et al., 1975). Deacylation of the lipoteichoic acid and certain lipids of Streptococcus faecalis ATCC 9790 removed the inhibitory action of these compounds on the autolytic enzyme (a muraminidase) of this organism (Cleveland, Wicken, Daneo-Moore & Shockman, 1976). Thus deacylation of lipoteichoic acid in vivo by the action of a deacylase may provide a mechanism for the control of autolytic activity.

E. PHOSPHATE LIMITATION OF GROWTH OF MICRO-ORGANISMS

The ability of a micro-organism to alter its structure and function in response to changes in its growth environment is a striking

one. In particular, the changes which often accompany phosphate limitation of growth are many and varied. Phosphorus is found as a structural component in nucleic acids, lipids, cell wall polymers and energy reserve polymers. It is therefore not surprising to find that much work has been carried out in investigating the effects of phosphate limitation on growth of micro-organisms.

One of the problems facing investigators in this field is that micro-organisms interact chemically with their environment, taking up nutrients and excreting end-products of metabolism. Consequently the environment in which the organism is being grown is changing continuously and, as the environment changes, so too will the parameter which is being studied. This difficulty can be avoided by growing the organism in a continuous-flow culture system. Only then are steady state conditions achieved, in which the properties of the organism and the environment of the organism do not change substantially with time.

1. Gram-positive bacteria

Phosphate limitation of growth produces major changes in both the cell-wall composition and lipid composition of many Gram-positive bacteria. Some of the effects have been reviewed by Ellwood & Tempest (1972). The changes in cell-wall composition which accompany phosphate limitation of Bacillus subtilis var niger grown in chemostat culture are particularly striking and these have been summarised in Table 4.

It would appear that, when a constraint is applied to the supply of phosphate, this organism can terminate the synthesis of wall teichoic acid and replace it with a non-phosphorus-containing anionic polymer, teichuronic acid (Ellwood & Tempest, 1968). The effect of

Table 4

The Effects of Phosphate Limitation on the Cell Walls of
B. subtilis var niger

1. The phosphate content of the cell wall falls 10-15 fold.
2. The teichoic acid content falls from 40-50% of the dry weight of the wall to < 3% of the dry weight.
3. The content of uronic acid increases from < 3% to 25% of the dry weight of wall.
4. The galactosamine content rises from < 3% to 17% of the dry weight of the wall.

phosphate limitation on synthesis of cell walls teichoic acid has now been examined in variety of other Gram-positive bacteria, and in each case the teichoic acid was replaced by teichuronic acid (Ellwood & Tempest, 1972). The list of organisms studied included B. subtilis W23, B. subtilis 168, B. licheniformis, B. megaterium KM and Staphylococcus aureus H.

The peptidoglycan component of walls of Gram-positive bacteria may also be changed phenotypically by substrate limitation in general and phosphate limitation in particular. When B. subtilis W23 for example is grown under conditions of phosphate limitation, the peptidoglycan contains significantly more muramic acid than glucosamine (Ellwood & Tempest, 1972). Such changes in peptidoglycan composition are paralleled by an altered sensitivity of the organism to lysozyme.

Rather less information is available on the effects of phosphate limitation of growth on the lipid composition of gram-positive bacteria. Minnikin, Abdolrahimzadeh & Baddiley (1971) claim that when B. subtilis W23 and B. cereus T are grown under conditions of apparent phosphate limitation, these organisms accumulate diglucosyldiglycerides at the expense of some of the phosphatidylethanolamine. B. cereus T also produces an acidic glycolipid which partially replaces the acidic phospholipids phosphatidylglycerol and diphosphatidylglycerol.

2. Gram-negative bacteria

The cell envelope composition of Aerobacter aerogenes grown under different conditions of substrate limitation has been extensively studied (Ellwood & Tempest, 1968). When phosphate limits the growth of this organism, the cell envelopes contain elevated amounts of lipopolysaccharide while the levels of protein, phosphorus and carbohydrate are correspondingly lowered.

Minnikin et al. (1974) have repeated their claim that acidic phospholipids may be replaced in part with acidic glycolipids. The organism under study in this case was Pseudomonas diminuta. Shaw (1975), however, disagrees with their conclusions and suggests rather that the phospholipids are largely replaced by neutral glycolipids.

3. Yeasts

Slodki and his co-workers have shown that the phosphate content of exocellular mannans of Hansenula capsulatum and H. holstii is decreased when the phosphate concentration of the growth medium is lowered. Substitution of β -linkages by α -linkages also occurs (Slodki, Safranski, Hensley & Babcock, 1970). The same workers additionally demonstrated that growth in medium containing low concentrations of phosphate increased the level of O-acetyl substitution of the exocellular phosphogalactan of Sporobolmyces sp. Recently, these results were confirmed with a different strain of H. holstii which had been grown batchwise in an undefined medium (San Blas & Cunningham, 1974a,b). It is interesting to note that these workers found that the mannans elaborated by the yeast, although deficient in phosphate residues, were still heterogeneous towards ion-exchange chromatography. They also detected changes in the structure of both the cell wall and the exocellular mannans and the apparent replacement of phosphate groups by O-acetyl groups. Some of these findings were confirmed by Ellwood (1975) using H. holstii grown in a chemostat.

Some effects of phosphate limitation on the lipid composition of Sacch. cerevisiae and Candida utilis have been briefly reported (Johnson, Brown & Minnikin, 1973). An increased total lipid content was reflected in increased synthesis of fatty acids and triacylglycerols.

Only minor, unspecified changes in the polar lipid fraction were reported. Similar changes in neutral lipid composition were also observed in C. utilis. However, the levels of the three major phospholipids were greatly diminished and they were replaced by three novel, phosphorus-free, lipids which were not characterised.

Although a detailed study of the effects of phosphate limitation on yeast fine structure has not been carried out, it has been reported that striated and crystalline inclusions are present in the nucleus and cytoplasm of Sacch. cerevisiae cultured in a basal medium of low phosphate content (Tanaka & Mizunaga, 1974).

OBJECT OF THE INVESTIGATION

The specific aims of this investigation were two-fold:

- (i) an analysis of cell walls and lipids of Sacch. cerevisiae NCYC 366 grown under conditions of phosphate limitation
- (ii) a search for teichoic acids in yeasts.

Substantial changes have been reported in both the cell-wall and lipid composition of a variety of bacteria when these organisms were grown under conditions of phosphate limitation. Similar detailed studies with yeasts have not yet been described, however. The primary objective of this work was to investigate whether related changes in the content and composition of these components occur when Sacch. cerevisiae is grown under phosphate-limiting conditions.

A secondary objective was to confirm the reported occurrence of teichoic acid in Candida intermedia and then to extend the survey for these polymers to other yeast species. Until recently teichoic acids were widely assumed to be confined to Gram-positive bacteria. An unambiguous demonstration of the presence of these compounds in eukaryotes might lead to further clarification of their function in micro-organisms.

MATERIALS AND METHODS

8

A. GROWTH OF YEAST

1. Strains

The strains of yeast used in this study were

- i. Saccharomyces cerevisiae NCYC 366
- ii. Candida intermedia CBS 572
- iii. Sacch. cerevisiae type I
- iv. C. utilis
- v. Baker's yeast.

Sacch. cerevisiae NCYC 366 and C. intermedia CBS 572 were maintained both in the freeze-dried state and on slopes of glucose-salts-vitamins agar (Table 5). Lyophilised cells of C. utilis and Sacch. cerevisiae type I were purchased from Sigma Chemical Company (London). Baker's yeast was obtained from Allinson Ltd. (London).

2. Growth media

Cultures of Sacch. cerevisiae NCYC 366 were grown in defined media based on that detailed in Table 5. For non-phosphate-limited and phosphate-limited cultures the concentrations of KH_2PO_4 were 3 g l^{-1} and 81.6 mg l^{-1} , respectively. Cultures of C. intermedia were grown in three different media, two of which (GPYS Types A and B) were undefined. Details of all three media are also given in Table 5.

3. Cultural conditions

i. Small-scale batch culture

Aliquots of culture medium (50 or 500 ml) were dispensed in dimpled Erlenmeyer flasks (250 ml or 2 l) and sterilised by autoclaving

Table 5 Growth media used for different yeast species

(a) For Saccharomyces cerevisiae NCYC 366

	<u>g/l</u>
Glucose	20
(NH ₄) ₂ SO ₄	3
KH ₂ PO ₄	3
MgSO ₄ ·7H ₂ O	0.25
CaCl ₂ ·6H ₂ O	0.25
L-Asparagine	1.0
Inositol	0.1
Pyridoxal HCl	0.01
Calcium-D-pantothenate	0.01
Thiamine	0.01
Biotin	2.2 x 10 ⁻⁶

(b) For Candida intermedia CBS 572

<u>GPYS Type A</u>		<u>GPYS Type B</u>		<u>Defined medium</u>	
	<u>g/l</u>		<u>g/l</u>		<u>g/l</u>
Glucose	20	Glucose	20	(NH ₄) ₂ SO ₄	3
Peptone	10	Peptone	10	KH ₂ PO ₄	3
Yeast extract	5	Yeast extract	5	MgSO ₄ ·7H ₂ O	0.25
KH ₂ PO ₄	1	KH ₂ PO ₄	3	CaCl ₂ ·6H ₂ O	0.25
MgSO ₄ ·7H ₂ O	0.50	MgSO ₄ ·7H ₂ O	0.25	Inositol	0.1
CaCl ₂ ·6H ₂ O	0.25	CaCl ₂ ·6H ₂ O	0.25	Pyridoxal HCl	0.01
				Calcium-D-pantothenate	0.01
				Thiamine	0.01
				Biotin	2.2 x 10 ⁻⁶
				Alkanes ¹	2% (v/v)

¹Alkanes' consisted of a mixture of equal volumes of dodecane, tetradecane, hexadecane and octadecane.

All media were adjusted to a pH value of 4.5. Distilled water was added to a final volume of 1 l.

at 10 psi for 10 min, or 15 psi for 15 min. The former sterilisation conditions were employed for the defined media. Culture medium (50 ml) was inoculated with an aliquot (0.1 ml) of a washed cell suspension (E_{1cm}^{650} 0.12 - 0.15) prepared from an overnight culture. Cultures were incubated at 30°C in an orbital incubator (Gallenkamp Ltd.). The platform was adjusted to shake at 125 rpm.

ii. Large-scale batch culture

Cultures (15 l) were grown in a fermenter (LH Engineering Co. Ltd., Stoke Poges, Bucks.). The fermenter vessel was sterilised, empty, by autoclaving at 15 psi for 30 min. Culture medium was pumped into the vessel through a sterile cellulose acetate membrane, pore size 0.45 μ (Millipore Corp. Ltd.). Mixing of the culture medium was accomplished using an impellar, revolving at 400 rpm. Sterile air was sparged through the culture at a rate of 10 l min⁻¹. The temperature of the culture medium was maintained at 30°C by thermostatically-controlled, water-filled, submerged heating elements.

Control cells were grown after inoculation of 0.5 l of an overnight culture into 14.5 l of medium. Cells were harvested during the middle of the exponential phase of growth ($E_{1cm}^{650} = 1.0$). Phosphate-limited cells were grown in a similar fashion. Culture medium was inoculated with a washed cell suspension (1 ml) of phosphate-limited cells. The culture was harvested towards the end of the logarithmic phase of growth ($E_{1cm}^{650} = 0.75$)

Most cultures (10 l) of C. intermedia were grown in a 20 l vessel. The medium was sterilised in the vessel by autoclaving at 15 psi for 30 min. An overnight broth culture (0.5 l) was used to inoculate the medium. Sterile air was supplied through a sparger at a rate of up to

5 l min⁻¹. Incubation at 30°C was continued until the latter part of logarithmic growth had occurred ($E_{1\text{cm}}^{410} = 3.0 - 4.0$).

iii. Continuous culture

Chemostat cultures of the yeast were grown in a 2 l vessel, fitted with a device for the control of temperature. Vortex mixing was accomplished using an impellar driven at a constant speed of approximately 1000 rpm. Sterile air was sparged into the culture at a flow rate of approximately 1 l min⁻¹. The dilution rate (which is numerically equal to the growth rate) was adjusted to 0.23 h⁻¹ by means of a Watson-Marlow peristaltic pump. At regular intervals samples were removed aseptically through a drain point in the floor of the chemostat vessel and used to check the purity, absorbance, ($E_{1\text{cm}}^{650}$) and pH value of the culture. The amount of orthophosphate (as phosphorus) present in cell-free filtrates of these samples was also determined. "Steady-state" cells were harvested either by draining the vessel to about 10% (v/v) of the initial volume or by recovery from the chilled, overflow receiver at short regular intervals.

4. Measurement of growth

Growth was followed by removing portions of cultures aseptically and measuring the absorbance in a Pye Unicam spectrophotometer at a wavelength of either 410 nm or 650 nm against a medium blank. Growth rates (μ) were calculated using the equation:

$$\mu = \frac{\log_e 2}{t_d} = \frac{0.69}{t_d} \quad \text{where}$$

t_d = culture doubling time.

B. PREPARATIVE METHODS

1. Preparation of cell walls

Cell walls were prepared by mechanical disruption of the yeast with glass beads. Cells were suspended in cold water (approximately 10 g wet weight plus 10 ml of water) and shaken with No. 10 Ballotini beads (10 g) for 75 - 90 s at 4000 rpm in a Braun MSK Homogeniser (B. Braun, Melsungen, West Germany). The temperature was maintained at 0 - 4°C throughout the disruption procedure. The extent of cell breakage was estimated by phase contrast microscopy and was usually found to exceed 90%. Beads were removed by filtration through a sintered glass filter (porosity grade 0) and the filtrate centrifuged at 750 x g for 20 min. Cell walls were recovered from the bottom layer of the pellet and washed ten times in 0.1 M tris-HCl buffer, pH 7.2, then four times in distilled water; the temperature remained below 4°C throughout. Wall preparations, which consisted of intact "wall hulls" and large fragments, were finally lyophilised and stored in vacuo over silica gel.

2. Treatment of cell-wall preparations with β -glucanase and α -mannosidase

To 6 ml of a cell-wall suspension (2 mg ml^{-1}) in 0.05 M sodium citrate buffer pH 5.0, containing 10 mM MgCl_2 and 10 mM ZnSO_4 , was added 0.05 ml (3.75 Units) of α -mannosidase and 0.15 ml (50 Units) of β -glucanase solution (10 mg/ml). The mixture was incubated at 30°C for 35 min. Aliquots (0.1 ml) were removed at time zero and after 35 min, diluted to 3 ml with distilled water and their absorbance at 650 nm measured in glass cuvettes of 1 cm pathlength against a distilled water blank.

3. Acid hydrolysis of enzymically-treated cell-wall suspensions

Cell-wall suspensions (1.8 ml) which had been pretreated with α -mannosidase and β -glucanase as described above were hydrolysed in HCl (final volume 2 ml) at 105°C in vacuo for periods of time ranging from 2 to 4 h. Hydrolysates were neutralised with 0.5 M KOH (4 ml) and transferred quantitatively to conical centrifuge tubes. After centrifugation at 1100 x g for 5 min to remove any insoluble material, hydrolysates were stored at -20°C. For qualitative examination of hydrolysis products by paper chromatography, aliquots of neutral hydrolysate (1 ml) were further concentrated by evaporation to dryness in vacuo over KOH followed by dissolution in a minimal volume of distilled water.

4. Acid hydrolysis of intact cell walls or trichloroacetic acid (TCA) extracts of cells prior to paper chromatography

Intact cell walls (10 - 15 mg) or TCA extracts (5 - 10 mg) of whole cells were hydrolysed in 2 M HCl (2 ml) at 105°C for 3 h in vacuo. Hydrolysates were evaporated to dryness in vacuo over KOH then resuspended in a minimal volume of distilled water.

5. Alkaline hydrolysis of cell walls

Cell walls (20 - 25 mg) were hydrolysed for 1 h in 0.1 M NaOH (20 ml) under nitrogen at 100°C (Owen & Salton, 1975). Insoluble material was removed by centrifugation at 1100 x g for 5 min and the hydrolysate made 0.5 M with respect to HCl then extracted four times with diethyl ether (100 ml). The ether extract was evaporated to dryness in vacuo and finally redissolved in a minimal volume of distilled water.

6. Lipid extraction

All solvents were distilled prior to use and stored over anhydrous sodium sulphate or calcium chloride.

Freeze-dried cells of Sacch. cerevisiae NCYC 366 were extracted according to the method of Hunter & Rose (1972) (Fig 8). The combined lipid extracts were evaporated to dryness on a Buchler Rotary-Evapomix. Non-lipid contaminants were removed in the following manner. The extract was redissolved in a small aliquot of chloroform-methanol (2:1, v/v), transferred to a conical centrifuge tube and the volume adjusted to 5 ml with washings from the rotary evaporator flask. KCl solution (0.88%, w/v; 1 ml) was added and the phases thoroughly mixed. Separation of the phases was achieved by centrifugation at 1100 x g for 5 min. The upper phase was removed and discarded and the surface of the lower phase washed three times with 1.5 ml of the upper phase of a chloroform-methanol-0.88% (w/v) KCl solution (8:4:3, v/v/v). The purified lipid extract was again evaporated to dryness and stored at -20°C under nitrogen.

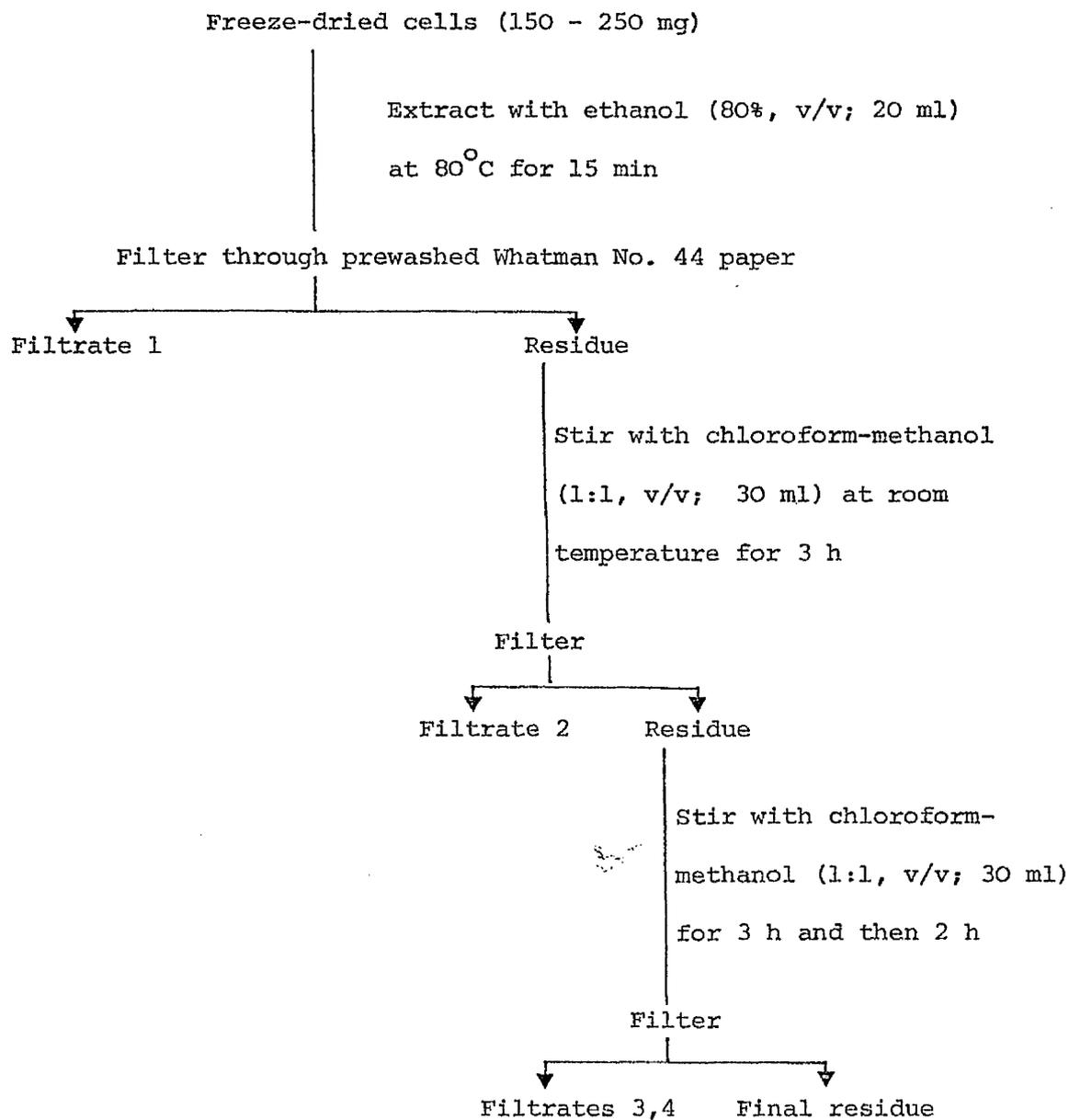
7. Thin layer chromatography

Scrupulously clean glass plates (20 cm x 20 cm) were coated with a layer (0.4 mm thick) of Kieselgel PF 254 + 366. The plates were prepared using a Shandon Unoplan Leveller model SA (Shandon Scientific Co. Ltd., Willesden, London).

Separation of the non-polar lipids was accomplished by developing the plates in petroleum spirit (BP 40° - 60°C)-diethyl ether-acetic acid (70:30:2, v/v/v). The different lipid classes were located by fluorescence at 254 and 366 nm using a UVSL-58 Mineralight multiband U.V. lamp (Ultraviolet Products Inc., San Gabriel, California). Bands

Figure 8

Lipid extraction schedule (Hunter & Rose, 1972)



Filtrates are combined and stored at -20°C

corresponding to the sterol ester and triacylglycerol fractions were scraped off together, eluted from the silica gel with three aliquots of chloroform-methanol (4:1, v/v; 5 ml) and rechromatographed in petroleum spirit (BP 40° - 60°C)-diethyl ether-acetic acid (90:10:1, v/v/v). Both solvent systems contained butylated hydroxytoluene (0.1%, w/v).

Acylglycerols were eluted from the gel with two portions of chloroform (3 ml) followed by two portions of diethyl ether (3 ml). Sterols and sterol esters were eluted with three portions of chloroform-methanol (4:1, v/v) and fatty acids with two portions (3 ml) of diethyl ether-methanol (9:1, v/v) followed by two portions of chloroform (3 ml).

Individual phospholipids were separated by chromatography in a two dimensional system. Glass plates were coated with a layer (0.4 mm thick) of Kieselgel PF 254, as previously described, prewashed overnight in chloroform and then activated by heating at 110°C for 1 h and allowed to cool for 30 min in a desiccator.

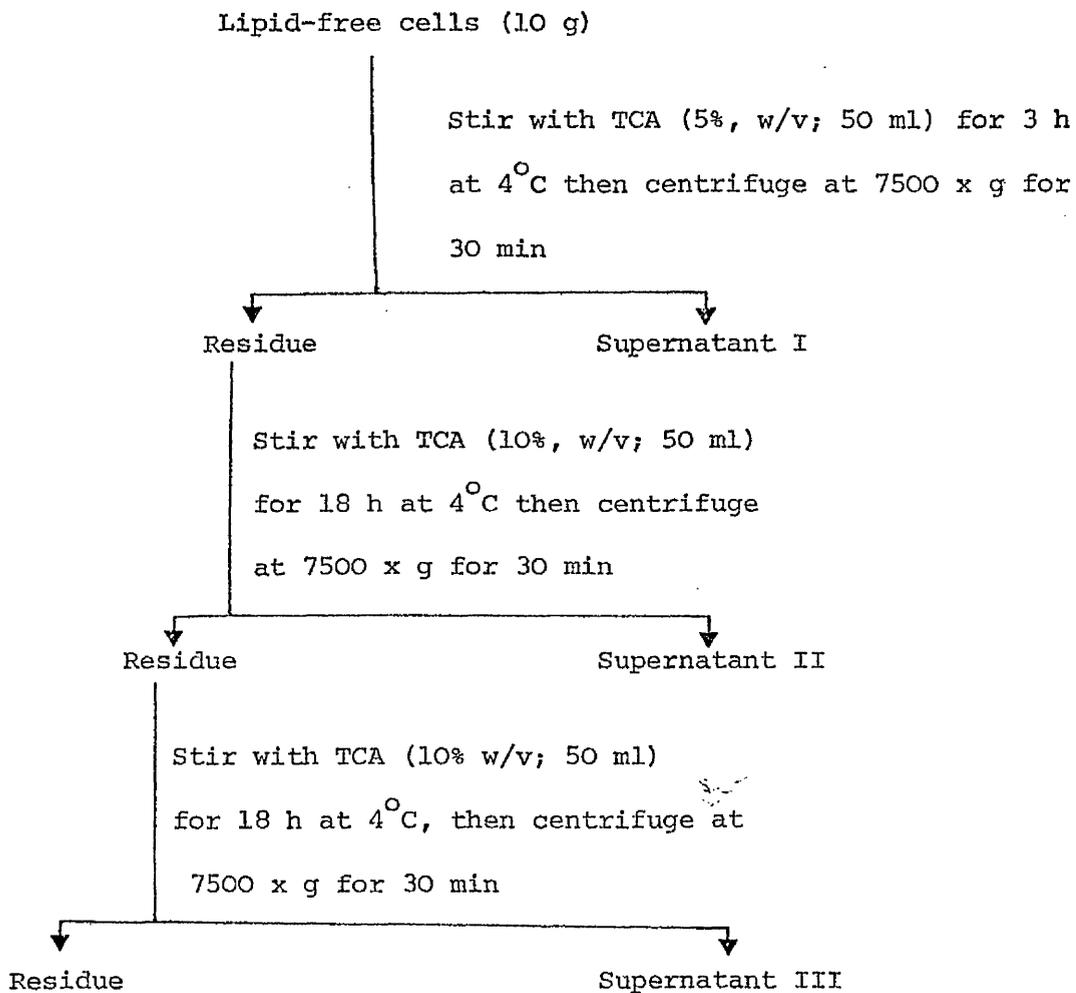
The plates were developed in the first direction in chloroform-methanol-ammonia (0.88 sp.gr.) (65:42.5:6.5, v/v/v) and in the second direction in chloroform-acetone-methanol-acetic acid-water (50:20:10:10:4, v/v/v/v/v). Phospholipids were detected by exposing the plates to iodine vapour.

8. Treatment of cells prior to trichloroacetic acid extraction

All cell crops (50 g dry weight) which were to be examined for teichoic acid were first extracted with chloroform-methanol (2:1, v/v; 2.5 l) for 18 h at 4°C. The defatted cells were recovered by filtration of the suspension through a sintered glass filter then washed extensively with large volumes (1 - 2 l) of chloroform-methanol (2:1, v/v).

Figure 9

Trichloroacetic Acid Extraction of Yeast (Armstrong et al., 1958)



9. Extraction of cells with trichloroacetic acid

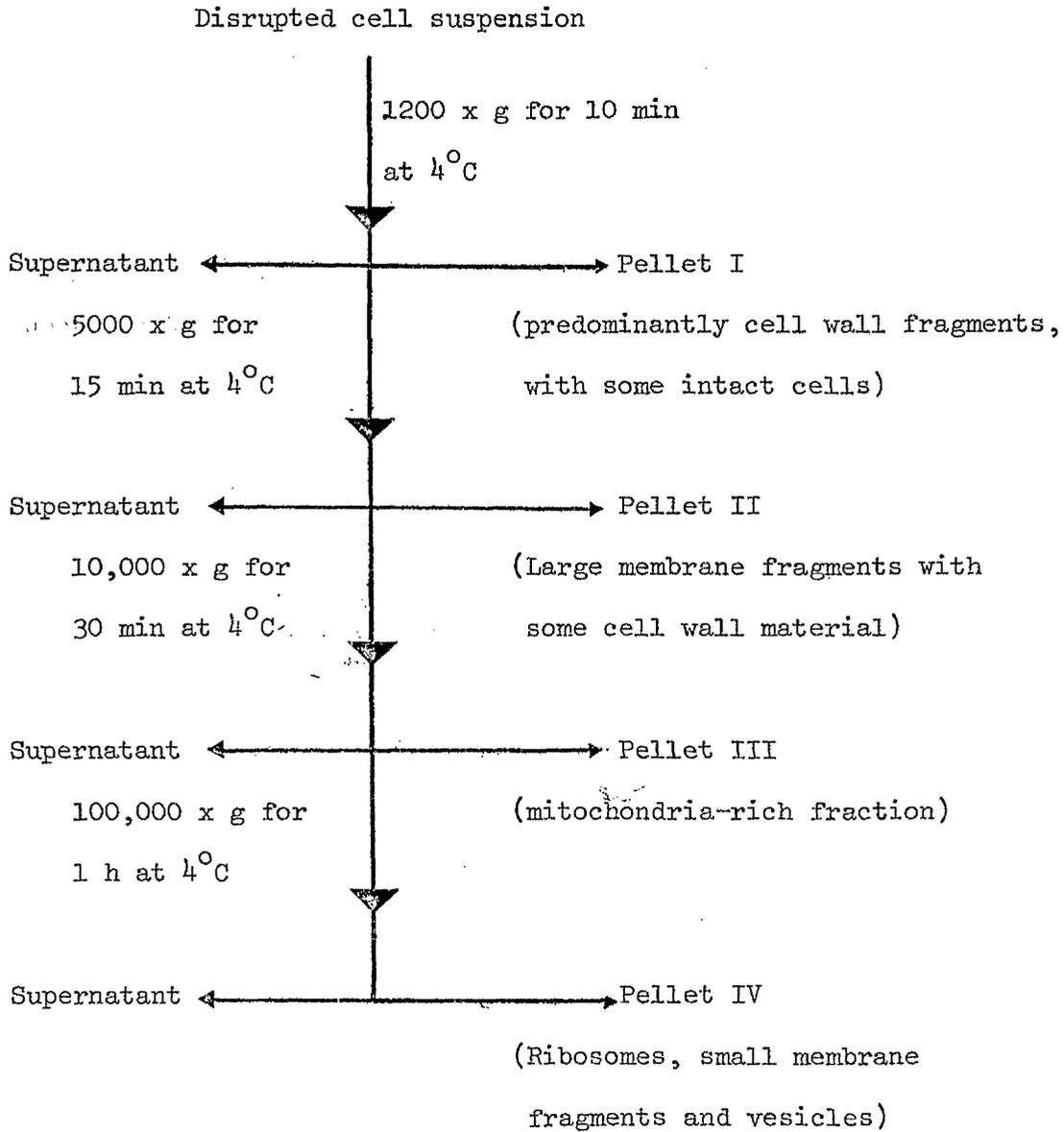
Lipid-free cells were extracted according to the schedule of Armstrong *et al.* (1958) as outlined in Fig 9. To supernatants I, II and III were added 2 volumes of cold absolute ethanol. The resulting precipitates were recovered by centrifugation at 12,000 x g for 1 h, then redissolved in a minimal volume of water. Cold, absolute ethanol (2 volumes) was added. Precipitates were recovered by centrifugation at 23,000 x g for 1 h then washed with ethanol, acetone, diethyl ether and finally dried in vacuo over solid KOH. The material precipitated in this way was designated extract I, II or III corresponding to supernatants I, II or III respectively.

10. Preparation of sub-cellular fractions of *C. intermedia*

Cells (10 g, wet weight), suspended in 0.1 M phosphate buffer pH 6.5, containing 0.25 M sucrose (10 ml), were disrupted by shaking with No. 10 Ballotini beads at a speed of 4000 rpm for 80 s in a Braun MSK homogeniser. Cooling was achieved using a flow of vapourising CO₂. The glass beads were removed from the disrupted cell suspension by filtration through a sintered glass filter (porosity grade 0).

Various sub-cellular fractions were prepared by differential centrifugation as outlined in Fig 10, a scheme adapted from that of Rose (1976). Pellet II was washed three times in 0.1 M tris-HCl buffer pH 7.4, containing 10 mM MgCl₂ and 1 mM EDTA. Pellet IV was washed once in this buffer. Pellet III, the fraction rich in mitochondria, was washed twice in 0.1 M phosphate buffer pH 6.5 containing 0.25 M sucrose then once in tris buffer. Each fraction was resuspended in tris buffer (2 ml) and stored at -20°C.

Figure 10 Preparation of sub-cellular fractions of C. intermedia



11. Gel-filtration of a trichloroacetic acid (TCA) extract of *C. utilis*

TCA extract II of *C. utilis* (200 mg) was dissolved in eluting buffer (2 ml) and centrifuged at 2000 x g for 5 min to remove any traces of insoluble, particulate material which remained. It was then applied to a column (57 x 2.6 cm) of Sephadex G-100 and eluted with an upward flow of 0.05 M tris-HCl buffer, pH 7.4, containing 0.1 M KCl and 0.02% (w/v) NaN_3 , at a rate of 20 ml h^{-1} . Fractions (2.3 ml) were collected using an automatic fraction collector (LKB Instruments Ltd., South Croydon, Surrey).

C. ANALYTICAL METHODS

Prior to analysis all cell-wall suspensions were routinely subjected to ultrasonic treatment for at least 1 min in a sonic water bath (Millipore Corp. Ltd.). This procedure resulted in the production of homogeneous suspensions and so removed a possible source of error in the subsequent assays.

1. Paper chromatography

Descending chromatography was carried out on Whatman No. 1 paper (unless stated otherwise) in the following solvent systems:

A: butan-1-ol - pyridine - water (6:4:3, v/v/v; Jeanes, Wise & Dimler, 1951)

B: ethyl acetate - pyridine - acetic acid - water (5:5:1:3, v/v/v/v;
Fischer & Nebel, 1955)

C: upper phase of butan-1-ol - formic acid - water (4:1:5, v/v/v;
Owen & Salton, 1975) in the ascending direction

D: propan-1-ol - aqueous ammonia (sp. gr. 0.88) - water (6:3:1, v/v/v;
Hanes & Isherwood, 1949)

E: ethanol - M - ammonium acetate, pH 3.8 (5:2, v/v; Paladini & Leloir,
1951) on Whatman No. 3MM paper.

Solvents A and B were used for the separation of sugars, solvent C for the separation of non-volatile acids and solvent D for the separation of polyols and phosphate esters. Solvent E was used to isolate polymeric product in the assay for poly (glycerol phosphate) synthetase.

Hydrolysis products were identified by co-chromatography with authentic standards and the use of the following specific spray and dip reagents:

- 1) alkaline silver nitrate for sugars and polyols (Trevelyan, Proctor & Harrison, 1950)
- 2) periodate - Schiff reagent for polyols (Baddiley et al., 1956)
- 3) perchloric acid - molybdate for phosphate esters (Hanes & Isherwood, 1949)
- 4) aniline - diphenylamine for neutral sugars and uronic acids (Davenport, 1969)
- 5) Bromophenol blue for non-volatile acids (Kennedy & Barker, 1951)
- 6) Bromocresol green - bromophenol blue - permanganate for non-volatile acids (Paskova & Munk, 1960).

2. Estimation of total carbohydrate

The total carbohydrate content of cell walls, wall hydrolysates and TCA extracts was determined spectrophotometrically using the phenol-sulphuric acid method of Dubois et al. (1956). Glucose was used as a standard.

3. Enzymic determination of glucose and mannose

The glucose and mannose contents of cell-wall hydrolysates

were determined enzymically. Glucose was determined using glucose oxidase blood-sugar kit (Boehringer Corp., Lewes, Sussex) while mannose was determined by the method of Gawehn (1974). Each method is absolutely specific for its respective substrate, glucose or mannose.

4. Estimation of phosphorus

The phosphorus content of cell-wall suspensions, lipid extracts, isolated phospholipids and cell-free culture filtrates was determined by a modification (Letters, 1964) of the micromethods of Allen (1940) and Bartlett (1959). Potassium orthophosphate was used as a standard. The phosphorus content of column fractions was determined by the method of Chen, Toribara & Warner (1956).

5. Estimation of glucosamine

The glucosamine content of cell-wall hydrolysates was determined by the method of Rondle & Morgan (1955). Glucosamine was used as a standard.

6. Estimation of protein

Protein (as bovine serum albumin) was determined by the method of Lowry et al. (1951).

7. Total lipid estimations

Lipid extracts were redissolved in a small aliquot of chloroform-methanol (2:1, v/v; 5 ml) and transferred to a tared flask which had been heated at 140°C for 30 min then allowed to cool over silica gel and KOH before weighing. The solvent was evaporated using a stream of

nitrogen gas and the flask returned to the desiccator. The lipid was dried to a constant weight \pm 0.05 mg over silica gel.

8. Phospholipid estimation

Total phospholipids were determined by assaying the phosphorus content of the lipid extract as previously described. Phosphorus contents were multiplied by a factor of 25 to give a value for the total phospholipid content.

Individual phospholipids, isolated by thin layer chromatography, were assayed while still adhering to the silica gel. A blank area of silica gel was incorporated into the reagent blank.

9. Estimation of the free sterol content of lipid extracts

A method adapted from that of Jatzkewitz & Mehl (1960) was used to assay free sterols in lipid extracts. Aliquots of sterol solution, containing up to 0.1 mg sterol, were evaporated to dryness using a stream of nitrogen. A mixture of sulphuric acid (sp. gr. 1.84) and glacial acetic acid (1:1, v/v; 2 ml) was added to each tube. The tubes were then placed in a water bath at 90°C for 15 min. After cooling, the extinction at 390 nm was determined spectrophotometrically against a reagent blank. Ergosterol was employed as a standard.

10. Estimation of the sterol ester content of lipid extracts

Sterol esters were assayed by the amount of free sterol liberated upon saponification (Hunter & Rose, 1972). Isolated sterol esters were evaporated to dryness in vacuo then redissolved in benzene (0.1 ml) and supplemented with ethanolic potassium hydroxide solution (10% (w/v) KOH in 90% (v/v) ethanol; 0.4 ml). A cold-finger condenser

was placed in each tube and the contents refluxed for 2 h at 100°C. Each hydrolysate was evaporated to dryness and the liberated sterol fraction isolated by thin layer chromatography in petroleum spirit (BP 40° - 60°C) - diethyl ether-acetic acid (70:30:2, v/v/v). The sterols were then eluted with three portions of chloroform-methanol (4:1, v/v; 3 ml) and assayed as described above.

11. Estimation of the acylglycerol content of lipid extracts

Acylglycerols were assayed using a chromotropic acid method (Van Handel & Zilversmit, 1957). Contents of triacyl-, diacyl- and monoacylglycerols (as mg tri-, di- and monopalmitin) were calculated from standard curves prepared using tripalmitin.

12. Estimation of the free fatty acid content of lipid extracts

Free fatty acids were assayed according to the method of Heinen & De Vries (1966), using palmitic acid as a standard.

13. Gas-liquid chromatography of fatty acids

Aliquots of lipid extract, containing 1 - 2 mg lipid, were evaporated to dryness in vacuo redissolved in benzene (0.1 ml) and saponified by refluxing in ethanolic potassium hydroxide (10% (w/v) KOH in 90% (v/v) ethanol; 0.4 ml) for 2 h on a steam bath. The fatty acids were subsequently isolated by thin layer chromatography in petroleum spirit (BP 40° - 60°C) - diethyl ether-acetic acid (70:30:2, v/v/v) then eluted from the gel as previously described.

Fatty acids were analysed using a Perkin Elmer F17 gas chromatograph with a flame ionisation detector. The fatty acids were

separated on a stainless steel column (1000 mm x 3.2 mm outside diameter) packed with 5% FFAP on Chromosorb G AW DMCS. The temperature of the column was programmed to increase by 4°C per min from 160°C to 230°C; thereafter the column was isothermal. The injection temperature was 275°C and the nitrogen carrier gas flow rate was 40 ml min⁻¹.

All samples were injected on to the column in 1 µl of chloroform. Peak areas were measured by triangulation (height of peak x width of peak at half the height).

14. Estimation of glycerol

Glycerol in acid hydrolysates of TCA extracts from C. intermedia was determined colourimetrically using a chromotropic acid method (Hanahan & Olley, 1958) following enzymic dephosphorylation of the hydrolysates. Dephosphorylation was achieved by incubation of hydrolysates (5 ml) with a solution of alkaline phosphatase in 0.1 M ammonium carbonate, pH 9.3 (2 mg/ml; 1 ml) for 24 h at 37°C under toluene.

15. Assay for poly (glycerol phosphate) synthetase activity

Incubation mixtures contained MgCl₂ (2 µmole), CDP [¹⁴C]-glycerol (1.4 nmole; 3.12 x 10⁴ cpm) and crude enzyme suspension (200 µl) in a total volume of 0.22 ml. Following incubation at 37°C for 1 h, reactions were terminated by boiling for 1.5 min. Zero-time mixtures contained crude enzyme suspension which had been boiled for 2 min.

Mixtures were applied as bands to Whatman No. 3MM paper and subjected to descending chromatography in solvent E for 18 h. Poly (glycerol phosphate) remains at the origin in this solvent. After

thorough drying of the paper, a strip (4 cm x 1 cm) corresponding to the origin of each track was cut out and counted in 5 ml NE 260 scintillant (Nuclear Enterprises Ltd., Edinburgh) in an automatic scintillation spectrometer (Model 8312, Nuclear Enterprises Ltd.).

16. Statistical analyses

Results were expressed as: sample mean \pm standard error of the mean. The 95% confidence limit was also calculated. The significance of differences in sample means was determined by the t-test and the level of probability (P) at which the null hypothesis be rejected was noted.

The reliability of the t-test depends on the standard deviations of the means being similar. The variance ratio test was employed to check the equality of these standard deviations. If, by the variance ratio test, the standard deviations were unacceptably dissimilar, then the result of the t-test was discarded.

D. CHEMICALS

Basidiomycete QM806 β -glucanase was kindly provided by Professor A.H. Rose, University of Bath and CDP ^{14}C -glycerol was a gift from Dr. L.J. Douglas. α -Mannosidase and the glucose oxidase blood sugar kit were obtained from The Boehringer Corporation Ltd. (London); fatty acids and lipid standards were from Sigma Chemical Co. Ltd. (London); Sephadex G-100 was from Pharmacia (Uppsala, Sweden); yeast extract was from Difco Laboratories (Detroit, Michigan, U.S.A.); silica gel was from Merck (Darmstadt, Germany). All other biochemicals and enzymes were purchased from Sigma Chemical Co. Ltd. (London). Solvents and other

chemicals were of analytical grade where available and were obtained from BDH Chemicals Ltd. (Poole, Dorset).

E. GLASSWARE

All glassware used in analytical determinations was cleaned by steeping in either chromic acid or a 2% (v/v) solution of detergent. Cleaning fluid was removed by thorough rinsing in tap water followed by two rinses in distilled water prior to drying in an oven at 105°C.

RESULTS

8

SECTION I THE EFFECT OF PHOSPHATE LIMITATION OF GROWTH ON THE CELL
WALL AND LIPID COMPOSITION OF Saccharomyces cerevisiae
NCYC 366

A. GROWTH OF Sacch. cerevisiae

1. Formulation of a chemically defined medium for the growth of
Sacch. cerevisiae

The defined medium of Rose & Nickerson (1956) which supports only scant growth of Sacch. cerevisiae NCYC 366 was used as a basis for further studies. Variation of the concentration of vitamins (Table 6) and/or supplementation of the medium with inositol had no marked effect on the growth of this strain of Sacch. cerevisiae. However, growth was greatly enhanced by the addition of asparagine (Tables 6 and 7). Basal medium supplemented with asparagine at a final concentration of 1 mg ml^{-1} was used for all subsequent experiments.

2. Effect of varying the concentration of KH_2PO_4 on the growth of
Sacch. cerevisiae

Preliminary experiments showed that lowering the concentration of KH_2PO_4 from 20 mM to 1 mM had little or no effect on the growth of this organism. Consequently media were prepared which contained a range of concentrations of KH_2PO_4 from 0.0 to 1.0 mM in uniform increments of 0.1 mM KH_2PO_4 . The results of a growth experiment using these media are shown in Table 8. The availability of phosphate in the medium appeared to limit the growth of the organism at a KH_2PO_4 concentration of around 0.8 mM

Table 6

The effect of different concentrations of vitamins,
inositol and asparagine on the growth of Sacch. cerevisiae

Concentration of vitamins	Concentration of inositol (mg ml ⁻¹)	Concentration of asparagine (mg ml ⁻¹)	Growth after 72 h
DS ¹	0.02	0	-
DS	1.02	0	-
DS	0.02	0.4	+++
DS	1.02	0.4	+++
TS ²	0.03	0	-
TS	1.03	0	-
TS	0.03	0.4	+++
TS	1.03	0.4	+++

¹DS = Double strength.

²TS = Triple strength.

Medium (50 ml) was inoculated with 0.1 ml of a washed cell suspension
(E₁⁶⁵⁰ = 0.12 - 0.15) prepared from an overnight culture.

Growth was assessed visually as follows: -, no growth; +/-, slight
growth; +, moderate growth; ++, heavy growth; +++, very heavy growth.

Table 7

The effect of different concentrations of vitamins
and asparagine on the growth of Sacch. cerevisiae

Concentration of vitamins	Concentration of asparagine (mg ml ⁻¹)	Growth after	
		48 h	90 h
DS ¹	1.0	++	+++
DS	0.5	+	+++
DS	0.25	+/-	++
DS	0.125	+/-	+
SS ²	1.0	++	+++
SS	0.5	+	+++
SS	0.25	+/-	++
SS	0.125	+/-	+

¹DS = Double strength;

²SS = Single strength.

The inoculum was prepared and growth was assessed as described in
Table 6 .

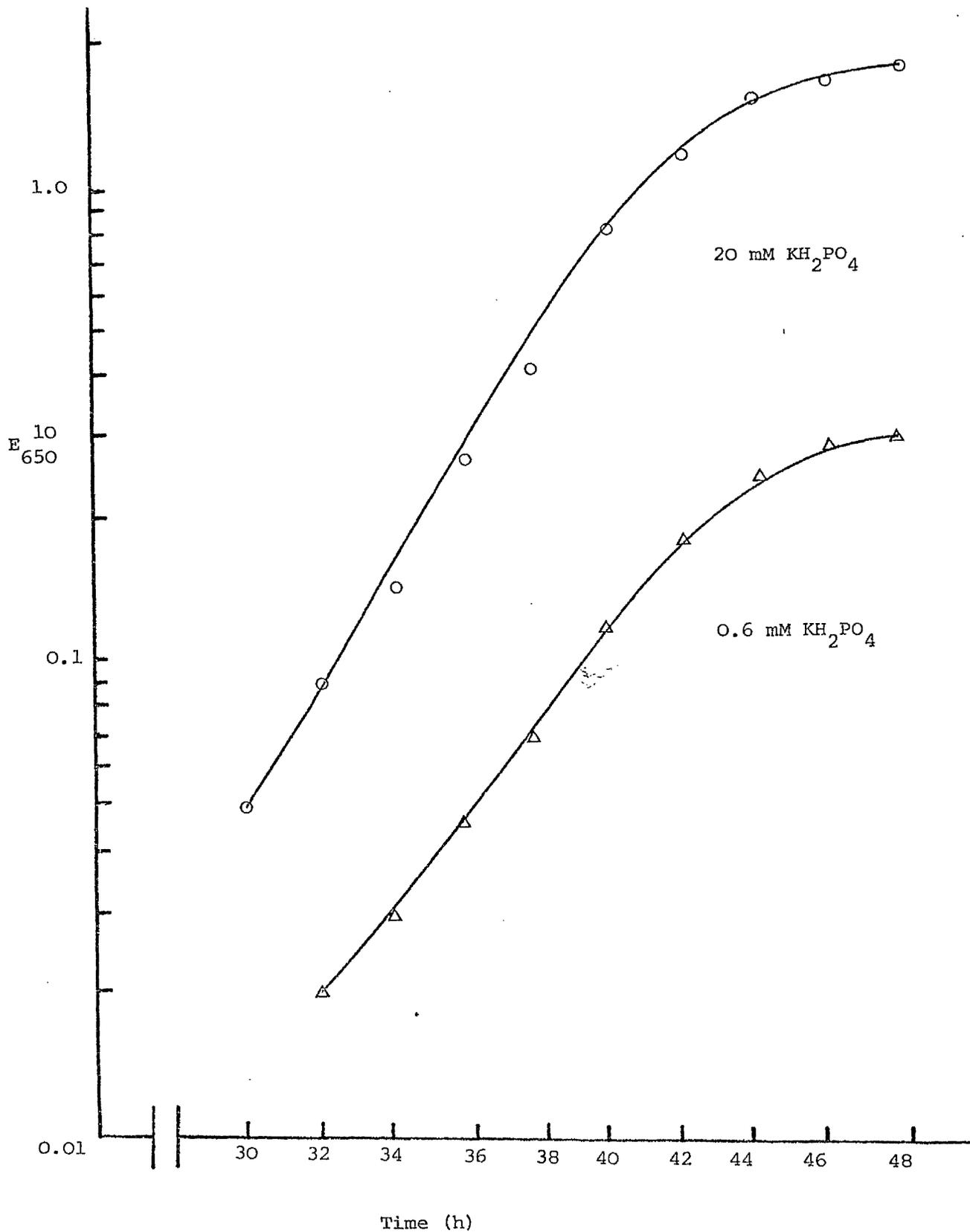
Table 8

The effect of different concentrations of KH_2PO_4
on the growth of Sacch. cerevisiae

Concentration of KH_2PO_4 (mM)	Growth after 40 h
20	+++
1.0	+++
0.9	+++
0.8	++
0.7	++
0.6	++
0.5	+
0.4	+
0.3	+/-
0.2	-
0.1	-
0	-

The inoculum was prepared and growth was assessed as described
in Table 6 .

Figure 11 Time-course of growth of *Sacch. cerevisiae* in medium containing 20 mM KH_2PO_4 (O) or 0.6 mM KH_2PO_4 (Δ). Triplicate cultures were inoculated with a washed cell suspension (0.1 ml; $E_{650}^{1\text{ cm}} = 0.2$) prepared from a mid-exponential phase culture grown at the low concentration of phosphate.



and growth was severely limited at 0.5 mM KH_2PO_4 . From these results, 0.6 mM KH_2PO_4 was selected as a growth-limiting concentration of phosphate suitable for further study.

3. Measurement of growth rate of *Sacch. cerevisiae* in media containing either 0.6 mM KH_2PO_4 (growth-limiting concentration) or 20 mM KH_2PO_4 (optimum concentration)

Growth curves for cultures in media containing 20 mM KH_2PO_4 and 0.6 mM KH_2PO_4 are shown in Fig 11. At the lower concentration of KH_2PO_4 there was a marked decrease both in exponential growth rate and in cell yield. The growth rates were 0.26 h^{-1} and 0.23 h^{-1} at 20 mM KH_2PO_4 and 0.6 mM KH_2PO_4 respectively.

4. The effect of a decreased concentration of K^+ on the growth rate of *Sacch. cerevisiae*

In order to determine whether the decreased growth rate at 0.6 mM KH_2PO_4 was a result of phosphate limitation or potassium limitation of growth, the concentrations of orthophosphate anion and potassium cation were varied independently by substituting NaH_2PO_4 and KCl for KH_2PO_4 in the growth medium. The results (Table 9) show that when the potassium ion concentration was lowered from 20 mM to 0.6 mM, the growth rate in media containing either 20 mM or 0.6 mM phosphate was unaffected. Thus, when KH_2PO_4 is used in the medium at a concentration of 0.6 mM, growth is limited solely by the availability of the orthophosphate anion.

5. Production of *Sacch. cerevisiae* NCYC 366 for analysis of the effects of phosphate limitation

Control cells (yeast grown at a KH_2PO_4 concentration of 20 mM)

Table 9

The effects of high and low concentrations of potassium and orthophosphate in the culture medium on the growth rate of Sacch. cerevisiae

Concentration of K^+ (mM)	Concentration of $H_2PO_4^-$ (mM)	Growth rate (h^{-1})
20	20	0.262 ± 0.003 (0.013)
0.6	20	0.266 ± 0.007 (0.030)
20	0.6	0.227 ± 0.003 (0.015)
0.6	0.6	0.230 ± 0.003 (0.015)

Triplicate cultures containing K^+ and $H_2PO_4^-$ at the concentrations shown were inoculated as described in the legend to Fig 11.

Results are expressed as mean \pm standard error of the mean.

95% confidence limits are shown in parentheses.

and some of the phosphate-limited ($0.6 \text{ mM KH}_2\text{PO}_4$) cells were grown batch-wise in a 15 l fermenter (see Materials & Methods, section A.3.iii for details). The growth rates under these conditions were calculated to be 0.34 and 0.28 h^{-1} respectively.

The remainder of the phosphate-limited cells were produced using a chemostat of 2 l working volume. The dilution rate was adjusted such that the growth rate was numerically equal to 0.23 h^{-1} . A typical growth profile showing three of the five parameters monitored daily is depicted in Fig 12. In the steady state, the culture had an absorbance ($E_{1\text{cm}}^{650}$) of 0.30 and a pH value of 3.5 . The phosphorus content of cell-free culture filtrates was $17 \mu\text{g ml}^{-1}$ as compared with an initial concentration of $22 \mu\text{g ml}^{-1}$ in the growth medium.

6. Total phosphorus content of whole cells

Aqueous cell suspensions (1 mg ml^{-1}) were prepared using lyophilised samples of control cells, batch-grown, phosphate-limited cells and chemostat-grown, phosphate-limited cells. Aliquots (0.2 ml) of each suspension were then used for the determination of total phosphorus by the method of Chen et al. (1956). The results (Table 10) show that the phosphorus contents of both types of phosphate-limited cells were similar and markedly lower than that of control cells.

B. CELL WALL ANALYSIS

1. Qualitative analysis of cell-wall preparations

Cell-wall preparations from control cells, batch-grown, phosphate-limited cells and chemostat-grown, phosphate-limited cells

Figure 12

Growth profile of phosphate-limited Sacch. cerevisiae
grown in a chemostat

- Phosphorus content of cell-free
culture filtrates ($\mu\text{g phosphorus ml}^{-1}$)
- ▲—▲ pH value of culture
- Absorbance ($E_{1\text{cm}}^{650}$) of culture

The arrows indicate times at which the chemostat vessel
was drained in order to harvest steady-state cells.

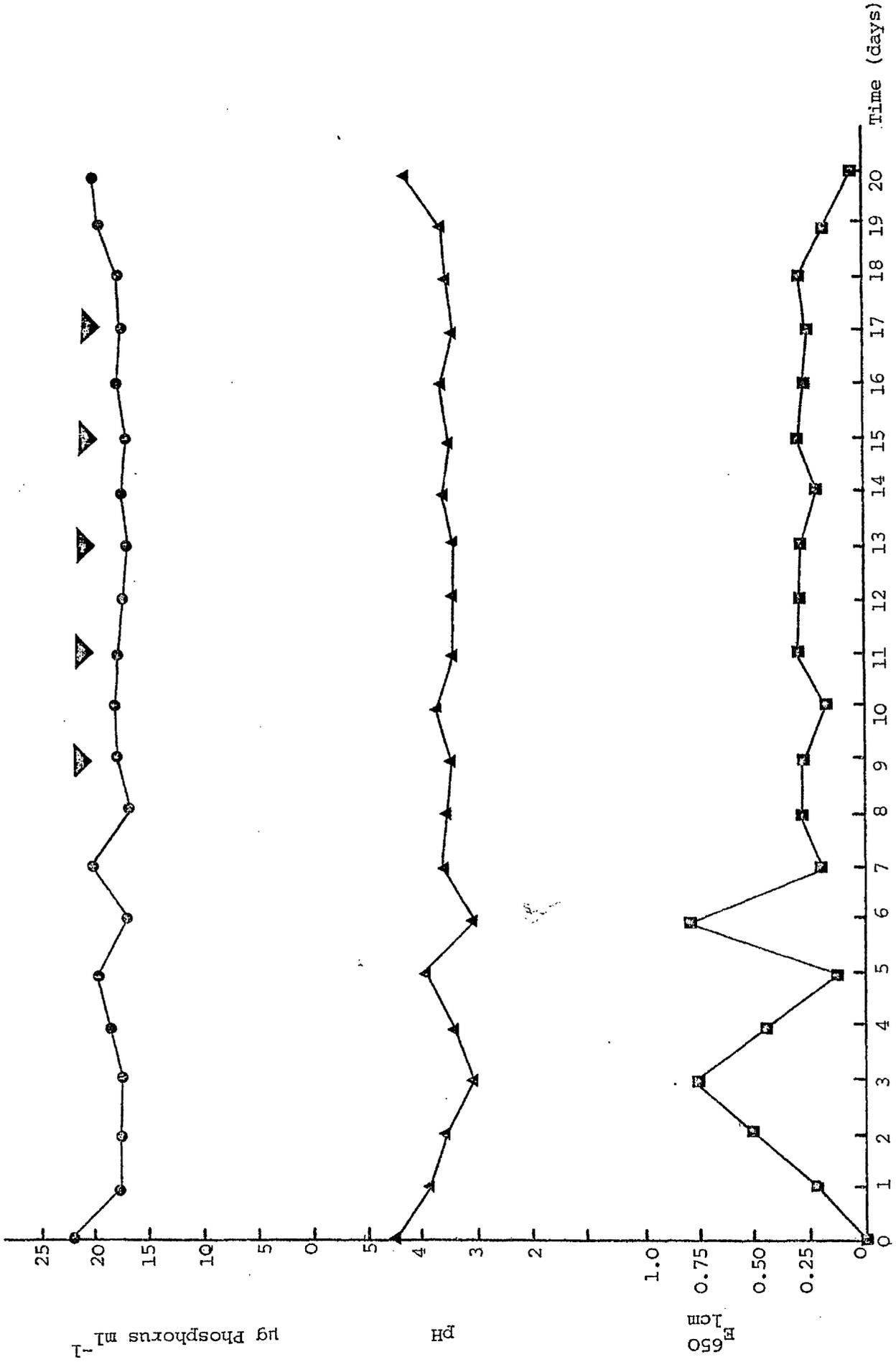


Table 10

Total phosphorus content of whole cells

<u>Cell preparation</u>	<u>Total phosphorus content</u> (mg/100 mg dry yeast)
Control	3.29 ± 0.08 (0.18)
Batch-grown, phosphate-limited	2.58 ± 0.08 (0.18)
Chemostat-grown, phosphate-limited	2.34 ± 0.14 (0.32)

Values quoted are means ± the standard error of the mean.

95% confidence limits are shown in parentheses.

(designated wall preparations A, B and C respectively) were hydrolysed in 2 M HCl at 105°C for 3 h in vacuo. Paper chromatography of the hydrolysis products in solvents A or B, followed by staining with the silver nitrate reagents revealed glucose, mannose, glucosamine and a white "salt" spot in all three hydrolysates. No evidence was obtained for the presence of uronic acid in any of the hydrolysates following chromatography in either solvent and staining with the aniline-diphenylamine reagent.

Treatment of the three wall preparations with β -glucanase and α -mannosidase, followed by acid hydrolysis under rather milder conditions, gave the products shown in Table 11. Glucose and mannose were again major hydrolysis products, together with smaller amounts of glucosamine. N-acetylglucosamine was also identified as a product of this combined enzymic and acid hydrolysis of wall preparations B and C, but could be detected only in trace amounts in the hydrolysate from preparation A.

The white "salt" spot revealed by the silver nitrate reagents following hydrolysis of walls in 2 M HCl was also present after the combined enzyme and acid treatment. Furthermore, the intensity of the spots obtained after staining indicated that this component might be present in rather greater amounts in the hydrolysates of wall preparations B and C than in that from preparation A. This hydrolysis product, which had an R glucose value of 0.86 in solvent A, also stained with a bromophenol blue spray and a complex spray reagent (Paskova & Munk, 1961) both of which are specific for non-volatile acids. Following spraying with the complex reagent this component appeared as a pink spot with a purple edge on a grey-brown background. The spray reagent of Paskova & Munk (1961) also revealed an additional component (R glucose = 0.60) in each hydrolysate

which appeared as a green spot within one minute of spraying. Hexoses and hexosamines are not visualised with this stain. Succinic acid, which was used as a reference compound, ran with the solvent front.

An attempt was made to isolate the acidic cell-wall component by alkaline hydrolysis and ether extraction (Owen & Salton, 1975). Ascending chromatography in solvent C of the ether extracts and unextracted hydrolysates of all three wall preparations, A, B and C, revealed the same pattern of components in each case. Resolution of the individual components was not good but three products could be clearly distinguished. The fastest running component ($R_F = 0.35$) stained pink in colour and appeared very rapidly during spraying. A second component ($R_F = 0.20$) appeared more slowly (about 1 min after spraying) and was green. Material remaining at the origin of the chromatogram stained pink and appeared rapidly during spraying. Succinic acid, palmitic acid and citric acid were co-chromatographed with the hydrolysates. All three standards stained pink although the fatty acid was much slower to appear than either succinic or citric acids. Succinic and palmitic acids chromatographed almost together ($R_F = 0.85 - 0.90$) while citric acid ran more slowly ($R_F = 0.55$). Chromatography of the alkaline hydrolysate in solvent A followed by staining with the silver nitrate reagents demonstrated that only a small amount of polysaccharide had been hydrolysed to monomeric constituents.

2. Quantitative chemical analysis of cell-wall preparations

The carbohydrate, protein and phosphorus contents of cell-wall preparations A, B and C are shown in Table 12. Walls prepared from

Table 11

Products of mild acid hydrolysis of cell walls prepared from Sacch. cerevisiae grown under conditions of phosphate limitation

<u>Component</u>	<u>Cell Wall Preparation</u> ¹		
	A	B	C
Glucose	+++	+++	+++
Mannose	+++	+++	+++
Glucosamine	+	+	+
N acetyl glucosamine	+/-	+	++
Salt spot (R _{glc} =0.86)	+/-	+	+
Slow-running component 1 (R _{glc} =0.64)	+	+	+
Slow-running component 2 (R _{glc} =0.46)	+	+	+

Following treatment with β -glucanase and α -mannosidase, cell-wall preparations were hydrolysed in 1 M HCl at 105°C for 2 - 4 h in vacuo. Hydrolysis products were separated by paper chromatography in solvent A and visualised using the silver nitrate reagents.

¹Cell wall preparations A, B and C are as explained in the text.

chemostat-grown, phosphate-limited cells (preparation C) contained markedly less total carbohydrate than either of the other two wall preparations. Perhaps surprisingly, walls from batch-limited cells contained the highest levels of carbohydrate. Statistical analysis showed that the differences in carbohydrate content between walls from chemostat-grown cells and walls from either control cells or batch-limited cells were highly significant ($P = 0.001$). Variation in glucosamine content, on the other hand, was very slight.

It would appear that the protein content of the cell wall may be complementary to the carbohydrate content. Walls prepared from the chemostat-grown cells contained the highest amount of protein (18.28%) while walls from batch-limited cells contained only 12.6% of protein. These differences were also highly significant ($P = 0.001$).

It was surprising to find that the phosphorus content of walls from the chemostat-grown cells (0.70%) was higher than that of walls from batch-limited cells (0.52%). However, the phosphorus contents of walls from batch-limited cells and continuously-limited cells were both lower than that of walls prepared from control cells ($P = 0.001$ and 0.05 respectively).

3. Quantitative chemical analysis of cell-wall hydrolysates

Wall preparations A, B and C were subjected to hydrolysis in M HCl at 105°C in vacuo for time periods ranging from 2 to 4 h. The glucose and mannose contents of the hydrolysates were then determined by enzymic methods specific for the free sugar.

Table 12 Gross composition of cell walls prepared from Sacch. cerevisiae grown under different conditions of phosphate limitation

Component	Percentage composition (w/w) of cell-wall preparation		
	¹ A	² B	³ C
Carbohydrate	83.55 ± 1.28 (2.95)	90.12 ± 1.24 (2.87)	72.20 ± 1.06 (2.45)
Protein	14.80 ± 0.37 (0.85)	12.60 ± 0.40 (0.92)	18.28 ± 0.43 (0.98)
Phosphorus	0.75 ± 0.01 (0.05)	0.52 ± 0.01 (0.05)	0.70 ± 0.01 (0.05)
Glucosamine	1.91 ± 0.14 (1.80)	2.65 ± 0.30 (3.86)	1.73 ± 0.02 (0.27)

Values quoted are means ± the standard error of the mean

95% confidence values are shown in parentheses

¹ Walls prepared from control cells.

² Walls prepared from batch-grown, phosphate-limited cells.

³ Walls prepared from chemostat-grown, phosphate-limited cells.

The results of these determinations are shown in Table 13. It is clear that a summation of the glucose and mannose contents of the hydrolysates does not approach the figure corresponding to total carbohydrate released from the walls during hydrolysis. One possible explanation for this would be that although the bulk of the cell wall carbohydrate is released during the hydrolysis procedure, it is in a form which does not react in the enzymic assays for free glucose and mannose. However, subsequent treatment of neutralised hydrolysates with a β -glucanase from Basidiomycete QM 806 had, in most cases, little effect on the glucose content (Table 13).

An alternative procedure involved treatment of the cell-wall suspensions with β -glucanase and α -mannosidase prior to hydrolysis in 1 M HCl. The enzymic treatment solubilised a considerable proportion of each cell-wall preparation such that the absorbance ($E_{1\text{cm}}^{650}$) of the control, batch-grown limited and continuously-grown limited yeast wall suspensions fell by 47.2%, 27.9% and 23.3% respectively. The total carbohydrate, glucose and mannose contents of the hydrolysates were determined as in the previous procedure (Table 14). This time all of the cell-wall carbohydrate was released, although significantly, after different time periods for each cell-wall preparation. The carbohydrate of walls from chemostat-grown, limited cells was released most readily and that from control walls least readily. The recovery of carbohydrate as glucose + mannose was also greatly improved and ranged from 66 to 82% of the carbohydrate released from the wall. The mannose content of walls prepared from control and continuously-grown limited cells were about the same (24%) while batch-limited yeast walls contained more

Table 13 Amount of total carbohydrate, glucose and mannose released during acid hydrolysis of Sacch. cerevisiae cell wall

Cell-wall preparation ¹	Duration of hydrolysis (h)	% (w/w) of cell-wall carbohydrate released	% (w/w) of cell wall released as		
			Carbohydrate	Mannose	Glucose ²
A	2	83.7	69.9	9.1	9.5 (22.2)
	3	75.3	62.9	14.2	14.8 (15.0)
	4	71.2	59.5	14.8	16.1 (16.3)
B	2	52.4	47.3	13.9	8.5 (9.3)
	3	82.2	74.1	17.9	11.2 (11.3)
	4	75.3	67.9	22.3	12.9 (12.8)
C	2	72.8	52.6	9.5	7.2 (11.2)
	3	65.7	47.4	14.0	9.7 (11.1)
	4	66.8	48.3	17.3	10.3 (10.0)

¹Nomenclature of the cell-wall preparations is as described in the footnote to Table 12 .

²Figures in parentheses refer to the amount of glucose released from the cell wall following acid hydrolysis and subsequent treatment of the hydrolysate with β -glucanase.

mannose (36%). The glucose content of control yeast walls (45%) was considerably higher than that of walls from cells grown under either type of phosphate limitation (26 - 28%).

C. LIPID ANALYSES

1. Total lipid content

Control cells contained more total lipid than either continuously-grown, phosphate-limited cells or batch-grown, limited cells (Table 15). The total lipid content of the continuously-grown, limited cells was higher ($P = 0.05$) than the total lipid content of the batch-grown limited cells.

2. Phospholipid content

Continuously-grown, phosphate-limited cells contained surprisingly more phospholipid than either type of batch-grown yeast (Table 16). Batch-limited cells contained least phospholipid. The differences in phospholipid content between continuously-grown, limited yeast and either control cells or batch-limited cells were highly significant ($P = 0.01$ and 0.001 , respectively).

3. Lipid Composition

i. Neutral lipids

A complex series of changes in lipid composition were observed, those in neutral lipids being particularly striking (Table 16).

Table 14 Amount of total carbohydrate, glucose and mannose released from cell walls of Sacch. cerevisiae during acid hydrolysis subsequent to treatment with β -glucanase and α -mannosidase

Cell-wall preparation ¹	Duration of hydrolysis (h)	% of cell-wall carbohydrate released	Carbohydrate	Mannose	Glucose
A	2	81.1	67.8	18.3	38.6
	3	95.2	79.5	20.7	42.4
	4	102.5	85.6	24.2	45.2
B	2	68.9	62.1	26.8	18.4
	3	103.5	93.2	28.9	24.3
	4	88.2	79.5	36.1	26.1
C	2	99.1	71.5	17.6	27.0
	3	96.8	69.9	23.8	27.9
	4	99.1	71.5	20.0	26.1

¹Nomenclature of the cell-wall preparations is as described in footnote to Table 12.

Table 15

The effect of phosphate limitation on the total lipid
content of Sacch. cerevisiae

<u>Type of culture</u>	<u>Growth rate</u> (h ⁻¹)	<u>Total lipid content</u> (mg/100 mg dry yeast)
Batch, control	0.34	14.73 ± 1.025 (3.26)
Batch, limited	0.28	10.28 ± 0.90 (11.50)
Continuous, limited	0.23	13.30 ± 0.39 (1.22)

Values quoted are means ± standard error of the mean.

95% confidence limits are shown in parentheses.

Table 16.

The effect of phosphate limitation on the lipid composition of Sacch. cerevisiae

Limitation	Content (mg/100 mg dry yeast) of					
	Phospho- lipid	Triacyl- glycerol	Diacyl- glycerol	Monoacyl- glycerol	Free fatty acid	Sterol Ester
None (control)	3.90±0.21 (0.89)	0.96±0.06 (0.19)	0.34±0.07 (0.22)	0.17±0.04 (0.12)	0.07±0.02 (0.05)	0.70±0.09 (0.29)
Batch	3.17±0.57 (7.24)	0.68±0.08 (0.32)	0.30±0.06 (0.27)	0.10±0.05 (0.22)	0.06±0.03 (0.12)	0.96±0.14 (0.62)
Continuous	4.79±0.10 (0.22)	0.58±0.02 (0.06)	0.20±0.02 (0.05)	N.D.	0.82±0.08 (0.19)	0.57±0.06 (0.15)

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N.D., Not detected.

Values quoted are means ± the standard error of the mean.
95% confidence limits are shown in parentheses.

Yeast grown under phosphate limitation synthesised much less triacylglycerol than did control cells, with the continuously-grown cells producing least of all ($P = 0.001$). A similar pattern was observed with diacylglycerol and monoacylglycerol levels although the differences were not statistically significant. Monoacylglycerols were only detected in lipid extracts from batch-grown cells.

The sterol-ester content of phosphate-limited cells was also markedly lower than that of control cells (Table 16). Batch-grown and continuously-grown, limited cells contained only 52% and 33% respectively of the amount of sterol ester present in control cells ($P = 0.05$ and 0.001). Differences in free sterol content were less striking, although continuously-grown, limited cells contained less than batch-grown, limited cells ($P = 0.05$).

Curiously, the free fatty acid content of continuously-grown, limited cells was 12 to 13 times higher than that of either type of batch-grown yeast. The differences appeared to be significant ($P = 0.001$), although the variance ratio test (a test for the validity of the t-test) rejected the t-test on the mean values for free fatty acid levels in control and continuously-grown, limited cells.

ii. Phospholipids

The phospholipid composition of yeast subjected to phosphate limitation of growth is shown in Table 17. The major differences in continuously-grown, limited cells as compared with control cells were an increased synthesis of phosphatidylcholine and phosphatidylethanolamine

Table 17 The effects of phosphate limitation of growth on the phospholipid composition of Sacch. cerevisiae

Nature of limitation	Content (mg/100 mg dry wt of yeast) of						
	PC	PE	PI	PS	PA	DMPE	BS
None (i.e. control cells)	1.30±0.04 (0.17)	0.98±0.12 (0.52)	0.97±0.07 (0.30)	0.28±0.06 (0.25)	0.19±0.02 (0.10)	0.03±0.02 (0.10)	0.07±0.03 (0.11)
Batch limited	1.00±0.23 (2.97)	0.85±0.15 (1.89)	0.76±0.02 (0.27)	0.19±0.03 (0.36)	0.09±0.02 (0.27)	0.17±0.02 (0.27)	ND
Continuous	1.90±0.05 (0.12)	1.35±0.03 (0.06)	1.04±0.02 (0.05)	0.15±0.01 (0.01)	0.20±0.03 (0.06)	ND	0.10±0.01 (0.01)

Cardiolipin and lysophosphatidylcholine were detected in trace amounts only in lipid extracts of control cells.

ND not detected.

Values are given as mean ± standard error of the mean. 95% confidence limits are shown in parentheses.

PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine;

PA = phosphatidic acid; DMPE = N,N dimethylphosphatidylethanolamine,

BS = base spot.

(P = 0.01) and a decreased synthesis of phosphatidylserine (P = 0.001). The phosphatidylinositol and phosphatidic acid contents were similar. However, compared with continuously-grown cells, the batch-grown, limited yeast contained less phosphatidylinositol and less phosphatidic acid (P = 0.01).

Very low levels of N'N dimethylphosphatidylethanolamine, lysophosphatidylcholine and cardiolipin were present in control and batch-grown, limited cells. These lipids were not detected in extracts from continuously-grown, limited cells. The amount of phosphorus remaining at the origin after thin-layer chromatography (base spot) was minimal in all samples.

iii. Fatty acids

The fatty acid composition of lipids extracted from continuously-grown, limited cells is shown in Table 18. Unsaturated fatty acids accounted for almost 49% of the total with C_{16:1} and C_{18:1} acids predominating. The saturated fatty acid content appeared to be relatively high with C_{14:0} and C_{16:0} acids present as major components.

SECTION II A SEARCH FOR TEICHOIC ACIDS IN YEASTS

1. Growth of yeasts

The first, and to date, only report of teichoic acid in yeasts involved a strain of Candida intermedia (Naumova & Streshinskaya,

Table 18 Fatty acid composition of the lipids of
 continuously grown, phosphate-limited
Sacch. cerevisiae

Fatty acid	Content (% molar composition)
C8:0	5.1
C8:1	trace ¹
C10:0	7.9
C11:0	trace
C12:0	2.4
C12:1	1.9
C14:0	12.9
C14:1	1.2
C16:0	18.5
C16:1	29.6
C18:0	4.3
C18:1	16.1

¹trace = <0.1%

1973). Since this particular strain is not available, attempts to confirm the results of the Russian workers were made using the type strain, C. intermedia CBS 572. Other yeasts examined were those for which lyophilised cell crops were commercially available.

Because the amount of teichoic acid in yeasts is likely to be small, it was important to adjust the growth conditions so as to achieve a maximum cell yield. C. intermedia was grown initially in a glucose-containing medium and harvested towards the end of the exponential phase (Fig 13). However, Naumova & Streshinskaya (1973) reported an improved yield of teichoic acid from yeast grown in a medium which contained alkanes as the carbon and energy source. Unfortunately, growth of C. intermedia CBS 572 in alkane-containing medium was rather poor, and the cell yield was much lower than that from glucose medium (Fig 14). Attempts to use this medium for large-scale growth of the yeast were therefore abandoned.

Details of the cell yields obtained from various batches of glucose-containing medium are given in Table 19. The best yield was obtained with GPYS type B medium which contained a higher ratio of phosphate to magnesium than did GPYS type A medium. However, this culture (culture D) was also the only one grown in a fermenter.

2. Preparation of TCA extracts of cells

The classical procedure for extracting teichoic acids from bacteria with TCA was modified slightly in accordance with the method employed by Naumova & Streshinskaya (1973).

Fig. 13 Growth of C. intermedia in GPYS
type A medium

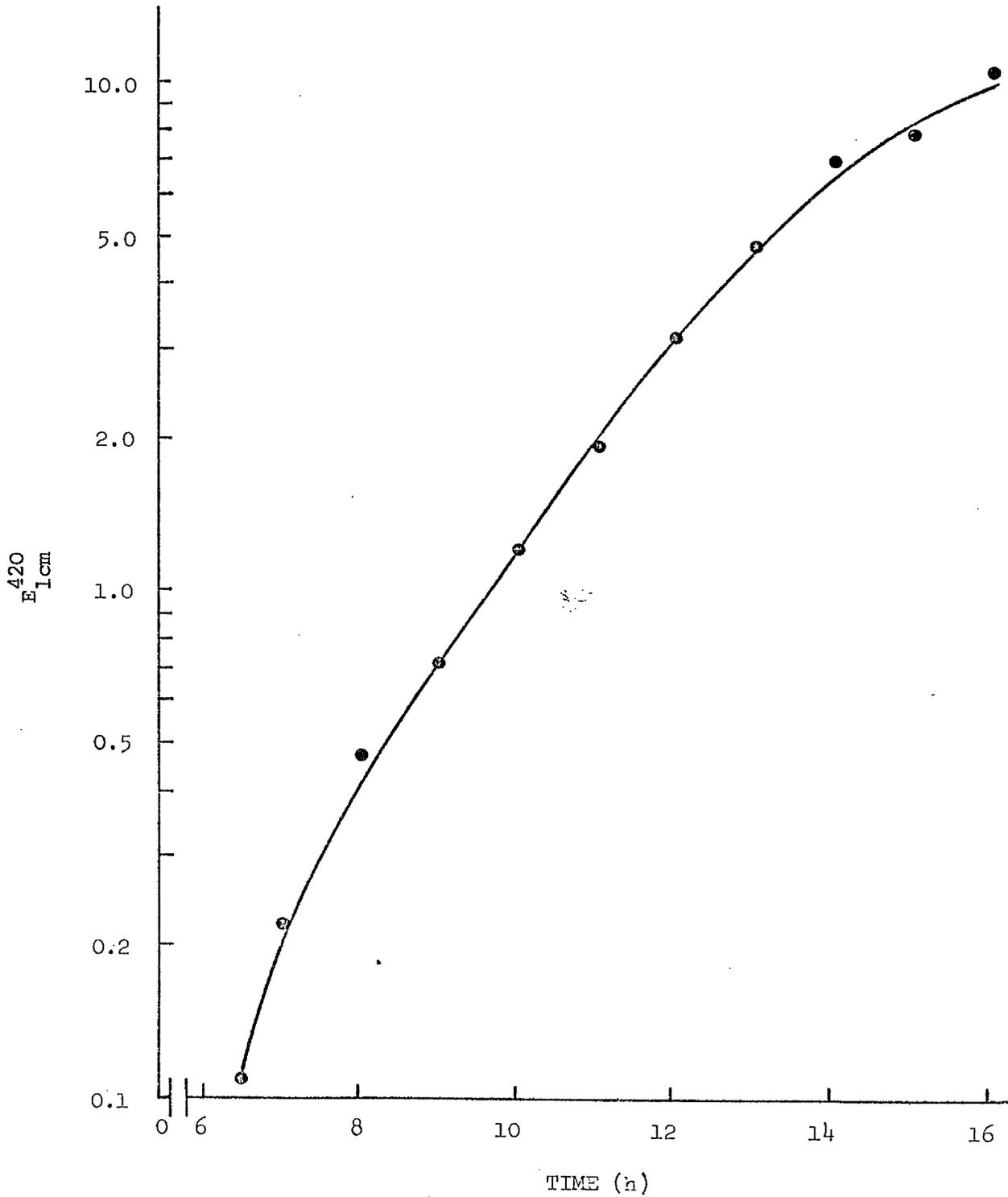


Fig. 14 Yield of C. intermedia in different growth media

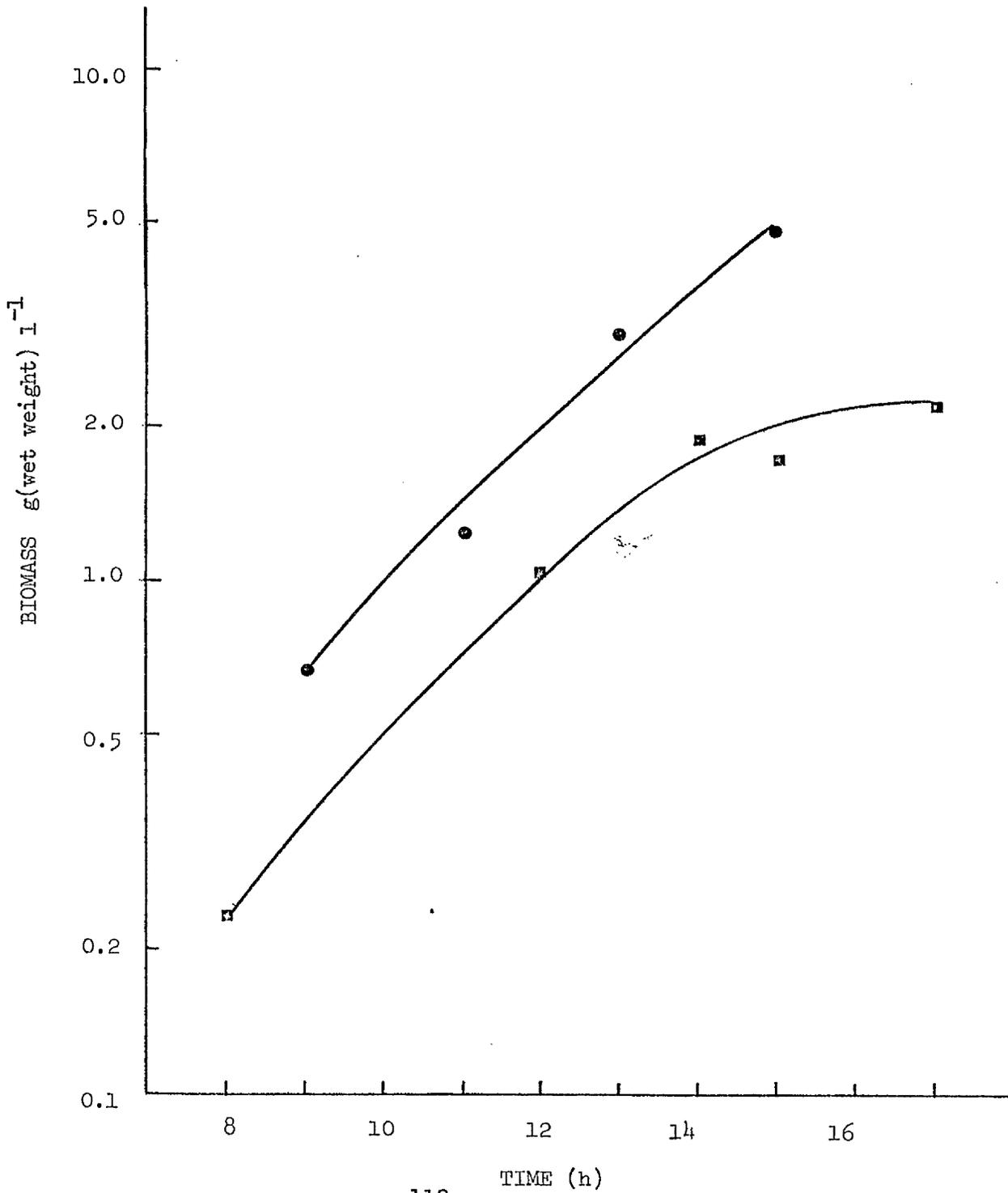


Table 19 Preparation of TCA extracts of different yeast species

Species	Culture No.	Culture volume (l)	Wet weight of cells (g)	Dry weight of fat-free cells (g)	Weight (mg) of		
					Extract I	Extract II	Extract III ¹
<u>C.intermedia</u> ²	A	10	45.0	9.6	39.5	31.0	-
<u>C.intermedia</u> ²	B	10	56.1	15.4	3.14	12.9	-
<u>C.intermedia</u> ²	C	2 x 10	103.4	26.1	26.2	10.4	-
<u>C.intermedia</u> ³	D	15	446	93.4	128.7	140.3	224.2
Baker's yeast ⁴	-	-	-	30.8	43.8	8.8	-
<u>S.cerevisiae</u> ⁵ (type 1)	-	-	-	77.1	180.4	50.6	-
<u>C.utilis</u> ⁵	-	-	-	75.0	75	520.4	162.4

¹Extracts I, II and III represent material recovered from 5%, 10% and 10% (w/v) TCA extractions of defatted cells.

²Grown in GPYs type A medium.

³Grown in GPYs type B medium.

⁴Obtained from Allinson Ltd. (London).

⁵Purchased from Sigma Chemical Co. Ltd. (London).

Washed cells were defatted with chloroform-methanol (2:1, v/v) prior to extraction with 5% (w/v) TCA (extract I), then two portions of 10% (w/v) TCA (extracts II and III). The yield of material recovered after each extraction was extremely low and highly variable (Table 19).

3. Acid hydrolysis of TCA extracts and qualitative analysis of hydrolysates

Poly (glycerol phosphate) teichoic acids are readily hydrolysed in dilute acid at 100°C. Characteristic hydrolysis products which may be identified on paper chromatograms include glycerol, glycerol phosphate and glycerol diphosphate.

TCA extracts of yeast were hydrolysed in 2 M HCl at 105°C for 3 h, subjected to paper chromatography in solvent A then examined for the presence of polyols using the periodate-Schiff reagents (Table 20). A product which co-chromatographed with glycerol was detected in some but not all of the extracts. Authentic standards of glycerol characteristically appear within 30 s of spraying with the periodate-Schiff reagents. In one case only (the hydrolysate of extract I from C. intermedia culture B) did the material co-chromatographing with glycerol appear within the prescribed time period. With other hydrolysates several hours were required for the visualisation of this product.

The acid hydrolysates were also chromatographed in Solvent B and products located with the alkaline silver nitrate reagent. The distribution of products revealed by this stain is shown in Table 21. It is apparent that a considerable amount of cell-wall material was

Table 20 Products of acid hydrolysis of TCA extracts separated by paper chromatography in solvent A

Species	C.intermedia		C.intermedia		C.intermedia		C.intermedia		Baker's yeast		Sacch. cerevisiae		C.utili	
	A	B	C	D	I	II	III	I	II	I	II	I		II
Culture No. ²	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Extract No. ²	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Compound	R glycerol value													
Glycerol	P	1.0	+	+	+	+	+	+	+	+	+	+	+	+
Ribitol	P	0.81												
Unknown product	1	P			+	+	+	+	+	+	+	+	+	+
2	YG		+		+					+	+	+	+	+
3	P		+		+					+	+	+	+	+
4	P				+	+				+	+	+	+	+
5	YG				+	+				+	+	+	+	+
6	P		+		+					+	+	+	+	+
7	P		+		+					+	+	+	+	+
8	P				+	+				+	+	+	+	+
9	YG				+	+				+	+	+	+	+
10	P				+	+				+	+	+	+	+
11	P				+	+				+	+	+	+	+
12	P				+	+				+	+	+	+	+

Hydrolysates were applied to Whatman No. 1 paper and subjected to descending chromatography in solvent A for 18 h. Products were visualised using the periodate-Schiff reagents.

¹ Colour produced by the periodate-Schiff reagents: P = purple; YG = yellow-green

² Nomenclature as described in Table .

solubilised during the extraction with TCA since both glucose and mannose were major hydrolysis products. Although the alkaline silver nitrate stain characteristically reveals the presence of reducing sugars, glycerol is also visualised. Again, material which co-chromatographed with glycerol was detected in some but not all of the hydrolysates.

All hydrolysates which contained material co-chromatographing with glycerol in both solvents were further chromatographed in Solvent A. This time products were located with the molybdate spray reagent for phosphate esters. Inorganic phosphate was present in all hydrolysates examined in this way. However, only the hydrolysate of extract II from C. intermedia culture B contained a product which co-chromatographed with 1-sn glycerol phosphate. No material corresponding to glycerol diphosphate was detected in any of the hydrolysates.

4. Gel filtration of a TCA extract of C. utilis

It seemed possible that very small amounts of teichoic acid might be masked during analysis by other components present in TCA extracts, particularly cell-wall polysaccharides. An attempt was therefore made to fractionate TCA-extracted material on a column of Sephadex G-100. Extract II from C. utilis was used since this extract was available in amounts sufficient for column chromatography.

Analysis of every third fraction collected from the Sephadex column (Fig 15) showed phosphorus-containing material of high molecular weight in the extract. Resolution of the phosphorus-containing

Table 21 Products of acid hydrolysis of TCA extracts separated by paper chromatography in solvent B

Species	<u>C.intermedia</u>			<u>C.intermedia</u>			<u>C.intermedia</u>			<u>Baker's Yeast</u>			<u>Sacch.cerevisiae</u>			<u>C.utilis</u>																																																																																																																																																																													
Culture No. ¹	A	B	C	D	I	II	I	II	I	II	I	II	I	II	I	II	I	II																																																																																																																																																																											
Extract No. ¹	I	II	I	II	I	II	I	III	I	II	I	II	I	II	I	II	I	II																																																																																																																																																																											
Compound	R glycerol value																																																																																																																																																																																												
Glycerol																			Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Unknown product 1																			2																			3																			4																			5																			6																			7																		
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Unknown product 1																			2																			3																			4																			5																			6																			7																																					
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Unknown product 1																			2																			3																			4																			5																			6																			7																																																								
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Hydrolsates were applied to Whatman No. 1 paper and subjected to descending chromatography for 18 h in solvent B. Products were visualised using the alkaline silver nitrate reagents.

¹Nomenclature as described in Table .

material into three discrete peaks (peaks I, IA and II; Fig 16) was achieved upon subsequent analysis of every fraction between numbers 40 and 50. Fractions corresponding to each of the three peaks were pooled, dialysed against water and freeze-dried. Samples of the dried material were then hydrolysed in 2 M HCl and the products analysed by paper chromatography as before. Glycerol and neutral sugars were present in each of the three hydrolysates. However, 1-sn glycerol phosphate could not be detected on any of the chromatograms.

5. Glycerol and carbohydrate contents of TCA extracts of *C. utilis*

Confirmation of the presence of glycerol in these extracts was sought using a chemical assay for glycerol. Aliquots of the crude extracts were hydrolysed in acid. Any glycerol phosphate released during hydrolysis was dephosphorylated using alkaline phosphatase. The glycerol content of each hydrolysate was then determined (Hanahan & Olley, 1958). The results obtained (Table 22) indicated that glycerol accounted for 2.3 to 4.9% (w/w) of the extracts. Considerable amounts of carbohydrate were also shown to be present in each extract.

6. Assay of poly (glycerol phosphate) synthetase activity in sub-cellular fractions of *C. intermedia*

Since the demonstration of teichoic acid in TCA extracts of *C. intermedia* was not wholly convincing, various sub-cellular fractions were examined for their ability to synthesise a labelled polymer from CDP- $[^{14}\text{C}]$ glycerol (a known precursor of poly (glycerol phosphate) teichoic acids in bacteria). The greatest amount of radioactive label

Fig. 15

Elution profile of extract II from C. utilis
separated on Sephadex G-100

(\triangle — \triangle) $E_{260}^{1\text{ cm}}$

(\odot — \odot) Phosphorus

(\square — \square) Carbohydrate

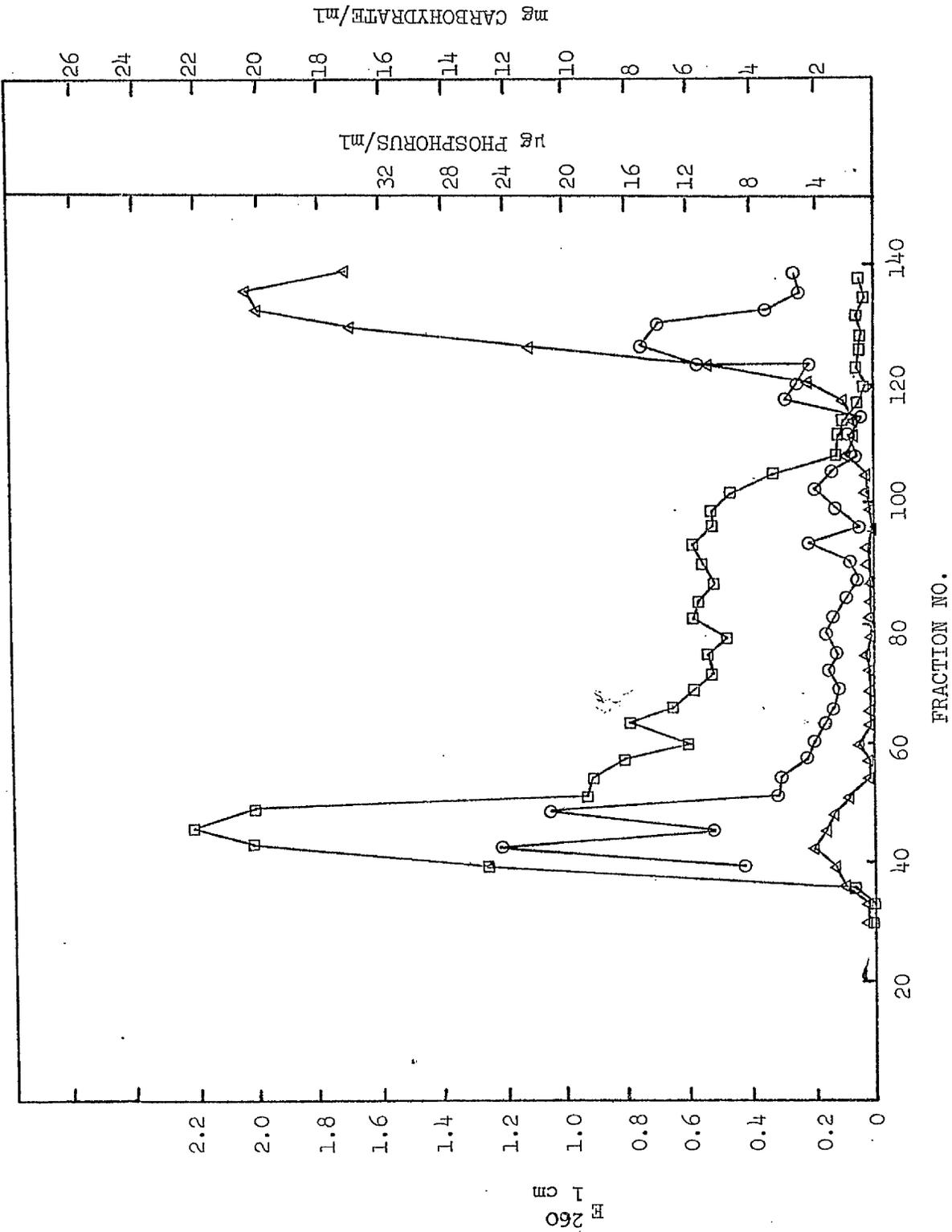


Fig. 16

Separation of extract II from
C. utilis on Sephadex G-100

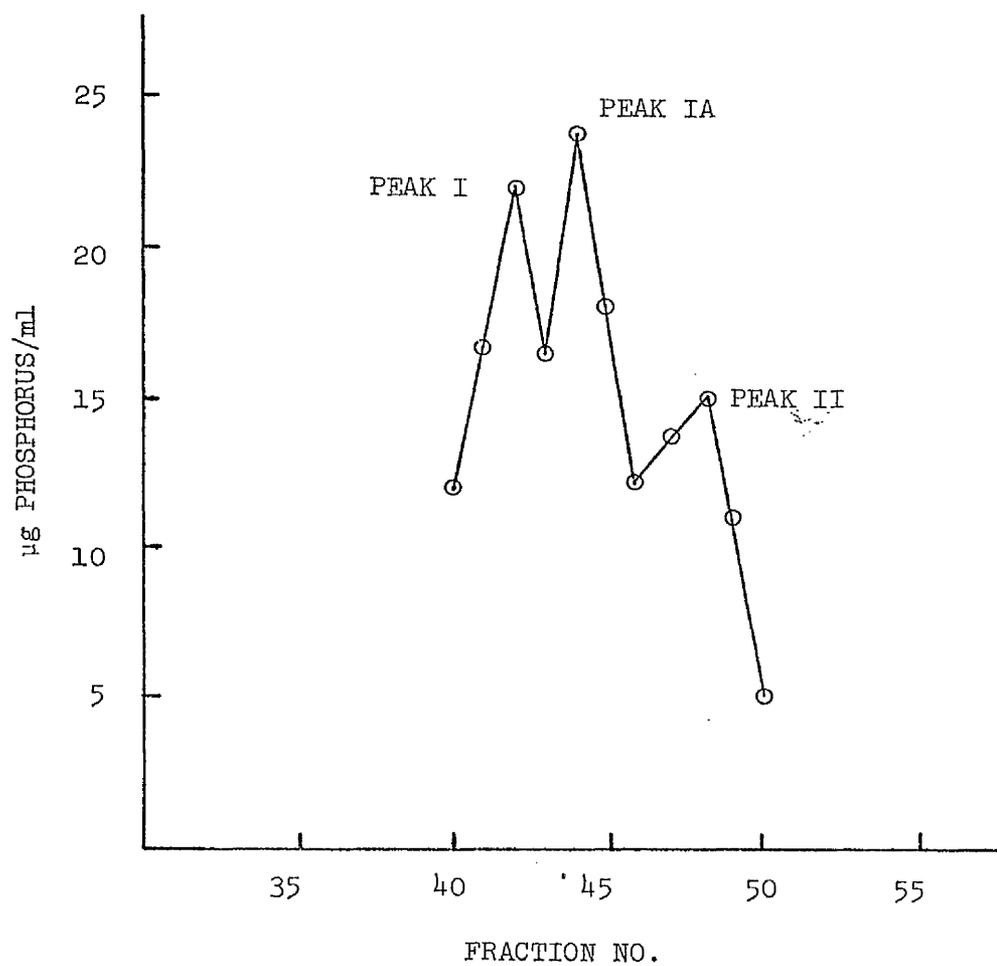


Table 22

Glycerol and carbohydrate content of TCA extracts
from C. utilis

Extract No.	Wt. hydrolysed (mg)	Glycerol ¹ (mg)	Carbohydrate (mg)	% (w/w) of glycerol in extract
I	10.4	0.51	1.5	4.9
II	9.5	0.22	1.83	2.3
III	9.4	0.34	1.0	3.6

¹These figures have been corrected for interference by carbohydrate in the colourimetric determination. Absorption due to carbohydrate is 2% of that due to glycerol on a weight for weight basis (Hanahan & Olley, 1958).

was converted to polymeric form (Table 23) when a 100,000 x g fraction was used as the source of enzyme. However, no determination of specific activity of any of the fractions was made and the amount of incorporation achieved was too low to allow a proper characterisation of the polymeric product.

Table 23 Incorporation of $[^{14}\text{C}]$ -glycerol from CDH $[^{14}\text{C}]$ -glycerol into a polymeric product
by sub-cellular fractions of C. intermedia

Enzyme Source	cpm of polymeric product	% incorporation of $[^{14}\text{C}]$ -glycerol into polymeric product
5000 x g fraction (pellet II)	462	0.53
5000 x g fraction boiled	296	
10,000 x g fraction (pellet III)	779	1.04
10,000 x g fraction boiled	453	
100,000 x g fraction (pellet IV)	1337	1.77
100,000 x g fraction boiled	786	

DISCUSSION

SECTION I THE EFFECT OF PHOSPHATE LIMITATION OF GROWTH ON THE
CELL-WALL AND LIPID COMPOSITION OF Saccharomyces
cerevisiae NCYC 366

A. GROWTH OF Sacch. cerevisiae UNDER PHOSPHATE-LIMITING CONDITIONS

Since micro-organisms are capable of rapidly modifying their chemical composition in response to changes in their environment it is difficult to study the physiology of growing, functioning organisms without culturing them in a rigidly controlled environment. This cannot be achieved in a closed system such as a batch culture where due to the uptake of nutrients and excretion of metabolic end-products by the growing cells, the environment changes continuously as growth proceeds. The only effective way of maintaining the chemical environment constant is to use an "open" system, that is, continuous-flow culture.

Of the various "open" culture systems available the "chemostat" type of continuous culture has the advantage of providing not only a range of controlled environments but also a range of unique environments which are never obtained in batch-type culture, or are obtained only transiently. With a chemostat the microbial population density is not controlled directly. Instead, the medium is compounded in a way such that all substances essential for the growth of the organisms, except one, are present in the culture at concentrations that are in excess of the microbial growth requirement. The one "growth-limiting" nutrient is present in the medium at a concentration sufficient to support only a limited amount of growth; thus, if the culture was allowed to develop as a batch culture, this nutrient would become depleted first, causing growth to cease. Under

continuous-flow conditions, however, the growth rate of the culture is prescribed by the rate at which this growth-limiting nutrient (in effect, fresh medium) is added. The chemostat therefore provides a means of studying the effects of strict limitation of a single essential nutrient on the chemical composition of micro-organisms. In the present study Sacch. cerevisiae NCYC 366 was grown in a chemostat under phosphate-limiting conditions in order to investigate the effects of this particular nutrient limitation on the cell-wall and lipid composition of the yeast. Cells grown batch-wise in the same medium, and harvested during the late exponential phase of growth (when the phosphate content of the medium was almost depleted), were also analysed.

Clearly, if the effects of phosphate limitation of growth on specific cellular components are to be determined, it is first necessary to establish the content and composition of those components in organisms grown in an environment containing an optimum concentration of phosphate. There are certain intrinsic problems associated with the selection of conditions suitable for the production of "control" cells, however. One approach frequently used is to compare cells grown in a chemostat under one nutrient limitation (say, phosphate limitation) with those grown similarly under another (K^+ or glucose limitation, for example). This method has the advantage that both cultures are grown in a controlled environment at the same growth rate but the disadvantage that effects due to a second nutrient limitation are introduced. An alternative strategy is to use for comparison cells grown batch-wise in a medium containing optimum concentrations of all nutrients. The disadvantages here are those associated with growth in a changing environment. However, during the early or mid-exponential growth phase of such a culture there would be an

excess of nutrients present and a minimal accumulation of toxic metabolites. Thus the cellular composition of organisms harvested during this period should correspond to that obtained under optimum growth conditions. This second approach was adopted in the present study: the composition of phosphate-limited cells was compared with that of cells grown in batch culture under conditions of nutrient excess and harvested during the mid-exponential phase of growth. The major draw-back of this procedure was that the growth rates of the cultures were not identical and therefore possible growth-rate effects had to be taken into account when the analytical data were interpreted (see Sections IB and IC).

The growth of Sacch. cerevisiae in media containing 0.6 mM KH_2PO_4 was limited by the availability of the phosphate anion and not by that of the potassium cation (Table 9). Nevertheless the potassium ion concentration may be of some relevance since potassium appears to play a vital, but as yet unexplained, role in the transport of phosphate into the yeast cell. Goodman & Rothstein (1957) for example, have shown that the uptake of phosphate by baker's yeast is stimulated at a K^+ concentration of 20 mM, particularly at low pH values.

Little information is available on the mechanism of phosphate uptake by Sacch. cerevisiae. Recently, however, some interesting data have been reported on phosphate uptake by Neurospora crassa (Lowendorf, Slayman & Slayman, 1974; Lowendorf, Bazinet & Slayman, 1975). There appear to be two transport systems for phosphate in this organism. The first is a constitutive, low-affinity system which is stimulated by K^+ . A second, high-affinity system is only derepressed during phosphorus starvation; no study of the K^+ -dependence of this system was made.

In "steady state" chemostat cultures of phosphate-limited Sacch. cerevisiae the level of phosphorus in the medium (as detected in cell-free culture filtrates) remained at about 75% of the input level. If the "steady state" cultures were allowed to grow batch-wise, however, by halting the flow of fresh medium into the chemostat vessel, then all of the phosphate was utilised. This anomalous situation might be explained by the existence of two systems for phosphate uptake similar to those reported for N. crassa. A constitutive, low-affinity and K^+ -dependent system would operate inefficiently in "steady state" cultures due to the relatively low concentration of potassium ion available. The phosphate concentration in the growth medium would therefore never fall to a level necessary for derepression of the second high-affinity system. Under conditions of batch culture on the other hand there would be a gradual diminution in phosphate concentration until eventually the second system was induced. The operation of this system would then result in the total depletion of phosphate in the medium.

B. EFFECT OF PHOSPHATE LIMITATION OF GROWTH ON THE CELL-WALL
COMPOSITION OF Sacch. cerevisiae

Perhaps the most striking change that phosphate limitation of growth induces in the cell-wall composition of many Gram-positive bacteria is the replacement of teichoic acid by an acidic polysaccharide containing uronic acid residues (teichuronic acid). It is apparent from the present study that no such drastic change in cell-wall composition occurs when Sacch. cerevisiae is similarly grown under conditions of phosphate limitation. The phosphorus content of the yeast wall is attributed to the presence of some phosphodiester linkages between adjacent mannose

residues in a phosphomannan thought to be located on the outer surface of the wall. This phosphomannan component is presumed to be largely responsible for the negative charge or zeta potential of the cell and thus may be considered analogous to teichoic acid in bacteria. While the phosphorus content of walls prepared from cells grown under phosphate-limiting conditions was lower than that of walls from control cells (Table 12) it was not dramatically so, and no evidence was obtained for the presence, in these walls, of uronic acid substituents. It may well be that, unlike Gram-positive bacteria, Sacch. cerevisiae is unable to incorporate uronic acids into polymers, even under conditions of phosphate limitation. Although uronic acids are known to be constituents of capsular polysaccharides in some yeasts (Phaff, 1971), their presence in cellular components of Sacch. cerevisiae has not been reported. Furthermore attempts to demonstrate the presence of UDP-glucose dehydrogenase (an enzyme unique to glucuronic acid synthesis) in batch-grown, phosphate-limited Sacch. cerevisiae have been unsuccessful (L.J. Douglas, unpublished observations).

Although uronic acid was not detected, it appeared that acid hydrolysates of walls prepared from phosphate-limited cells contained a larger quantity of one or more unidentified anionic components than did hydrolysates of walls from control cells. The anionic nature of these components was demonstrated by positive staining on chromatograms with reagents used for the detection of non-volatile acids. Thus it seems that under conditions of phosphate limitation, cell-wall phosphate may be partially replaced by an increased proportion of other negatively-charged constituents. This conclusion is supported by the work of San Blas and Cunningham (1974) who studied the effect of phosphate deprivation

on the composition of mannans produced by Hansenula holstii. When this yeast was grown in a culture medium from which KH_2PO_4 had been omitted the cell-wall mannan contained only trace quantities of phosphorus but nevertheless bound in appreciable amounts to a column of DEAE-cellulose. The acidic constituent was not identified although, as in the present work, analysis for uronic acid proved negative.

M. lysodeikticus is rare among Gram-positive bacteria in lacking a lipoteichoic acid (Powell et al., 1974). Membranes of this organism do however contain a mannan which is negatively-charged due to succinyl residues (Owen & Salton, 1975). A possible analogy with the mannan of phosphate-limited Sacch. cerevisiae prompted a search for succinic acid in cell-wall hydrolysates of the yeast. No succinate was detected, however, and the identity of the anionic component(s) remains to be established.

Significant quantitative changes in gross composition were observed in walls prepared from yeast grown under phosphate-limiting conditions (Table 12). Such changes must be examined carefully in light of the finding by McMurrough & Rose (1967) that the overall wall composition of Sacch. cerevisiae is affected by changes in growth rate. These workers showed that the protein and phosphorus contents of the wall increased as the growth rate was decreased. The total carbohydrate content, on the other hand, was largely unchanged, as were the relative proportions of glucan and mannan.

If wall carbohydrate content is independent of growth rate then the observed decrease from 83.5% (walls of control cells) to 72% (walls

of chemostat-grown cells) can be attributed to a specific effect of phosphate limitation of growth. Changes in protein and phosphorus content, however, may be partially accounted for by variations in the growth rate. Thus, while the protein and carbohydrate contents of walls appear to some extent to be complementary (Table 12), the increased protein content of walls from chemostat-grown, phosphate-limited cells may be a reflection of the lower growth rate of this culture. The values obtained for both protein and carbohydrate content of walls from batch-grown, phosphate-limited cells seem anomalous and no explanation can be offered for these at present.

As might have been expected, the amount of phosphorus in walls prepared from batch-grown, phosphate-limited cells was substantially less than that in walls from control cells, a decrease of some 28%. Surprisingly, however, the phosphorus content of walls from chemostat-grown, limited cells fell by only 4%. Again this relatively high figure may reflect a balance between the opposing effects of phosphate limitation, which would tend to decrease the phosphorus content and a low growth rate, which would tend to increase it.

In order to investigate fully the effect of phosphate limitation of growth on the carbohydrate composition of cell walls, a simple and specific method had to be found for the independent assay of the two major polysaccharides. Early procedures involved lengthy fractionations of cell walls followed by a non-specific assay for hexose (Kessler & Nickerson, 1959; Korn & Northcote, 1960). More recently, the glucose content of cell-wall hydrolysates has been determined enzymically, and the

mannose content calculated by subtracting this value from that for total hexose content (Fleet & Manners, 1976). In the present study, an attempt was made to determine both glucose and mannose in cell-wall hydrolysates by specific enzymic methods.

Initially, conditions were sought which produced maximum hydrolysis of the wall polysaccharides and at the same time, minimum degradation of the monosaccharides released. The results in Table 13 show that mild acid hydrolysis alone released approximately 60 to 80% of the polysaccharide. However, very little of this material was recovered as either the glucose or mannose monosaccharide. Pretreatment of the cell-wall suspensions with β -glucanase and α -mannosidase prior to acid hydrolysis solubilised considerable amounts of the cell wall. Subsequent acid hydrolysis conditions released 100% of the polysaccharide, mainly in monosaccharide form (Table 14).

It is always difficult to achieve complete hydrolysis of a polysaccharide to its constituent monosaccharides while avoiding destruction of the sugar or acid reversion to oligosaccharides. The results shown in Table 14 therefore represent minimum values for glucose and mannose contents of the different cell-wall preparations. If these figures are extrapolated to account for the total hexose present in the cell wall then the results shown in Table 24 are obtained. Such an extrapolation assumes not only that glucose and mannose together account for the total hexose present, but also that glucan and mannan are hydrolysed to an equivalent extent, and may not therefore be completely valid. Nevertheless it is clear from either set of figures that walls prepared from both types of phosphate-limited yeast contained considerably

less glucan than did walls from control cells. In the case of the batch-grown, phosphate-limited cells this decreased glucan content appears to have been compensated for by an increased synthesis of mannan.

The conclusion that phosphate-limited cells have a decreased proportion of glucan in their walls is supported by the finding that enzymic treatment of wall suspensions prior to acid hydrolysis solubilised more of the walls from control cells than those from phosphate-limited cells. The Basidiomycete β -glucanase used in this pretreatment is known to hydrolyse β - linked yeast glucan rapidly, where Jack-bean α -mannosidase has a rather restricted action on yeast mannan (Ballou, 1976). Thus walls containing a high proportion of glucan would be expected to be more readily solubilised by this procedure. It is possible, however, that the different susceptibilities of the wall preparations to enzymic hydrolysis also reflect differences in the chemical structure of the cell-wall polysaccharides.

C. EFFECT OF PHOSPHATE LIMITATION OF GROWTH ON THE LIPID COMPOSITION OF *Sacch. cerevisiae*

The lipid composition of micro-organisms is very responsive to changes in the chemical and physical properties of the environment. Among the environmental factors that have been reported to affect the lipid composition of yeasts are growth rate (Brown & Rose, 1969; Hunter & Rose, 1972), composition of the medium (Brown & Rose, 1969; Ratcliffe et al., 1973), growth temperature (Hunter & Rose, 1972) and dissolved oxygen tension in the culture (Brown & Rose, 1969). Of these various parameters

Table 24

Estimated glucose and mannose contents of cell-wall
preparations

Walls prepared from	% (w/w) content of	
	Glucose	Mannose
Control cells	55.8	29.8
Batch-grown, phosphate-limited cells	42.6	50.6
Chemostat-grown, phosphate-limited cells	43.3	28.2

Values quoted in Table 14 for glucose and mannose released during acid hydrolysis were extrapolated to account for the total carbohydrate content of each cell wall preparation.

only changes resulting from different growth rates are directly relevant to the results of the present study.

Hunter & Rose (1972) have compared the lipid composition of Sacch. cerevisiae NCYC 366 grown in a chemostat at growth rates of 0.25 h^{-1} and 0.05 h^{-1} . They found that phospholipid synthesis was decreased at the lower growth rate and that this was reflected in lowered levels of phosphatidylethanolamine and, to a much lesser extent, phosphatidylinositol. The phosphatidylserine content was unaffected while the phosphatidylcholine content increased by some 37%. Thus the only change in phospholipid composition of chemostat-grown, phosphate-limited cells which may be attributed to the lower growth rate of this culture is the increased content of phosphatidylcholine. The increased level of phosphatidylethanolamine and decreased level of phosphatidylserine in these cells, and more especially, the wholly unexpected increase in total phospholipid content are in no way accounted for by these observations.

Phosphate limitation of growth induces quite profound changes in the polar lipid composition of some bacteria. Minnikin, Abdolrahimzadeh & Baddiley (1974) have shown that when Pseudomonas diminuta is grown in a chemostat under phosphate-limiting conditions the phospholipid content of the organism falls almost to zero and is replaced by an increased content of neutral glycolipids and acidic glycolipids containing uronic acid residues. By comparison, Sacch. cerevisiae would again appear less versatile in responding to this particular growth constraint. This conclusion is supported by the preliminary report of Johnson, Brown & Minnikin (1973) which states that only small differences in polar lipid

composition were detected when Sacch. cerevisiae NCYC 712 was grown under conditions of phosphate limitation. The phospholipid composition of Sacch. cerevisiae can be manipulated to some extent. For example, the inclusion of low concentrations of ethanolamine in the growth medium induces an increased synthesis of phosphatidylethanolamine (Ratcliffe et al., 1973). However, while this yeast may be relatively amenable to changes in phospholipid composition it would appear from the present work to be intolerant of any appreciable diminution in overall phospholipid content.

Perhaps the most striking change in the lipid composition of chemostat-grown, phosphate-limited cells was their decreased content of sterol esters and triacylglycerols. Moreover this was unlikely to be a growth-rate effect since Hunter & Rose (1972) demonstrated that lowering the growth rate of Sacch. cerevisiae in a chemostat at 30^oC caused an increased synthesis of sterol esters while the triacylglycerol content of the cells was unaffected. It is likely that inter-relationships exist between the pathways for sterol ester, triacylglycerol and phospholipid biosynthesis involving the operation of complex control mechanisms. Thus, if the decreased synthesis of sterol esters and triacylglycerols is a primary effect of phosphate limitation of growth, an increased synthesis of phospholipid may necessarily be a secondary effect. In this connection it is interesting that some of the results reported by Hunter & Rose (1972) for glucose-limited yeast, when collated (Table 25), reveal a remarkably similar series of changes to those observed in phosphate-limited yeast. Here glucose limitation of growth resulted in a decreased synthesis

Table 25

Effect of glucose limitation on the lipid composition of Sacch. cerevisiae NCYC 366 (Results taken from Hunter & Rose, 1972).

Growth conditions	Growth rate (h^{-1})	Lipid Composition (mg lipid/100mg cells)			
		Phospho- lipid	Sterol	Sterol esters	Triacyl- glycerols
Cells grown batch-wise at 30°C and harvested in mid-exponential growth phase	0.34	3.76	0.23	1.32	1.22
Cells grown in a chemostat at 30°C under conditions of glucose limitation	0.25	4.92	0.18	1.09	0.46

of sterols, sterol esters and triacylglycerols, and this was accompanied by an increased synthesis of phospholipid. The same workers also demonstrated that when the growth temperature of cells growing in a chemostat at fixed rate was lowered from 30 to 15°C, the synthesis of sterols and sterol esters decreased while that of phospholipids increased. The existence of control mechanisms operating between the various biosynthetic pathways might also partially explain the somewhat anomalous results obtained for the lipid composition of batch-grown, phosphate-limited cells (Table 16).

Sterol esters and triacylglycerols are two classes of lipid which because of their extreme hydrophobic nature cannot be accommodated in a membrane system. They are rather located exclusively in vesicles (Hossack, Wheeler & Rose, 1973; Cartledge & Rose, 1973; Clausen et al., 1974). There are two types of vesicle (large and small) present in Sacch. cerevisiae. Electron microscope studies on budding cells provided the first insight into their function. Sentandreu & Northcote (1969) showed that the large vesicle (or vacuole) multiplies by fission during the cell cycle and that the progeny of this fragmentation (small vesicles or sphaerosomes) are distributed between the mother and daughter cells. The small vesicles were observed to congregate at the neck of the budding cell, a region of active envelope growth.

Following the development of reliable methods for the isolation of vesicles, several workers reported a variety of lytic enzyme activities in these organelles, including β -glucanase, N-acetyl- β -glucosaminidase and esterase activities (Matile et al., 1971; Cortat, Matile & Wiemken, 1972; Cartledge & Rose, 1973). On the basis of these findings it was suggested

that small vesicles may function in cell-envelope biogenesis either by transporting envelope components to the growing region or by modification of the envelope prior to acceptance of these precursors (Cartledge & Rose, 1973).

The decreased content of sterol esters and triacylglycerols in Sacch. cerevisiae grown under phosphate limitation implies a diminished population of one or both types of vesicle in these cells. If small vesicles are indeed involved in cell-wall biogenesis this would explain the disturbances in wall composition, notably glucan content, apparently caused by phosphate limitation of growth. It would be interesting to know whether vesicles contain a relatively high proportion of phosphatidylserine since the content of this particular phospholipid was substantially decreased in phosphate-limited yeast.

Electron microscopy of thin sections or replicas of frozen fracture planes would confirm that phosphate-limited cells contain a diminished population of vesicles. Both techniques were attempted in this study but the micrographs obtained were of insufficient resolution to allow any conclusions to be drawn. A lack of resolution in electron micrographs of fungi in general is a fairly common problem and seems to be caused mainly by the relatively poor penetration of fixatives and stains through the cell walls of these organisms.

The changes observed in the levels of phosphorus-containing components in walls and lipids of Sacch. cerevisiae subjected to conditions of phosphate limitation would not appear to account for the appreciable decrease in total phosphorus content of these cells (Table 10). No attempt was made in this study to investigate the nucleic acid content and

composition of phosphate-limited yeast and changes in these components cannot be discounted. However it seems more likely that the decrease in total phosphorus content can be attributed to a decreased synthesis of polyphosphate, a compound known to be accumulated by Sacch. cerevisiae (Dawes & Senior, 1973). The exact role of polyphosphate in yeast remains to be established although it is presumed to provide a reserve of phosphorus and/or energy. In light of the present work it may be of some significance that polyphosphate appears to be located in the large vesicle or vacuole (Indge, 1968).

SECTION II THE SEARCH FOR TEICHOIC ACIDS IN YEASTS

The presence of teichoic acids in walls and membranes of Gram-positive bacteria is well established (Archibald, 1974) but, prior to the report of Naumova & Streshinskaya (1974), these polymers had been widely assumed to be absent from other groups of micro-organisms. The Russian workers described the isolation and partial characterisation of a poly (glycerol phosphate) teichoic acid from the yeast Candida intermedia H-30. In the present study attempts were made to confirm these findings and to extend the survey to other yeast species. However, since C. intermedia H-30 is not available outside the Soviet Union, and since the Russian workers did not respond to a request for the culture, the initial confirmatory work was undertaken with the type strain C. intermedia CBS 572.

Because the amount of teichoic acid present in yeast is likely

to be small, isolation of the polymer in quantities sufficient for chemical characterisation requires the provision of a relatively large weight of cell starting material. Thus it was important to adjust the growth conditions so as to achieve a good cell yield and, if possible, maximum teichoic acid production. No reference to the growth medium (Rieder's medium) used by the Russian workers could be found in the literature. C. intermedia CBS 572 was therefore grown in a conventional undefined medium (GPYS type A) which supported good growth of the yeast. A second medium (GPYS type B) was also used in which the ratio of phosphate to magnesium was higher. The relative proportions of these two constituents are known to influence the extent of teichoic acid synthesis in bacteria (Ellwood & Tempest, 1972).

Naumova & Streshinskaya (1973) claimed an improved yield of teichoic acid from yeast grown in a medium containing a mixture of alkanes as the carbon and energy source. When a similar medium was used in the present work, problems were encountered in stabilising the alkane-aqueous phase emulsion and cell yields were poor. The use of this medium was therefore discontinued. General difficulties in obtaining quantities of yeast sufficient for analysis also prompted the use of commercially-produced lyophilised preparations of other yeast species.

The procedure used to investigate the presence of teichoic acids in yeasts was similar to that of early investigators in the bacterial field (Armstrong et al., 1958). Material recovered from trichloroacetic acid extracts of defatted cells was hydrolysed in acid and chromatograms of the hydrolysates were examined for compounds

characteristically produced from a glycerol teichoic acid, namely glycerol and its mono- and diphosphates. Trace amounts of material which co-chromatographed with glycerol and stained similarly with specific reagents were recognised in most trichloroacetic acid extracts of the species examined. Attempts to establish conclusively the identity of this compound as glycerol by an enzymic method were unsuccessful. Chemical analysis of hydrolysates of C. utilis extracts on the other hand, indicated relatively large amounts of glycerol (Table 22). These results, however, must be viewed with some caution since the assay method used was rather non-specific and there was considerable interference from carbohydrates.

Glycerol phosphate was demonstrated in a hydrolysate of one trichloroacetic acid extract only. Glycerol diphosphates were never detected. Thus, the results, overall, from the examination of crude extracts were ambiguous and the evidence for the presence of teichoic acids not compelling.

Part of the problem appeared to be the heterogeneous nature of the extracts which contained large proportions of carbohydrate and nucleic acid. Obviously, the isolation and identification of minor hydrolysis products from such preparations is extremely difficult. Indeed, it is possible that even the hydrolysis conditions used - those normally adopted for pure teichoic acid - were inappropriate for these crude extracts. An attempt was therefore made to fractionate the extract from C. utilis by gel filtration. Phosphorus-containing material of high molecular weight was eluted from the column and acid

hydrolysates of this material were shown to contain glycerol. Again, neither glycerol phosphate nor glycerol diphosphate were detected in the hydrolysates.

The results of this study do not support nor refute the claim that teichoic acids are present in yeasts. The identification of glycerol and phosphorus in a component of high molecular weight in the extract from C. utilis might be considered reasonably good presumptive evidence. However, the possibility that traces of lipid were associated with polymeric material in this extract cannot be discounted. An unequivocal demonstration of teichoic acid would require the isolation of sufficient material for a complete characterisation of the products of acid and alkaline hydrolysis. Naumova & Streshinskaya (1973) were able to identify glycerol phosphate and glycerol diphosphate following both acid and alkaline hydrolysis of their material. However, the presence of diglycerol triphosphate, a product considered diagnostic for a 1,3-linked glycerol teichoic acid (Archibald & Baddiley, 1966) was not reported.

The maximum yield of teichoic acid obtained from C. intermedia H-30 by the Russian workers was 0.3 mg/g dry yeast. A demonstration of such low levels of a compound previously unreported in yeast inevitably gives rise to the question of bacterial contamination of the yeast culture. A rough calculation can be made which shows that the degree of contamination necessary to produce this yield of teichoic acid would be 0.7% (w/w) or approximately 1 bacterium per 14 yeast cells. It seems unlikely that such a level of contamination would escape notice.

In bacteria, glycerol teichoic acids are synthesised from CDP-glycerol, a nucleotide precursor unique to teichoic acid synthesis. Thus, an alternative approach to the problem of identifying these polymers as yeast components would be to demonstrate incorporation of $[^{14}\text{C}]$ -glycerol from CDP $[^{14}\text{C}]$ -glycerol into poly (glycerol phosphate) by subcellular fractions. The results of such experiments (Table 23) indicated that some synthesis had taken place although the amount of polymeric product formed was too small to permit its characterisation. Again, these results are somewhat equivocal. The assay conditions used were those which have been found optimal for teichoic acid synthesis in bacteria. It is possible, for example, that the pH value and divalent cation concentration used were inappropriate for the yeast system and responsible, at least in part, for the low levels of incorporation observed. Further investigation of these parameters might prove fruitful.

After this work was completed, the identification of glycerol teichoic acid in extracts of a Gram-negative bacterium Butyrivibrio fibrisolvens was reported (Sharpe et al., 1975). It has recently been characterised as a lipoteichoic acid (Hewett et al., 1976) and is present in an amount comparable to that found in most Gram-positive bacteria. This polymer was initially detected by serological methods although it was subsequently isolated and characterised chemically. A similar use of specific antisera to poly (glycerol phosphate) might prove helpful in any future search for teichoic acids in yeasts.

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