REGULATION OF MANNAN SYNTHESIS IN SACCHAROMYCES CEREVISIAE

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To Kathleen, and my parents

"Everyone is free to declare his

judgement, in things which have

not been fully discovered"

                    Antonie van Leeuwenhoek (1685)

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DECLARATION

This thesis is the original work of the author. Part of the results in Section IV have been published in the <u>Journal of Bacteriology</u> and, as an abstract, in the <u>Society for General Microbiology Quarterly</u>.

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SUMMARY

Although a great deal is known about the chemical structure and enzymatic mechanisms involved in the assembly of yeast mannoproteins, little is known about the regulation of mannan biosynthesis. The aim of this study, therefore, was to investigate the control mechanisms involved. Two approaches were used. Firstly, the potential role of low molecular-weight effectors of mannosyltransferases was investigated. Secondly, the possible existence of an activation-inactivation mechanism of control, analogous to that which operates during chitin synthesis was explored.

Mannosyltransferase activity in sphaeroplast lysates and washed membranes prepared from Saccharomyces cerevisiae X2180 was measured by following the incorporation of $[^{14}C]$mannose from GDP-$[^{14}C]$mannose into material precipitable with cold 0.3M perchloric acid. Mannosyltransferase activity was optimal at low enzyme protein concentrations (1 mg/ml), 30°C and neutral pH values; with 7.5 mM $MnCl_2$; it did not require exogenous mannoprotein acceptors.

Mannosyltransferase activity from both sphaeroplast lysate and washed membrane preparations was not markedly affected by sugars, nucleoside monophosphates, UDP, UDP-N-acetylglucosamine, ADP-, CDP-, TDP- and UDP-glucose. UDP- and ADP-mannose, GDP-glucose, GDP and GTP all inhibited mannosyltransferase activity from both preparations while activity was enhanced by ADP and ATP. UTP enhanced activity from the washed membrane fraction but inhibited that from the sphaeroplast lysate. The stimulatory effects of ATP and UTP were dependent upon the exact method of sphaeroplast lysate preparation. These results suggest that UTP and ATP levels may function in the control of mannosyltransferase activity. GDP-glucose acted as a potent competitive inhibitor of mannosyltransferase activity.

When enzyme activity was assayed at high concentrations of sphaeroplast lysate protein (10 mg/ml), with 7.5 mM $MnCl_2$, a severe inhibition was observed. This inhibition could be relieved by pre-incubation of the sphaeroplast lysate or by omission of $MnCl_2$ from assay mixtures. The addition of EDTA or monovalent cations removed inhibition in the presence of Mn^{2+}. No similar inhibition was observed when a washed membrane fraction was substituted for sphaeroplast lysate as the source of mannosyltransferase activity. The supernatant fraction obtained by centrifuging sphaeroplast lysate at 100,000 x g, when added to assay mixtures containing either sphaeroplast lysate preincubated at $4^{\circ}C$ or washed membrane fraction, also caused inhibition of enzyme activity. This inhibition required 7.5 mM $MnCl_2$, was destroyed by heating the supernatant fraction at $60^{\circ}C$ for 10 min, or by trypsin or pepsin treatment at $30^{\circ}C$, and was gradually lost when the supernatant fraction was stored at $4^{\circ}C$. The supernatant fraction caused inhibition of synthesis of both β-eliminable oligosaccharides and polysaccharide chains of mannan. These results indicate the existence of a protein inhibitor of mannan synthesis whose inhibitory activity in sphaeroplast lysates may be modulated by preincubation at low temperature or by varying the available Mn^{2+} concentration. These findings indicate that activation-inactivation processes exist in the regulation of mannan as well as chitin biosynthesis. A model, proposed by Farkaš (1979), for yeast cell-wall formation is discussed in which the results of this study on mannan synthesis have been integrated.

ABBREVIATIONS

A_{600}	absorbance value of a solution in a cell of 1 cm light path at a wavelength of 600 nm
AMP, ADP, ATP	5'-mono-, di- and tri-phosphates of adenosine
ATPase	adenosine 5'-triphosphatase
Asn	asparagine
^{o}C	degrees Celsius
$\left[^{14}C\right]$	all radioactive sugars and sugar nucleotides purchased were uniformly labelled in the sugar moiety
CDP	cytidine 5'-diphosphate
Ci	Curie $(3.7 \times 10^{10}$ disintegrations/second$)$
cm	centimetre
cpm	counts per minute
cyclic AMP	adenosine-3',5'-phosphate
DEAE-cellulose	diethylaminoethylcellulose
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EC	enzyme commission (with number)
EDTA	ethylenediaminetetraacetate
Expt	experiment
g	gravitational field (after multiplication sign; 9.81 ms^{-2})
g	gram
GlcNAc	N-acetylglucosamine
$(GlcNAc)_2$	di-N-acetylchitobiose
GMP, GDP, GTP	5'-mono-, di- and tri-phosphates of guanosine
GTPase	guanosine 5'-triphosphatase

h	hour
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonate
K_m	Michaelis constant
kcpm	10^3 counts per minute
l	litre
m	metre
M	molar
min	minute
mnn	genetic locus concerned with mannoprotein biosynthesis
mol	mole
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
No.	number
o-	*ortho*-
p-	*para*-
p., pp.	page, pages
pH	hydrogen ion concentration, minus log of
*p*HMB	*p*-hydroxymercuribenzoate
Pi	inorganic phosphate
PMSF	phenylmethylsulphonyl fluoride
PP_i	inorganic pyrophosphate
R_F	$\dfrac{\text{distance moved by solute}}{\text{distance moved by advancing front of solvent}}$
s	second
Ser	serine
SD	standard deviation
SF	solvent front
sugars	all sugars were of the D-configuration

TAME	p-tosyl-L-arginine methyl ester
dTDP	thymidine 5'-diphosphate
Thr	threonine
t.l.c.	thin-layer chromatography
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
Tris	tris(hydroxymethyl)aminomethane
UMP, UDP, UTP	5'-mono-, di- and tri-phosphates of uridine
UTPase	uridine 5'-triphosphatase
V	maximal enzyme reaction velocity
vol	volume
v/v	volume per volume
WGA	wheat germ agglutinin
wt	weight
w/v	weight per volume
w/w	weight per weight
%	per cent
>	greater than

INTRODUCTION

I. GENERAL REMARKS

Yeasts comprise a group of single-celled fungi that were
first observed microscopically exactly three hundred years ago (in
1680) by the Dutchman Antonie van Leeuwenhoek. Two centuries elapsed
before yeast microbiology progressed further, when Louis Pasteur demon-
strated sugar fermentation by living cells. The conflict that developed
between Pasteur's 'vital' interpretation of fermentation and Gay-Lussac's
chemical theory of organic change was finally resolved by the Buchner
brothers. By grinding yeast with a mixture of sand and Kieselguhr,
they obtained a cell-free extract that fermented sugar, so showing that
fermentation results from the action of substances contained within the
yeast cell (Rose, 1977a). With this discovery of enzyme activity, the
Buchner brothers had given birth to the science of biochemistry and,
since then, yeasts have been used to elucidate many biochemical path-
ways. Due to the commercial availability of large quantities of
brewer's and baker's yeast, most of the work has been done with
Saccharomyces species. The importance of yeasts as pathogens (e.g.
Candida and Cryptococcus species) has also been an incentive for
research with these eucaryotic microorganisms (Odds, 1979).

II. STRUCTURE OF THE CELL WALL OF SACCHAROMYCES CEREVISIAE

A. Wall architecture

Early investigations on the structure of the cell wall have
been reviewed by Phaff (1971). The thick, rigid wall represents
15-16% of the dry weight of the cell (Bacon et al, 1969). 80-90%
(w/w) of the wall is composed of polysaccharide, the remainder consist-

ing of protein (6-13%) and lipid (0.8-13.5%; Bartnicki-Garcia, 1968).

Discrepancies in values reported for lipid content (Phaff, 1971) may

simply reflect different levels of contamination by lipid-rich plasma

membrane. The lipids found are principally triglycerides, sterol

esters, free sterols and lower glycerides (Työrinoja et al, 1974;

Nurminen et al, 1975). Differences in isolation procedure and in the

interpretation of cytological and analytical evidence has led to many

inconsistencies in the literature concerning the composition, thickness,

stratification and appearance of the yeast cell wall (see Phaff, 1971;

Farkaš, 1979).

The thickness of the wall depends upon growth conditions, but

in adult cells varies from 70-180 nm (e.g. Moor and Mühlthaler, 1963;

Bowden and Hodgson, 1970; Cassone, 1973). Little evidence of strati-

fication was found by Houwink and Kreger (1953) or by Moor and Mühlthaler

(1963). However, other workers have observed two, or more layers in

washed walls of mechanically disrupted baker's yeast (Northcote and

Horne, 1952) or in ultrathin sections of fixed cells (Agar and Douglas,

1955; Vitols et al, 1961; Marquardt, 1962; Matile et al, 1969;

Cassone, 1973). Mundkur (1960) detected two layers by periodate

staining; his evidence, plus that from immunochemical studies (Hansen-

clever and Mitchell, 1964), indicates a surface covering of mannan.

The existence of chemically distinct layers, however, now seems

unlikely and the splitting of layers in thin sections (Agar and Douglas,

1955) is considered to be an artefact caused by polymerisation damage

during embedding procedures (Bowden and Hodgson, 1970). The appearance

of a fibrous, microcrystalline array of glucan, reported in boiled cells

and acid-treated walls (Houwink and Kreger, 1953), is also considered

artefactual due to the destruction of glucan crosslinking during

preparative procedures (Bowden and Hodgson, 1970).

Several hypothetical models have been proposed for cell-wall structure. Lampen (1968) proposed a model in which an inner, insoluble glucan layer was linked, via protein, to the outer-wall layer, composed of mannoprotein molecules joined by phosphodiester bridges. Manno-protein enzymes, such as invertase and acid phosphatase, were held within the wall either by phosphodiester linkages or by hydrogen-bonding or by both. Kidby and Davies (1970), however, found that invertase was not chemically bound to the cell wall and suggested that the enzyme was retained in the wall by a permeability barrier composed of mannan molecules linked by phosphodiester and disulphide bridges. A permea-bility barrier was also suggested by Arnold (1972), although he found invertase within the periplasmic space.

Two pieces of evidence which would substantiate these models are lacking. Firstly, the existence of phosphodiester bridges linking two macromolecular subunits in yeast mannan has not been shown. Secondly, although close association of glucan and mannan has been observed (Kessler and Nickerson, 1959), it has not yet been established whether they form a covalently linked unit. In contradiction to Lampen's model (Lampen, 1968), Linnemans et al (1977) and Cassone (1973) have observed an inner layer composed of mannoprotein.

B.   Glucans

The mainly insoluble, wall polysaccharide, glucan, was first prepared as early as 1894 by Salkowski, who wrongly called it 'achroocellulose'. The early chemical studies on glucan structure have been reviewed by Phaff (1971). There are three main difficulties associated with the interpretation of analytical data on glucan structure. Firstly, glycogen may be present as a major contaminant.

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It can be removed by extracting cells with acetic acid and subsequently autoclaving the residue with sodium acetate buffer, then water (Peat et al, 1958). However, the preferred method of removing glycogen is now to prepare clean, well-washed cell walls. Secondly, early extraction procedures were drastic and led to degradation and loss of some glucan components, a fact which was only recognised later. Finally, baker's yeast glucans are heterogeneous (Bacon and Farmer, 1968; Bacon et al, 1969; Manners and Masson, 1969). Three glucan components have now been isolated and a commonly-used fractionation scheme is that of Manners et al (1973a).

(i) Alkali-insoluble, acetic acid-insoluble β-(1→3)glucan

The alkali-insoluble glucan, which is the rigid, structural component of the cell wall, has been extensively studied. Prior to 1968, several contradictory structures have been proposed for this polymer. Bell and Northcote (1950) suggested that the molecule was a branched β-(1→3)glucan with β-(1→2)-glucosidic linkages. Misaki et al (1968), on the other hand, postulated a branched structure containing a backbone of β-(1→6)-linked glucose residues to which linear chains of about eight β-(1→3)-linked residues were attached. However, the structure proposed more recently by Manners et al (1973a), who removed the β-(1→6)glucan component, represents the best available at present. They subjected the alkali-insoluble material to 27 extractions with 0.5M acetic acid for 3-hour periods before finally treating the residue with a fungal endo-β-(1→6)glucanase to hydrolyse any residual, contaminating polysaccharide to soluble oligosaccharides. The purified polysaccharide represents about 85% of the crude, alkali-insoluble glucan and was shown to be a branched β-(1→3)glucan of high molecular weight (approximately 250,000). It contains 3% of β-(1→6)-linked

branch points and has a degree of polymerisation of about 1,500.

Due to the heterogeneity of glucans in different samples prepared from commercial baker's yeast, both the degree of branching and macromolecular structure of the β-(1→3)glucan are still unknown (Manners et al, 1974). Manners et al (1973a), however, have suggested two possible structures for the alkali-insoluble glucan. The first is a comb-type structure with a long backbone chain to which are attached, at intervals of unknown distance, linear side chains containing, on average, about 30 glucose residues. The second structure is a two-dimensional, randomly-branched, tree-type structure of the kind originally postulated for amylopectin. Assuming the tree-type structure to be the correct one, Villa et al (1977) have shown that the outer chains are relatively short (about 6 residues) whereas the solubilised inner chains are too large to show mobility on paper chromatograms.

It is thought that the rigidity and insolubility of the β-(1→3)glucan is due to hydrogen bonding and other forces of attraction (Manners et al, 1973a). Rees (1973) has suggested that the linking together of β-(1→3)glucan chains may occur by the formation of a triple-helix state similar to that occurring with an algal β-(1→3)xylan.

(ii) Alkali-insoluble, acetic acid-soluble β-(1→6)glucan

A minor component of the alkali-insoluble glucan was extracted with acetic acid and found to be a β-(1→6)glucan with some β-(1→3)-linked residues and a degree of polymerisation of about 140-150 (Manners and Masson, 1969). Manners et al (1973b) analysed this component in more detail and showed that it was a highly-branched structure accounting for 15% (dry wt) of the alkali-insoluble glucan. About 19% of the linkages were β-(1→3). Although a small number of these were found as interresidue bonds, about 75% were present at

interchain linkages. The proportion of this β-(1→6)glucan component is greater in several yeast species other than $\underline{S}$. $\underline{cerevisiae}$ (Manners $\underline{et}$ $\underline{al}$, 1974). Its function is not yet known. It may be a reserve material like glycogen or it may serve as a filling material or plasticizer within the rigid wall. Its function could be physically to prevent excessive hydrogen bonding of essentially linear segments of β-(1→3)-linked chains, allowing flexibility for wall extension.

(iii) Alkali-soluble β-(1→3)glucan

Several authors have reported that some of the cell-wall glucan can be extracted from yeast cells with hot alkali and that this glucan is complexed with mannan and protein (Roelofson, 1953; Falcone and Nickerson, 1956; Kessler and Nickerson, 1959). Eddy and Woodhead (1968) extracted cells of $\underline{Saccharomyces}$ $\underline{carlsbergensis}$ with cold 3% (w/v.) sodium hydroxide under nitrogen. They separated glucan and mannan components by first neutralising the alkali extract to pH values of about 10; the glucan formed a gel-like precipitate at this pH, and the mannan could be precipitated with Fehling's reagent. The glucan represented about 20% of the original cell-wall glucan and had a molecular weight of about 520,000.

Fleet and Manners (1976) purified the polymer to homogeneity and showed that it contained 80-85% β-(1→3)-linkages, 8-12% β-(1→6)-linkages and 3-4% branch residues joined via carbon atoms 1, 3 and 6. It had a molecular weight of 250,000 and a similar degree of polymerisation (1,500) to the alkali-insoluble β-(1→3)glucan. It appears to be the presence of the 150 or so β-(1→6)-linked residues that alters the solubility properties of the molecules. Fleet and Manners (1977) examined the finer structural details of the molecule by using bacterial endo-β-(1→3)- and endo-β-(1→6)-glucanases. Their results

indicate that the alkali—soluble glucan is made up of a $\beta-(1\rightarrow3)$glucan

type 'core', with a low degree of branching. The 'core' also contains

additional $\beta-(1\rightarrow6)$-linked residues. Attached to the core are mainly

$\beta-(1\rightarrow3)$-linked side chains which may contain secondary $\beta-(1\rightarrow3)$-linked

branching.

Alkali—soluble glucomannan complexes were found in yeast by

Kessler and Nickerson (1959) although no structures were reported until

Fleet and Manners (1977) showed that the alkali—soluble glucan always

contains a fragment of cell—wall mannan. Their results, from studying

glucanase action on both glucan and isolated cell walls of S. cerevisiae,

indicate that $\beta-(1\rightarrow6)$-linked glucose residues are involved in this

association.

C.   Chitin

Chitin is a linear polysaccharide composed of $\beta-(1\rightarrow4)$-linked

N—acetylglucosamine residues. Small amounts of chitin in yeast were

first conclusively demonstrated by Houwink and Kreger (1953). They

compared the X—ray diffraction patterns of granular material, obtained

by successive boiling of cells in acid and alkali, with those of

crustacean chitin. A milder extraction procedure was used by Bacon

et al (1966) to obtain a residue consisting almost exclusively of bud

scars. They extracted cells repeatedly with 3-6% (w/v) sodium hydroxide,

followed by sodium acetate buffer. The bud scars contained about 50%

(w/w) chitin. Cabib and Bowers (1971) examined bud scars, under the

electron microscope, after treatment with chitinase and found that a

chitin disc with a raised rim was embedded in the bud scar. The bud

scar, before extraction from the cell, also contains glucan (Bacon

et al, 1966) and mannan (Bauer et al, 1972; Bush and Horisberger,

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1973), presumably in the secondary septa (Cabib, 1975). Although mainly found in the bud scars, chitin and its oligomers have also been detected in the lateral cell wall and in the cytoplasm near the plasma membrane using gold-labelled wheat germ agglutinin (WGA) (Horisberger and Vonlanthen, 1977). Molano et al (1980) have recently combined this technique with specific labelling of chitin using $[^{14}C]$-N-acetyl-glucosamine and the effect of different enzymatic treatments on the release of label. Their results proved that the WGA-binding material in the lateral wall is actually chitin which amounts to less than 10% of the total chitin.

One further method for determining the location of chitin is to use fluorescent brighteners such as primulin or Calcofluor, which preferentially bind to chitin (Hayashibe and Katodha, 1973; Cabib and Bowers, 1975). Altered chitin distribution has recently been observed in yeast in two studies using such brighteners. Sloat and Pringle (1978) studied a temperature-sensitive mutant that was unable to form buds at the non-permissive temperature. Under these restrictive conditions, the pattern of synthesis became disorganised and chitin was deposited over the entire cell surface. Chitin also accumulates in a type cells treated with the soluble mating pheromone, α-factor, in the absence of division septa (Schekman and Brawley, 1979). Therefore, although more than 90% of chitin is usually found in the bud scar, its specific location can be altered under certain conditions.

D. Mannoproteins

The structure of cell-wall mannan, or mannoproteins, in yeast has been extensively reviewed by Phaff (1971) and Ballou (1974,

1976).   Yeast mannan is a covalently-linked polysaccharide-protein

complex comprising a mixture of many structurally and functionally

differentiated molecules with molecular weights ranging from 25,000

to at least 10^6 (Ballou, 1974).   Apart from the 'structural' mannan,

which contains on average 5-10% (w/w) protein (Ballou, 1974) there

are a number of mannoprotein enzymes (invertase, acid phosphatase,

glucamylase, endo-β-(1→3)glucanase and α-galactosidase), probably in

the periplasmic space, which contain 30-60% (w/w) protein (Lampen,

1968; Biely et al, 1976; Lazo et al, 1978).

Mannan was first termed 'yeast gum' when discovered by

Salkowski in 1894 (Phaff, 1971).   It was studied in detail by Haworth

et al (1937), who extracted yeast cells with 6% (w/v) sodium hydroxide

and precipitated the mannan by copper-ion complexation.   They sub-

sequently showed that (1→6)-, (1→2)- and (1→3)-linkages are present in

the molecule (Haworth et al, 1941).   Kessler and Nickerson (1959)

demonstrated glucan-mannan-protein associations in complexes extracted

from lipid-free cell walls with alkali at room temperature under

nitrogen.   Two milder isolation procedures have been adopted to

minimise mannan degradation by hot alkali.   Korn and Northcote (1960)

extracted walls for 3 days at 37^oC with ethylenediamine and Peat et al

(1961) obtained mannan by autoclaving yeast cells in neutral citrate

buffer.   The structure of baker's yeast mannan is described in section

III, together with that of S. cerevisiae X2180, which has been extensively

studied by Ballou and his coworkers (Ballou, 1974, 1976).

III.   STRUCTURE OF SACCHAROMYCES CEREVISIAE MANNOPROTEINS

A.   Nature of the protein component

The protein moiety of mannan has been given scant attention.

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Kessler and Nickerson (1959) analysed the amino acids present in two glucomannan-protein complexes. In both fractions aspartate (including asparagine) was the most abundant amino acid (13% and 31% of the total amino acids), while serine and threonine each accounted for between 4% and 6%. Sentandreu and Northcote (1968) isolated a high molecular-weight mannan, containing about 4% (w/w) amino acids. These amino acids contained much larger proportions of serine and threonine, accounting for over 40% of the total amino acid in the peptide. The external, mannan invertase and the internal, carbohydrate-free invertase of S. cerevisiae similarly contain high proportions of aspartate, serine, threonine and glutamate (Gascón et al, 1968).

B. Analysis of the carbohydrate portion

The complex carbohydrate moiety of mannan has been extensively studied. Detailed analysis of this portion requires that the macromolecule be first digested to smaller fragments which can subsequently be analysed by classical carbohydrate procedures (methylation analysis or periodate oxidation) that are described, for example, by Sharon (1975).

Ballou (1974, 1976) has developed a scheme, which combines both chemical and enzymatic degradation of mannan, to elucidate mannan structure. There are three types of chemical degradation. Firstly, mild acid hydrolysis (0.01M HCl at 100°C for 30 min) can be used to remove mannose residues attached to side chains by phosphodiester linkages (Rosenfeld and Ballou, 1974a). Secondly, β-elimination (0.1M NaOH at room temperature for 18 h) releases mannose and mannooligo-saccharides from their linkage to serine and threonine (Sentandreu and Northcote, 1968). In this reaction, sodium borohydride can minimise further degradation of the oligosaccharides by reduction (Yen and

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Ballou, 1974). The large polysaccharide chain is only released from asparagine by M sodium hydroxide at $100^{\circ}C$ for 5 h (Nakajima and Ballou, 1974a). Thirdly, selective acetolysis of mannan can be used (Gorin and Perlin, 1956). This reaction converts yeast mannans to smaller oligosaccharides by splitting $\alpha-(1\rightarrow6)$-linkages preferentially to $\alpha-(1\rightarrow2)$-, $\alpha-(1\rightarrow3)$- and $\alpha-(1\rightarrow4)$-linkages, reflecting the higher electron density at the $\alpha-(1\rightarrow6)$-glycosidic bond. The attacking species in the reaction is the acetylium ion, $[CH_3CO]^+$, that is produced from a mixture of acetic anhydride, acetic acid and concentrated sulphuric acid (Rosenfeld and Ballou, 1974b).

A variety of enzymes have been used to degrade the mannan macromolecule. A bacterial exo-α-mannanase hydrolyses $\alpha-(1\rightarrow2)$- and $\alpha-(1\rightarrow3)$-linkages faster than $\alpha-(1\rightarrow6)$-linkages (Jones and Ballou, 1969a). Consequently most of the mannan side chains can be removed by the action of this enzyme, leaving the backbone essentially intact (Jones and Ballou, 1969b). Nakajima and Ballou (1974b) isolated two enzymes secreted by a mannan-degrading bacterium: firstly an endo-α-(1$\rightarrow$6)mannanase which can degrade the $\alpha-(1\rightarrow6)$-linked backbone and secondly, an endo-β-N-acetylglucosaminidase capable of releasing the polysaccharide from its attachment to asparagine. Both of these enzymes were used by Nakajima and Ballou (1975a) to isolate the 'inner core' fragment of the mannan for analysis. Tarentino et al (1974) also reported a similar endo-β-N-acetylglucosaminidase which released the polysaccharide from the external invertase protein of S. cerevisiae.

C. Detailed structure of Saccharomyces cerevisiae X2180 mannan

The detailed structure of S. cerevisiae X2180 mannan, as determined by Ballou and coworkers (Ballou, 1976), is illustrated in

figure 1.   The mannan consists of α-D-mannose units linked to poly-

peptide chains in two ways.   First, there are short, β-eliminable

oligosaccharides, O-glycosidically attached to serine or threonine

residues (Sentandreu and Northcote, 1968).   This fraction represents

about 11% (w/w) of the total mannose (Collona and Lampen, 1974) and

the fragments released by mild alkali treatment have the same

structures as the side-chains of the mannan outer chain (Nakajima and

Ballou, 1974a), except that they have not yet been shown to contain

phosphate.   Secondly, there are long, highly-branched polysaccharides

attached via a di-N-acetylchitobiose bridge to asparagine.   The

asparagine-linked polysaccharide is differentiated into a core oligo-

saccharide component, composed of about 15 mannose and 2 N-acetylglucos-

amine residues (Nakajima and Ballou, 1975a) and a branched outer chain

of about 250 mannose residues, to which is attached an occasional

mannosylphosphate unit (Rosenfeld and Ballou, 1974a).   The side-chains

in the core are linked to the α-(1→6)-backbone mainly by α-(1→3)-linkages,

whereas in the outer chain the side chains are uniformly attached to

the backbone by α-(1→2)-linkages.   Moreover, the core has not been

shown to possess mannosylphosphate substituents.

        Several mutants that are defective in synthesis of the

outer chain have been isolated, based upon the failure of mutant cells

to be agglutinated by specific antiserum raised against different

mannan acetolysis products (Raschke et al, 1973; Ballou et al, 1973).

The structures of the outer chains produced by these mutants is shown

in Figure 2.   Only one of these mutations (mnn 1) affects synthesis

of the inner core; this mutation alters the structure of the tetra-

saccharide chains attached to the mannan outer chain (Raschke et al,

1973), to serine threonine residues (Nakajima and Ballou, 1974a) and

Figure 1.   Detailed structure of the mannan from Saccharomyces

cerevisiae X2180 (from Ballou, 1976)

All anomeric linkages have the α-configuration except for the

trisaccharide unit, βMan(1→4)βGlcNAc(1→4) βGlcNAc, linked to

asparagine.   Mannose residues attached directly to serine and

threonine are now also known to be α-linked (Bause and Lehle, 1979).

M	Mannose	Asn	asparagine
GlcNAc	N-acetylglucosamine	Ser	serine
P	phosphate	Thr	threonine

Asn

GNAc → Asn

$\xrightarrow{1\ 4}$ GNAc

$\xrightarrow{1\ 4}$ GNAc

Ser (Thr)

Inner core

Outer chain

Base-labile
oligosaccharides

$[M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1}]_n \xrightarrow{6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 6} M \xrightarrow{1\ 4}$ GNAc
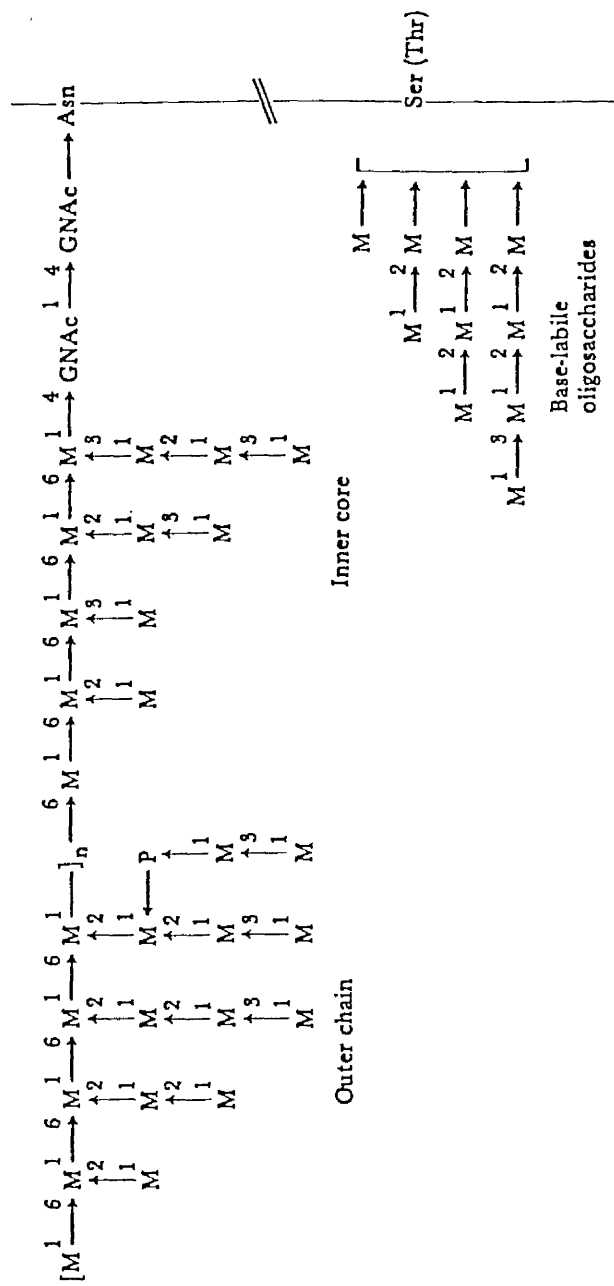
Figure 2.   Outer chain mannan structures for the various

Saccharomyces cerevisiae X2180 mannan mutants

(from Ballou, 1976).

The assumed enzymic defects that lead to the changes

are given in parentheses.   All backbone structures have

the α-$(1\rightarrow6)$-linkage.

```
        —→ M —→ M —→ M —→ M —→ M —→ M —→
            ↑2      ↑2      ↑2      ↑2      ↑2
         6  |1      |1      |1      |1      |1
    P —→ M      M      M      M      M
    ↑           ↑2      ↑2      ↑3      ↑2
    |1          |1      |1      |1      |1
    M       M      M      M      M
        ↑3      ↑3      ↑3
        |1      |1      |1
    M       M      M
```

Saccharomyces cerevisiae X2180 'wild type'

mnn1 [α-1→3-mannosyltransferase⁻]

mnn1, mnn4 [α-1→3-mannosyltransferase⁻ and mannosyl phosphate transferase⁻]

```
      —→ M —→ M —→ M —→ M —→              —→ M —→ M —→ M —→
          ↑2      ↑2      ↑2                   ↑2      ↑2
       6  |1      |1      |1                   |1      |1
  P —→ M      M      M                 M      M
  ↑         ↑2      ↑2                         ↑2
  |1        |1      |1                         |1
  M      M      M                       M
```

mnn2 [α-1→2-mannosyltransferase I⁻]

```
    —→ M —→ M —→ M —→
```

mnn4 [mannosyl phosphate transferase⁻]

mnn3 [α-1→2-mannosyltransferase II⁻]

```
                                      —→ M —→ M —→ M —→ M —→ M —→
                                          ↑2      ↑2      ↑2      ↑2
                                          |1      |1      |1      |1
                                      M      M      M      M
                                          ↑2      ↑3      ↑2
                                          |1      |1      |1
                                      M      M      M
                                          ↑3
                                          |1
                                      M
```

```
    —→ M —→ M —→
        ↑2
        |1
    M
```
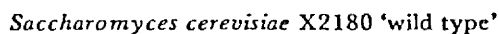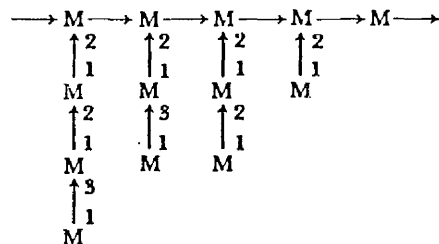
to the inner core (Nakajima and Ballou, 1975a) as well as the presence

of mannosylphosphate units (Rosenfeld and Ballou, 1974a). All of

these mutants are able to form a complete α-(1$\rightarrow$6)-backbone and none

of them appear to have any dramatic effect on the cell (Ballou, 1976).

The mutations do not alter the activity or stability of external

invertase, cause no change in cell viability or in osmotic fragility

and have no obvious effect on morphology. Only the mnn 1/mnn 1 (diploid)

strain showed a decreased tendency to form spores. (Ballou, 1976).

Recently, Ballou et al (1980) have obtained mutants in which the manno-

protein outer chain is greatly shortened, but which still possess a

typical inner core. Alteration in mannoprotein structure, in this

case, is also accompanied by dramatic changes in cellular physiology.

The cells are slow in growing and tend to form clumps by not dividing

cleanly. Cells also have distorted shapes and can lyse during growth.

Finally, homozygous diploids often form defective spores.

IV. YEAST CELL-WALL FORMATION

Wall growth in yeast cells can be regarded as the result of

wall material deposition, firstly around the whole periphery in non-

budding cells, and secondly at the tip of the daughter cell or at the

cross-wall that separates dividing cells. In other words, cell-wall

material is either uniformly deposited or is somehow directed to

specific growth regions. Johnson observed polar incorporation of

tritiated glucose into glucan forming at the bud apex in S. cerevisiae

(Johnson and Gibson, 1966; Johnson, 1968). By exposing cells to

fluorescein-conjugated concanavalin A, and allowing them to grow in

the absence of the label, Tkacz and Lampen (1972) showed that mannan

is also inserted at the distal end of the bud tip. Chung et al (1965),

however, used fluorescent antibodies against whole yeast cells and obtained contradictory results, indicating that new wall growth occurred at the base rather than the apex of the bud. Originally both glucan and mannan components were thought to be synthesised continuously throughout the cell cycle (Sierra et al, 1973). However, reevaluation of these results in the light of contradictory evidence has indicated that this is not so and that the rate of synthesis of mannan and glucan is considerably reduced at the time of cell division and in the prebudding phase (Biely, 1978). The almost exclusive location of chitin in the bud scar region (section IIC) and its discontinuous synthesis throughout the cell cycle (Cabib and Farkaš, 1971) imply that chitin synthesis is directed (Cabib, 1975).

Synthesis of the three polysaccharide components of the cell wall has been studied by several experimental approaches. Firstly, the overall synthesis and location of polymers has been investigated by various labelling techniques, for example pulse-chase autoradiography or specific lectin-labelling. However, this method has an inherently low resolving power. Secondly, the formation and secretion of intact wall components by yeast protoplasts (Nečas, 1971) or by whole cells, and inhibition of this process by compounds that prevent protein or polysaccharide synthesis has, so far, only shown that protein synthesis is coupled to mannan but not glucan synthesis (Elorza and Sentandreu, 1969; Farkaš et al, 1969). The third approach has been used extensively to determine the molecular mechanisms involved in synthesis of the cell-wall components. It involves incorporation of radioactivity from labelled precursors into endogenous and exogenous acceptors, using cell-free extracts.

Information on the synthesis of the three individual wall

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components, chitin, glucan and mannan, is discussed here, while regulatory aspects of cell-wall formation at the molecular and cellular levels are summarised in section V. Due to the wealth of information on fungal cell-wall synthesis, most of the discussion will relate to the processes in S. cerevisiae. Further literature on cell-wall synthesis in other yeasts and fungi can be found in reviews by Cabib (1975), Ballou (1976) and Farkaš (1979).

A.   Chitin biosynthesis

The first description of fungal chitin biosynthesis, in vitro, was by Glaser and Brown (1957) who used a cell-free extract of Neurospora crassa. Since then, chitin synthesis has been detected in twelve diverse genera representing all five major groups of fungi as well as in different developmental forms (examples listed by Ryder and Peberdy, 1977 and by Cabib et al, 1979). A single enzyme, chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxy-glucosyltransferase, EC 2.4.1.16), is involved and the general equation for the reaction is

$$\text{UDP-GlcNAc} + \left[\beta\text{-}(1\rightarrow4)\text{-GlcNAc}\right]_n \longrightarrow \left[\beta\text{-}(1\rightarrow4)\text{-GlcNAc}\right]_{n+1} + \text{UDP}$$

$$\text{primer} \qquad\qquad\qquad \text{product}$$
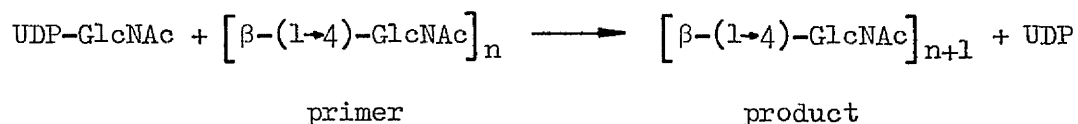
Chitin synthesis in Saccharomyces is discontinuous throughout the cell cycle (Cabib and Farkaš, 1971) and concomitant with bud emergence (Hayashibe and Katodha, 1973; Hasilik, 1974). The properties of chitin synthase from a particulate preparation of S. carlsbergensis were described by Keller and Cabib (1971). The reaction requires divalent cations, free N-acetylglucosamine and is optimal at pH 6.2. The enzyme can be inhibited by mono- and poly-valent cations and by the antibiotic polyoxin D, a structural analogue of UDP-GlcNAc, that acts as a strong competitive inhibitor.

Chitin synthase exists in cells largely in a latent or zymogenic state (Cabib and Farkaš, 1971). Using purified plasma membranes and intact sphaeroplasts, Durán et al (1975) showed that zymogen was only associated with the inner surface of the plasma membrane. 'Solubilisation' of the synthase with digitonin yielded an enzyme with a molecular weight of about 500,000 (Durán and Cabib, 1978). This enzyme was still inactive, which suggested that the latency of the synthase was not merely due to shielding by a membranous structure. Fluorescence microscopy and autoradiography revealed that the zymogen is uniformly distributed over the entire inner surface of the plasma membrane (Durán et al, 1979). However, evidence has been presented that the chitin synthase zymogen is not exclusively located on the plasma membrane. Bartnicki-Garcia et al (1978) have found chitin synthase activity in intracellular microvesicles, termed chitosomes, which measure 40-70 nm in diameter. The enzyme is present in the zymogen state and, after addition of UDP-GlcNAc and trypsin, chitosomes synthesise chitin microfibrils in vitro. Similar chitosomes, isolated from Mucor rouxii, can be dissociated into smaller sub-units (molecular weight of about 500,000 and 7-12 nm in diameter) by treatment with digitonin (Ruiz-Herrera et al, 1980). These subunits are also capable of synthesising microfibrils, some aggregates of which are similar to the 'lozenge'-shaped chitin particles described by Durán and Cabib (1978). The subunits can reassemble upon removal of digitonin (Bartnicki-Garcia et al, 1979). It was therefore suggested by Ruiz-Herrera et al (1980) that the 'solubilised' enzyme prepared by Durán and Cabib (1978) was, in fact, still particle-bound to such chitosome subunits. Schekman and Brawley (1979), using the same method described by Durán et al (1975), found that lysates of S. cere-

visiae contain chitin synthase activity associated not only with a plasma-membrane fraction but also with a microsomal fraction.

In crude sphaeroplast lysates from yeast, the zymogen is contaminated by an activating factor that converts inactive synthase to the active form. The activating factor is associated with a vacuolar fraction (Cabib et al, 1973) and can be released by subjecting the sphaeroplast lysate to mild sonic oscillation, followed by centrifugation (Cabib and Farkaš, 1971). When properties of the purified activating factor were compared with those of the known yeast proteinases (reviewed by Holzer et al, 1975), it was concluded that the activating factor was identical to the neutral, serine proteinase B (EC 3.4.4.8) (Cabib and Ulane, 1973; Hasilik and Holzer, 1973). Although other yeast proteases are unable to activate chitin synthase, many proteases from different sources - trypsin, chymotrypsin, subtilisin and papain, for example - are capable of doing so (Ulane and Cabib, 1976). Chitin synthase is also activated by α-mating factor. This pheromone increases the rate of synthesis of zymogen and leads to activation of a plasma-membrane fraction synthase (Schekman and Brawley, 1979).

The supernatant fraction, obtained after lysis of yeast sphaeroplasts contains a heat-stable protein which was originally believed to be an inhibitor of chitin synthase itself (Cabib and Keller, 1971) but which is now known to bind to the activating factor, thus rendering it ineffective (Cabib and Farkaš, 1971). The inhibitory protein, purified to homogeneity by Ulane and Cabib (1974) has a molecular weight of about 8,500 and lacks methionine, cysteine, arginine and tryptophan. A protein capable of inhibiting the in vitro synthesis of chitin in extracts of Mucor rouxii has also been shown (McMurrough and Bartnicki-Garcia, 1973; Ruiz-Herrera and Bartnicki-Garcia, 1976). This protein has been purified by López-Romero et al (1978) and shown

to inhibit activated chitin synthase. It also inhibits enzyme activity in dissociated chitosome subunits from M. rouxii (Ruiz-Herrera et al, 1980).

A working hypothesis for chitin synthesis in yeast was first proposed by Cabib and Farkaš (1971) and further developed by Cabib and his coworkers (Cabib, 1975, 1976; Cabib et al, 1979). In this model, chitin synthase, a zymogen, is uniformly distributed over the inner surface of the plasma membrane. Vesicles carrying the activating factor coalesce with the plasma membrane at the site of chitin deposition, permitting activation of the zymogen in a restricted area. The inhibitor functions as a safety device to inactivate any vacuolar protease that might spill into cytoplasm thus preventing random activation of the enzyme. Prolonged contact of the activating factor with the zymogen has been proposed by Hasilik (1974) as a mechanism for termination of chitin synthesis. He found that prolonged incubation of the zymogen and activating factor leads to a slow inactivation of chitin synthase. Similarly, a decrease in chitin synthase activity in toluenised cells and particulate fractions from S. cerevisiae occurs after prolonged incubation with trypsin or pronase (Dominguez et al, 1980).

Support for Cabib's hypothesis was recently provided by Braun and Calderone (1979), who obtained similar results with Candida albicans. Nevertheless, doubts remain concerning the role of proteases in the activation of chitin synthase. Mutants of S. cerevisiae, deficient in proteinase B, but not in proteinases A or C, are able to grow, divide and form apparently normal septa containing chitin (Wolf and Ehmann, 1979; Zubenko et al, 1979). This, however, does not rule out the possibility of a proteolytic mechanism for chitin synthase activation. Farkaš (1979) recently suggested that the real activating

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factor of chitin synthase might be a minor protease that would be difficult to detect in the usual protease assays. Dominguez et al (1980) have investigated the action of trypsin and pronase on the activation and degradation of chitin synthase and the effect of inhibition of protein and RNA synthesis on the regulation of its activity. They suggested from their results that exogenous proteases may act indirectly by altering the membrane environment of chitin synthase and that regulation of chitin synthase in vivo may take place independently of proteases.

B. Glucan biosynthesis

The molecular mechanism of glucan synthesis is poorly under-stood and uncertainty still exists concerning both the cellular location and regulation of glucan synthase. Farkaš (1979) has listed four factors which may have hindered research in this area. (i) Severe mechanical treatment of cells might disturb the spatial relationship of individual components in the glucan-synthesising machinery leading to a loss of activity. (ii) Glucan synthase could be inactivated by non-specific proteolysis. (iii) Enzyme expression could be prevented by combination of the synthase with various effectors during the isolation procedure. (iv) There might be hydrolysis of products or substrates during the assay by enzymes present in the crude particulate enzyme preparations (Sonnino et al, 1966).

Early studies on the nature of glucan synthesis involved cell-wall regeneration in yeast protoplasts. The fibrillar wall component synthesised by S. cerevisiae protoplasts in liquid media consists of crystallised glucan (Nečas, 1971). Microfibril formation, unlike the formation of mannoprotein matrix, is independent of protein synthesis (Nečas et al, 1968) while both are sensitive to inhibition

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of polysaccharide synthesis by 2-deoxyglucose (Farkaš et al, 1969).
Similarly glucan synthesis by intact cells, incubated with [14C]glucose,
is initially independent of protein synthesis (Elorza and Sentandreu,
1969).

That UDP-glucose could act as a precursor for glucan synthesis
in S. cerevisiae was first demonstrated using cells treated with a
toluene-ethanol mixture to make them permeable to small molecules
(Sentandreu et al, 1975). When the treated cells were incubated with
UDP-[14C]glucose, about 60% of the incorporated label was in β-(1-3)glucan.
No glucosyltransferase activity, however, was found in membrane particles.

Bálint et al (1976) reported the synthesis of β-glucans from
both GDP- and UDP-[14C]glucose, using particulate preparations of
S. cerevisiae. Following digestion with different partially-purified
β-glucanases, the products were tentatively characterised as mainly
β-(1→3)- and β-(1→6)- glucans for UDP- and GDP-glucose respectively.
Positive identification of products of β-glucan synthase activity was
obtained using cell-free extracts incubated with UDP-[14C]glucose
(López-Romero and Ruiz-Herrera, 1977). The polymer synthesised
contained mainly β-(1→3)glucan with a small but significant proportion
of β-(1→6)-glucosidic bonds. Contrary to the findings of Bálint
et al (1976), the highest specific activity of glucan synthase was
associated with the cell wall fraction and the amount of radioactivity
incorporated from GDP-[14C]glucose was negligible.

The kinetics of β-glucan synthase have been further studied
in the same system by López-Romero and Ruiz-Herrera (1978). Optimal
activity occurred at pH 6.7 and 24°C and activity was slightly
stimulated by the addition of Mg^{2+} or Mn^{2+} ions. UDP acted as a
competitive inhibitor, while glucano-ƍ-lactone was a strong non-com-

petitive inhibitor. The crude enzyme was unstable at 0° and $28^{\circ}C$,
activity being lost rapidly at the higher temperature. Since neither
pepstatin A nor PMSF protected the β-glucan synthase from this
destruction, inactivation does not appear to be due to the action of
proteinases A or B. Part of the β-glucan synthase from Phytophora
cinamoni exists in a zymogen form that can be activated by trypsin
(Wang and Bartnicki-Garcia, 1976). However it seems unlikely that
β-glucan synthase exists as a zymogen in S. cerevisiae since acid
proteases had no effect on the enzyme and neutral proteases were
slightly destructive at high concentrations (López-Romero and Ruiz-
Herrera, 1978).

Recently Shematek et al (1980) achieved an 80-fold increase
in specific activity for β-(1→3)glucan synthase over that reported by
López-Romero and Ruiz-Herrera (1978). Their system differed from
that of the latter workers in that there was no synthesis of β-(1→6)-
glucosidic linkages and the enzyme was optimal at pH 8. Although
insoluble glucan was found in the particulate enzyme (Shematek et al,
1980), all chains were apparently synthesised de novo, without a primer.
This was shown by analysing the products for the location of $\begin{bmatrix}^{14}C\end{bmatrix}$-
labelled sugars. No radioactivity was incorporated from GDP-$\begin{bmatrix}^{14}C\end{bmatrix}$glucose
and synthase activity was rapidly lost at $30^{\circ}C$ in the absence of
substrate. Enzyme activity was localised on the inner surface of the
plasma membrane, as has also been demonstrated for the chitin synthase
zymogen (Durán et al, 1975; Section IVA). Enzyme activity was
stimulated by ATP, GTP or by a heat-stable activator, purified from
yeast, whose active component appears to be GTP. This was further
investigated by Shematek and Cabib (1980). GTP is rapidly and
irreversibly bound to the enzyme, at $0^{\circ}C$ with EDTA, where it is

converted to GDP. Although ATP acts at the same site, an incubation period at higher temperature is required to stimulate enzyme activity. ATP does not stimulate enzyme preparations which have been centrifuged or treated with alkaline phosphatase but its ability to stimulate activity can be restored by the addition of supernatant fluid prepared by incubation of the particulate enzyme followed by centrifugation. The supernatant factor can also prevent the loss of synthase activity at 30°C and this protective activity is reduced by alkaline phosphatase treatment. The exact nature of the supernatant factor is not known. It is heat-stable and of low molecular-weight and while Shematek and Cabib (1980) suggest that it may be GDP or GMP, its action cannot be mimicked by certain compounds including GMP, GDP, GTP, ADP and ATP.

Incorporation of $\left[^{14}C\right]$glucose from UDP-$\left[^{14}C\right]$glucose into a lipid fraction is stimulated by yeast dolichol phosphate (Palamarczyk and Chojnacki, 1973). Baguley (1977) also reported that dolichol phosphate can be glucosylated by a membrane-bound enzyme incubated with UDP-glucose. This reaction was specifically inhibited by the antibiotic, papulacandin B, whereas the mannosylation of dolichol phosphate remained unaffected. A later report (Baguley et al, 1979) indicates that inhibition of glucan synthesis by papulacandin B in vitro only occurs at very high drug concentrations, or at low protein concentrations as with those required for in vivo inhibition. In this report, no evidence for a lipid intermediate was presented. Similarly no such intermediate was detected by Sentandreu et al (1975) or Shematek et al (1980).

C. Mannan biosynthesis

(i) Incorporation studies with radioactive precursors

Mannan synthesis in growing cells has been studied by

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following the incorporation of $[^{14}C]$ glucose and $[^{14}C]$ threonine into the polysaccharide and peptide moieties of mannan respectively (Sentandreu and Northcote, 1969b). Sentandreu and Lampen (1970) used this method to show that mannan synthesis, _in vivo_, is inhibited by cycloheximide, presumably by preventing formation of the polypeptide mannose acceptor (see regulation of mannan synthesis, section IV C, iii).

All mannosyl units in the mannan molecule originate from GDP-mannose. This was shown by following the incorporation of $[^{14}C]$ mannose from GDP-$[^{14}C]$ mannose into endogenous acceptors, using sphaeroplast lysates of S. carlsbergensis as the enzyme source (Algranati et al, 1963; Behrens and Cabib, 1968). The enzyme preparation had a K_m for GDP-mannose of 0.5 mM and an absolute requirement for Mn^{2+}; no lipid intermediate was detected by paper chromatography. Tanner (1969), on the other hand, incubated S. cerevisiae membranes with GDP-$[^{14}C]$ mannose and detected the formation of a substance with the properties expected of a mannosyl-monophosphate-lipid, which could be separated from reaction mixtures by paper chromatography. The reaction required either Mn^{2+} or Mg^{2+} ions and the product behaved kinetically like a mannan precursor. A similar product was extracted from reaction mixtures with chloroform-methanol (2:1, v/v; Sentandreu and Lampen, 1971); its formation was stimulated by yeast dolichol phosphates, which also acted as acceptors for mannosyl residues from GDP-mannose (Tanner et al, 1971). The lipid intermediate was subsequently identified as an alkali-resistant, mild acid-labile mannosyl-1-phosphoryl polyprenol (Sentandreu and Lampen, 1972) and the polyprenol has been isolated and characterised as a mixture of five dolichols with 14-18 unsaturated α-isoprene units (Jung and Tanner, 1973). The structure of dolichol monophosphate is

$$CH_3 \Big\rangle C{=}CHCH_2{-}\Big[CH_2\overset{\overset{\displaystyle CH_3}{|}}{C}H{=}CHCH_2\Big]_{13-17}{-}CH_2\overset{\overset{\displaystyle CH_3}{|}}{C}H{-}CH_2CH_2O\overset{\overset{\displaystyle O}{||}}{\underset{\underset{\displaystyle O^-}{|}}{P}}OH$$
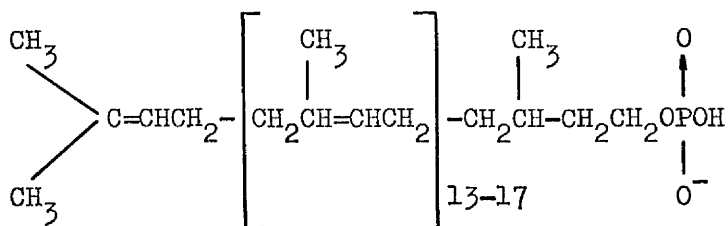
The involvement of lipid intermediates has been demonstrated in the biosynthesis of both carbohydrate moieties of mannan (figure 1, p. 31). The participation of dolichol monophosphate mannose in O-glycosylation of mannan was shown using a crude particulate fraction from yeast (Babczinski and Tanner, 1973; Sharma et al, 1974). The short, β-eliminable side-chains are synthesised by the following sequence of reactions in which dolichol monophosphate mannose is a precursor only for those residues attached directly to serine or threonine:

1. GDP-mannose + dolichol-P $\xrightarrow{\;Mn^{2+}\ or\ Mg^{2+}\;}$ dolichol-P-mannose + GDP

2. dolichol-P-mannose + (Ser/Thr)-protein $\xrightarrow{\;Mn^{2+}\ or\ Mg^{2+}\;}$ dolichol + mannose-(Ser/Thr)-protein

3. nGDP-mannose + mannose-(Ser/Thr)-protein $\xrightarrow{\;Mn^{2+}\;}$ nGDP + (mannose)$_{n+1}$-(Ser/Thr)-protein
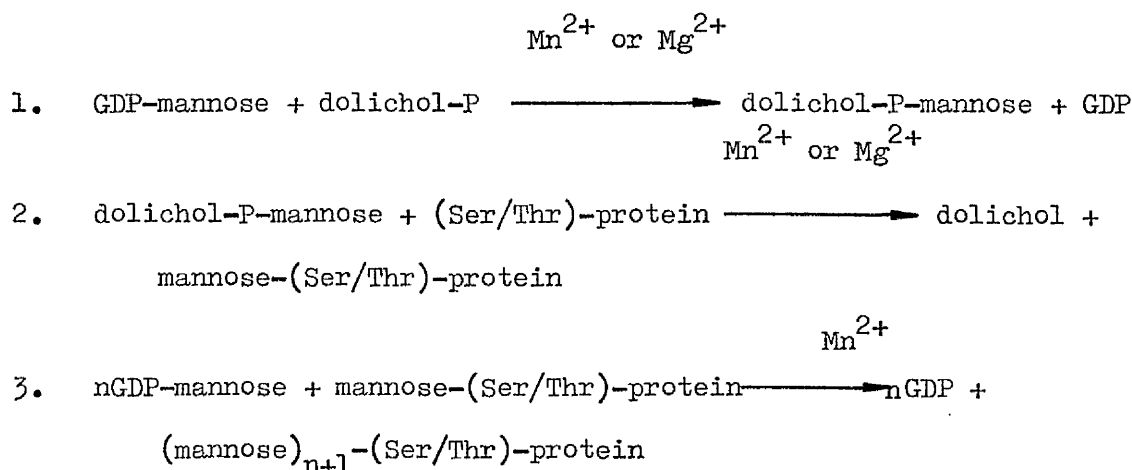
where n varies between 1 and 3.

The enzymes involved in steps 1 and 2 have been solubilised by Babczinski et al (1980). Recently Lehle and Tanner (1978b) have shown that mannose is linked to dolichol as the β-anomer. Its transfer to protein therefore requires an inversion of configuration to give an α-D-mannosyl-peptide (Bause and Lehle, 1979).

Formation of the N-glycosidically-linked inner core of

mannan requires a similar, but more complicated reaction sequence involving lipid intermediates. The outer chain, however, is synthesised directly from GDP-mannose without the participation of a lipid intermediate (Parodi, 1979a). In the case of the inner core, N-acetyl-glucosamine-1-phosphate is initially transferred from UDP-GlcNAc to form a lipid diphosphate N-acetylglucosamine that is soluble in the lower phase of chloroform-methanol-water (3:2:1, by vol.) mixtures (Lehle and Tanner, 1975). The characterisation of the lipid moiety as dolichol was confirmed by Reuvers et al (1978a). Subsequently, a series of more hydrophilic lipid intermediates have been extracted with more polar chloroform-methanol-water (10:10:3, by vol.) mixtures.

Yeast particulate enzyme preparations and, more recently, solubilised enzymes (Parodi, 1979a; Palamarczyk et al, 1980) have been shown to synthesise in vitro, dolichyldiphosphate derivatives containing di-N-acetylchitobiose (Lehle and Tanner, 1975; Palamarczyk, 1976; Reuvers et al, 1977; Welten-verstegen et al, 1980; Palamarczyk et al, 1980) or di-N-acetylchitobiose bearing one (Lehle and Tanner, 1975; Nakayama et al, 1976; Palamarczyk, 1976; Lehle and Tanner, 1978a) or several mannose residues (Parodi, 1978, 1979a; Lehle and Tanner, 1978a). Glucosyl units have also been detected in these intermediates (Parodi, 1976, 1977, 1979b; Dominguez et al, 1978c). Although large lipid-bound oligosaccharides, consisting of up to 20 hexose units, can transfer the carbohydrate portion to endogenous and exogenous proteins in vitro (Lehle and Tanner, 1978a,b; Parodi, 1977, 1978, 1979a), Lehle and Tanner (1978b) have shown that full elongation of the lipid-bound oligosaccharide is not an absolute requirement for its transfer to protein. They found that with increasing carbohydrate chain length, the transfer reaction rate increases. Dolichol derivatives are

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effective as glycosyl donors only after incorporation of at least a
second N-acetylglucosamine residue and preferably also a mannose
residue (Pless and Palamarczyk, 1978).   It is possible that, in vivo,
the small lipid-bound oligosaccharides are rapidly converted to
larger ones, thus reducing the likelihood of disaccharide or tri-
saccharide transfer to protein.

The presence of glucose in these lipid-bound oligosaccharides
(Parodi, 1976, 1977; Dominguez et al, 1978c) is intriguing since it is
not normally found in mannans isolated from yeast (see section III). Gluc-
ose may be added via a further lipid intermediate, dolichol monophosphate
glucose (Palamarczyk and Chojnaki, 1973; Parodi, 1976, 1977).   Recent
results of Parodi (1979b) indicate that the addition of glucose is not an
artefact produced in vitro.   He pulsed cells with $[^{14}C]$glucose and
then chased, for different time periods, with medium containing
unlabelled monosaccharide.   He found that up to three glucose
residues were attached to lipid-bound oligosaccharides as well as to
those oligosaccharides attached to asparagine.   Samples taken after
short chase periods revealed that these oligosaccharides gradually
lost their glucose residues.   This "processing" of the oligosaccharide,
and assembly of large dolichol diphosphate oligosaccharides in yeasts,
is analagous to mechanisms involved in the biosynthesis of N-glyco-
sylated mammalian and plant glycoproteins;   these have been reviewed
recently by Parodi and Leloir (1979) and Elbein (1979).

The structural requirements of the peptide for N-glycosyl-
ation have been studied.   Khalkhali et al (1976) have investigated
the glycosylation site by incorporation of GlcNAc from UDP-GlcNAc into
pancreatic ribonuclease by yeast membrane preparations.   Bause and
Lehle (1979) studied, in more detail, the transfer of $(GlcNAc)_2$ to

synthetic peptides using dolichyldiphosphate-di-N-acetylchitobiose as a glycosyl donor. Their results indicate that the tripeptide Asn-X-(Ser/Thr) (where X is any amino acid) is the minimal requirement for N-glycosylation and that the rate of glycosylation increases with longer chain peptides.

Syntheses of the two carbohydrate moieties in the mannan molecule proceed independently and the β-eliminable saccharides attached to serine or threonine do not serve as precursors in the formation of the polysaccharide part of the mannoprotein (Farkaš et al, 1976a). It has also been shown recently that the transferases involved in the addition of mannose to the outer chain are quite distinct from those required for inner core synthesis (Parodi, 1979a). Figure 3 summarises the intricate steps involved in mannan synthesis; this scheme does not, however, detail the reactions leading to the formation of individual glycosidic linkages.

The specific order of formation of different glycosidic linkages between the mannosyl units and the sizes of the side chains are most probably determined by the substrate specificities of individual mannosyltransferases. Evidence for this comes from studies with enzyme systems where mannose and short, defined oligosaccharides were used as exogenous acceptors. Membrane preparations could use mannose as an acceptor for the $\alpha(1\rightarrow2)$mannosyltransferase while $\alpha-(1\rightarrow6)$mannooligosaccharides served as substrates for both the $\alpha-(1\rightarrow6)-$ and $\alpha-(1\rightarrow2)$mannosyltransferases (Lehle and Tanner, 1974; Farkaš et al, 1976b). Similar results were obtained by Nakajima and Ballou (1975b) using a soluble multiglycosyltransferase preparation obtained by treating S. cerevisiae membranes with Triton X-100 and urea. They also found that reduced $\alpha-(1\rightarrow2)$-linked mannobriose acts

Figure 3.   Schematic representation of the role of dolichol

derivatives in yeast mannoprotein biosynthesis.

Dol, dolichol; GlcNAc, N-acetylglucosamine; Glc,

glucose; Man, mannose; P, phosphate; PP, diphosphate;

GDP and UDP, guanosine and uridine diphosphate;

Asn-X-(Ser/Thr), polypeptide acceptor sequence containing

asparagine, any amino acid and serine or threonine.

GDP-Man ⟶ Dol-PP-(GlcNac)$_2$ (Man)$_{2-15}$

Dol-P

Dol-P-Man   Dol-P-Glc

Dol-PP-(GlcNAc)$_2$ Man   Dol-P

GDP-Man   GDP-Man   Dol-PP-(GlcNAc)$_2$ (Man)$_{15}$(Glc)$_3$

Dol-PP-(GlcNAc)$_2$

UDP-GlcNAc   UDP-Glc

Dol-PP   (Ser/Thr)-X-Asn

Dol-PP-GlcNAc

P

UDP-GlcNAc   Dol-P   (Ser/Thr)-X-Asn-(GlcNAc)$_2$(Man)$_{15}$(Glc)$_3$

GDP-Man

Asn-X-(Ser/Thr)   (Glc)$_3$

Dol-P-Man

Dol-P   (Ser/Thr)-X-Asn-(GlcNac)$_2$(Man)$_{15}$

Asn-X-(Ser/Thr)

GDP-Man   GDP-Man

Asn-X-(Ser/Thr)-(Man)$_{2-4}$ (Ser/Thr)-X-Asn-[(GlcNAc)$_2$(Man)$_{15}$](Man)$_{100-150}$

β-eliminable   alkali-stable
oligosaccharide   polysaccharide

as an acceptor for the α-(1$\rightarrow$3)mannosyltransferase. The order of the

affinities of the transferases for the donor, GDP-mannose, is

α-(1$\rightarrow$6) > α-(1$\rightarrow$3) > α-(1$\rightarrow$2) (Nakajima and Ballou, 1975b). Evidence

for different mannosyltransferases can also be drawn from the synthetic

capacity of mutants that produce defective mannoproteins due to the

absence of specific mannosyltransferase activities (Nakajima and

Ballou, 1975b; Farkaš and Bauer, 1976). Karson and Ballou (1978)

have recently solubilised a mannosylphosphate transferase which requires

Mn^{2+} or Co^{2+} ions for activity. This enzyme catalysed the transfer

of mannosyl-1-phosphate from GDP-mannose to the carbon atom at

position 6 in the penultimate mannosyl unit of reduced α-(1$\rightarrow$2)-linked

mannotetraose. As with the other mannosyltransferases involved in

the synthesis of the outer chain (Parodi, 1979a), this enzyme does not

require a lipid intermediate.

Although mannosyltransferases with no requirement for a lipid

intermediate have been solubilised with Triton X-100 (Nakajima and

Ballou, 1975b), those enzymes involved in the dolichol pathway of

mannan biosynthesis have been studied mainly in crude, particulate

systems. Several of these enzymes have now been successfully

solubilised but, as yet, most of the solubilised preparations have

not been tested on defined substrates. Palamarczyk (1976) treated a

yeast particulate fraction with Triton X-305 to obtain an enzyme capable

of transferring GlcNAc from UDP-$[^{14}C]$GlcNAc to lipid. The transfer-

ases responsible for the formation of dolichol monophosphate mannose

and dolichol diphosphate oligosaccharides have been solubilised with

Triton X-100 and purified on a DEAE-cellulose column (Parodi, 1979a).

Babczinski et al (1980) have recently solubilised and purified the

two enzymes involved in the formation of dolichol monophosphate mannose

and the subsequent transfer of the mannose residue to serine or threonine. The yeast enzymes transferring GlcNAc from UDP-GlcNAc to dolichol phosphate and the chitobiosyl group from dolichol diphosphate-$(GlcNAc)_2$ to a hexapeptide (Bause and Lehle, 1979), containing the Asn-X-Ser sequence, have also been solubilised by Palamarczyk et al (1980). These two solubilised enzymes and the two involved in O-mannosylation (Babczinski et al, 1980) show an obligatory requirement for α-saturated polyprenols of a minimum chain length (greater than 35 carbon atoms).

All four enzymes solubilised by workers in Tanner's laboratory (Babczinski et al, 1980; Palamarczyk et al, 1980) have been extremely unstable at 0° and -25^oC due to enzyme inactivation by detergents. Palamarczyk et al (1979) showed that non-ionic detergents have a strong inhibitory effect on the formation of dolichol diphosphate-GlcNAc, dolichol diphosphate-$(GlcNAc)_2$ and dolichol monophosphate mannose. This inhibition can be prevented to a large extent when the enzyme is saturated with dolichol phosphate. Although it should be possible to protect enzymes during solubilisation by dolichol phosphate, this is not commercially feasible. Enzymes that do not involve dolichol steps are much more stable at 4^oC (Sharma et al, 1974; Parodi, 1979a).

(ii) Localisation of mannan synthesis

Behrens and Cabib (1968) considered that mannosyltransferase activity might be plasma membrane-associated. However, the evidence since then implies that mannoprotein formation is a vectorial process involving the glycosylation of nascent polypeptides along the rough and smooth endoplasmic reticula, followed by translocation across the plasma membrane to the cell wall. Cortat et al (1973) using the same assay procedure as Behrens and Cabib (1968), found that the highest mannosyl-

transferase activity was localised in a light-membrane fraction obtained by urografin density-gradient centrifugation. These membranes were considered to be derived from fragments of the endoplasmic reticulum (Moor, 1967). Smaller amounts of activity were also associated with plasma-membrane fragments and glucanase-containing, golgi-like vesicles (Cortat et al, 1972). Using 'pulse-chase' techniques with tritiated mannose, Košinová et al (1974) showed by autoradiography that the initial sites of mannose incorporation were on intracellular membranes and that the incorporated tritium was eventually translocated to the periphery of the cell.

Mannan biosynthesis commences with the formation of a poly-peptide on ribosomes on the rough endoplasmic reticulum. Initial incorporation of mannose and smaller amounts of glucose and glucosamine occurs while the nascent polypeptide chains are still attached to polysomes (Ruiz-Herrera and Sentandreu, 1975). Larriba et al (1976) similarly found that O-glycosylation of the hydroxyamino acids, via dolichol monophosphate mannose occurs at the polysomal level. Using the urografin-gradient fractionation procedure of Cortat et al (1973), Lehle et al (1977) studied the synthesis of both the β-eliminable saccharides and the alkali-stable portion of the mannoprotein. Their results indicated that dolichol-dependent steps occur on the endo-plasmic reticulum. Moreover, as the mannose chains elongate, mannosyl-ation appears to occur increasingly on the more peripheral membranes (i.e. on the golgi-like vesicles and the plasma membrane). A similar, but more detailed, study was made (Marriott and Tanner, 1979) in which sucrose density gradients were used to allow the inclusion of divalent cations during fractionation. S. cerevisiae sphaeroplast homogenates were fractionated into six membrane bands and 75% of the total manno-

syltransferase activity involved in O- and N-glycosylation was found in an endoplasmic reticulum-associated fraction. Less than 3% of the activity was detected in plasma membrane-rich fractions. Transferase activity has also been found in mitochondrial (Palamarczyk, 1976; Palamarczyk and Janczura, 1977; Marriott and Tanner, 1979) and nuclear (Palamarczyk and Janczura, 1977) membrane fractions. Santos et al (1978) reported that a large part of the mannosyltransferase activity found in the cell (80%) is associated with internal membranes. However, they found that the remaining activity (20%) was located in a purified plasma-membrane fraction. Part of this activity was located on the outer surface of the plasma membrane and was termed ectomannosyltransferase activity (Santos et al, 1978). Welten-verstegen et al (1980) have isolated a highly-purified plasma-membrane fraction from S. cerevisiae capable of glycosylating endogenous proteins by N-glycosylation with little, if any, O-glycosylation.

The mechanism of glycoprotein transport is still a subject for speculation. Mannan might be transported to the cell periphery by two methods: firstly, secretion of polysaccharides by reverse pinocytosis of vesicles and secondly, production of mannan in close association with the plasma membrane. The latter method is supported by Meyer and Matile (1975) who failed to find invertase secretion associated with vesicles in S. cerevisiae sphaeroplasts. They concluded that the glycoprotein was transported through the membrane. Others, working with intact cells, have concluded that exocytosis is the mechanism for invertase secretion (Holly and Kidby, 1973) and secretion of mannoproteins in general (Cortat et al, 1973).

Electron-microscopy studies on acid phosphatase deposition (Linnemans et al, 1977) and studies on the subcellular location of

mannosyltransferases (Santos et al, 1978) now tend to indicate that both

the above methods of mannan deposition are involved in cell-wall

formation. Firstly, the cell wall has to be built up during bud

formation and this might occur by reverse pinocytosis of golgi-type

vesicles (granulocrine mode). Such vesicles can be seen to accumulate

at the site of bud formation in the mother cell and are also found at

the tip of the bud during its growth (Moor, 1967; Sentandreu and

Northcote, 1969a). In the second method, thickening or ageing of the

mother cell-wall would occur by secretion of materials directly through

the plasma membrane and further glycosylation would occur at the cell

surface by ectomannosyltransferases (ecrine mode). Evidence supporting

the existence of both mechanisms in the secretion of glucan at different

stages during the cell cycle has been reported by Rodriguez et al

(1979), who investigated wall formation in S. cerevisiae by electron

microscopy. Cells were grown under conditions in which RNA and

protein synthesis were inhibited but glucan synthesis remained

unaffected; this allowed an examination of wall thickening due to the

accumulation of glucan.

(iii) Regulation of mannan synthesis

A great deal is known about the chemical structure (section

III) and enzymatic mechanisms (section IV C, i) in the assembly of

mannoproteins in S. cerevisiae. There is also some evidence regarding

the location of individual mannosyltransferases. However, our knowledge

of the control of these biosynthetic pathways is relatively patchy and

insubstantial. There is evidence for the control of mannoprotein

synthesis at two levels. Firstly, regulation can occur at the level

of transcription or translation of both the mannosyltransferases and

the mannoprotein polymers themselves. Secondly, post-translational

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control can act at the level of enzyme activity, both that of glycosyl-
transferases and of enzymes involved in sugar activation.   The
important role that compartmentalisation plays in the regulation of
eucaryotic glycoprotein synthesis, through physical separation of the
biosynthetic machinery, is discussed in section V.

(a) Regulation of mannoprotein and mannosyltransferase
synthesis

Periodic synthesis affords cells a method of controlling
protein concentration.   Synthesis of many enzymes in yeast, including
invertase (Gorman et al, 1964), and wall mannan synthesis (Biely, 1978)
are discontinuous throughout the cell cycle.   Mannan synthesis depends
intimately on protein synthesis and can be prevented by cycloheximide
(Sentandreu and Northcote, 1969b).   Cycloheximide does not affect
mannosyltransferase activity in a cell-free system (Sentandreu and
Lampen, 1972) nor does it influence the relatively low turnover of the
enzymes involved in both glucan and mannan synthesis (Elorza et al,
1976).   These results suggest that cycloheximide blocks mannan
synthesis by depleting the pool of peptides serving as mannosyl
acceptors.   Inhibition of RNA synthesis in intact cells (Elorza et al,
1976) or regenerating protoplasts (Kopecká and Farkaš, 1979) has shown
that RNA messengers of wall mannan peptides have a slow decay rate,
indicating that regulation of synthesis of the mannan peptides occurs
at the level of translation rather than transcription.   Elorza and
her co-workers have similarly studied the mechanisms of regulation
for the catalytic mannoproteins invertase and acid phosphatase.
Invertase synthesis is repressed by glucose and this is not due to
catabolite repression.   Rather, it is due to interference with both
transcription and translation and to activation of mRNA, or polysome,

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degradation (Elorza et al, 1977b). Repression of acid phosphatase synthesis by inorganic phosphate, however, is not caused by interference with mRNA translation, but may be due to the action of phosphate at the level of transcription, either by altering the gene configuration or the RNA polymerase (Elorza et al, 1978).

(b) Control of mannosyltransferase activity

It is likely that mannosyltransferase activity is controlled by several different mechanisms. Firstly the specificity of the enzymes catalysing the individual transfer steps provides an obvious regulation mechanism. Such specificity may relate to the sugar transferred, the acceptor or the position and anomeric configuration of the linkage formed (Ginsburg, 1978). Activity may also be regulated by the availability of certain factors. For instance, the extent of O-glycosylation of S. cerevisiae mannoproteins will depend upon the ionic environment. Transfer of the mannose unit attached directly to serine or threonine requires Mg^{2+} or Mn^{2+}, whereas the subsequent residues are transferred only with Mn^{2+} (Sharma et al, 1974).

The importance of dolichol phosphate in yeast mannoprotein synthesis is now well established (see section IV C, i) and the availability of this carrier to those mannosyltransferases requiring its participation may provide a further control mechanism. Such a role has been proposed for the analogous polyisoprenoid intermediate, undecaprenol phosphate, which functions in the synthesis of bacterial cell-wall components (Anderson et al, 1972). A range of dolichols, containing 60-80 carbon atoms is known to be present in yeast (Jung and Tanner, 1973) although the homologue containing 15 isoprene units is the most abundant (Reuvers et al, 1978a). The existence of distinct pools of dolichol remains to be demonstrated although there

is some evidence for these, possibly located in different internal membrane fractions, in plant (Ericson et al, 1978; Bailey et al, 1979) and mammalian systems (Oliver and Hemming, 1975; Kerr and Hemming, 1978; Godelaine et al, 1979a,b). In addition, Palamarczyk et al (1980) have recently found that four solubilised mannosyltransferases, involved in O- and N-glycosylation of yeast mannoproteins, exhibit variations in their activity with polyprenols of different chain length.

Mannosyltransferase activity may also be modulated by other lipid components. Douglas et al (1975) reported that mannan synthesis in cells and sphaeroplast lysates of S. cerevisiae decreases when the proportion of unsaturation in the membrane lipids is increased. Similarly, mannan synthesis in plasma membranes of Candida albicans is sensitive to changes in the lipid environment (Marriott, 1977). Dominguez et al (1978a) found that enzymes involved in the synthesis of both glucan and mannan in S. cerevisiae NCYC 86, an inositol-requiring strain, were more active in inositol-starved cells than in cells supplemented with inositol. This might be the result of changes in the phospholipid composition of the membranes. Similarly, Hanson and Lester (1980), using an inositol auxotroph of S. cerevisiae, have recently shown that phosphatidylinositol synthesis is required for the synthesis or secretion of glycans. In this connection, it is interesting to note that chitin synthase, when solubilised from particulate fractions of S. cerevisiae, is activated by phospholipids and inhibited by fatty acids (Durán and Cabib, 1978).

Feedback inhibition, preventing over-production of nucleotide precursors for wall components, may play a role in control of cell-wall synthesis. However, the accumulation of GDP-mannose and, to a lesser extent, UDP-N-acetylglucosamine in cells grown with cycloheximide (Sentandreu and Lampen, 1970) indicates that there is little

or no regulation by a feedback mechanism on the metabolic routes leading to the formation of these compounds. On the other hand, there is evidence that mannosyltransferases are regulated by nucleotide pools. ADP, UDP and GDP all competitively inhibit the lipid-independent mannosyltransferases in cells made permeable to GDP-mannose by toluene-ethanol treatment (Elorza et al, 1977a). Changes in the concentration of these nucleotides could therefore effect a rapid modulation of the rate of mannoprotein formation independent of gene transcription and translation.

Finally, mannoprotein synthesis may be regulated by the extent of glycosylation. The importance of glycosylation in manno-protein synthesis and secretion has been studied using inhibitors such as sugar analogues or certain antibiotics. 2-Deoxy-D-glucose and fluoro-sugars indirectly inhibit glycosylation through their UDP- and GDP- derivatives. GDP-2-deoxy-D-glucose blocks further protein glycosylation by transferring its sugar moiety into both β-eliminable (Lehle and Schwarz, 1976) and polysaccharide (Schmidt et al, 1978) chains via the dolichol monophosphate derivative. The antibiotic, tunicamycin – a lipophilic analogue of UDP-N-acetylglucosamine – inhibits mannan synthesis (Kuo and Lampen, 1974) by blocking the transfer of GlcNAc-1-phosphate from UDP-GlcNAc in the synthesis of lipid-bound oligo-saccharides (Lehle and Tanner, 1976). Bacitracin, a cyclic peptide antibiotic, inhibits the incorporation of GlcNAc into lipid-bound oligosaccharides and glycoprotein, as well as the incorporation of mannose into dolichol monophosphate mannose in yeast sphaeroplasts (Spencer et al, 1978). Reuvers et al (1978b) found that bacitracin differs from tunicamycin, in its mode of action, by inhibiting synthesis of dolichol diphosphate-$(GlcNAc)_2$ and the incorporation of GlcNAc into a protein fraction. Under conditions of inhibited glycosylation,

there is no accumulation of carbohydrate-free forms of extracellular mannoprotein enzymes in the cytoplasm (Liras and Gascón, 1971; Kuo and Lampen, 1974) which does indicate the existence of some type of feedback control.

V.   ROLE OF COMPARTMENTALISATION IN MODELS FOR YEAST CELL-WALL FORMATION

A.   Models for cell-wall formation

Wall growth in yeast is generally considered to be the combined result of wall synthesis, wall lysis and turgor pressure (Johnson, 1968).   When this balance is upset, morphological aberrations and cell lysis may ensue.   Cell lysis can occur for cells grown with 2-deoxy-D-glucose (Johnson, 1968) or 2-deoxy-2-fluoro-D-glucose (Biely et al, 1973) which inhibit glucan synthesis (see section IV C, iii b) and, in budding cells, with polyoxin D (Cabib and Bowers, 1975) which inhibits chitin synthesis.   Delocalised synthesis of cell-wall components, resulting in changes in wall morphology, has been found in a cell-cycle mutant (Sloat and Pringle, 1978), an inositol-starved strain (Dominguez et al, 1978b) and α-pheromone-induced a-type cells of S. cerevisiae (Lipke et al, 1976;   Schekman and Brawley, 1979) as well as cells blocked for RNA and DNA synthesis (Rodriguez et al, 1979).   Lysis of the rigid glucan component presumably results from the concerted action, at the growth region, of β-(1→3)glucanases which are found in the periplasmic space and cell wall (Cortat et al, 1972; Farkaš et al, 1973) and in intracellular vesicles (Cortat et al, 1972). Although mannanases have been found in S. carlsbergensis (Maddox and Hough, 1971) and S. cerevisiae (Cortat et al, 1972) these would appear

to be involved mainly in degrading mannan to allow for the insertion of new cell-wall material during growth since cell-wall mannan is metabolically inert once incorporated into the cell wall (Krátký et al, 1975).

Several models have been proposed to describe the mode of construction of the cell wall. These models, in the case of the ellipsoidal yeast cell, eventually have to explain the participation of both polarised growth and spherical extension, as well as the regulatory mechanisms involved in the activity of the individual polysaccharide synthases. Bartnicki-Garcia (1973) proposed a unitary model of wall growth in fungi in which cellular morphology would be determined by the distribution pattern of hypothetical growth units containing both synthetic and lytic enzymes. However, the mechanism of distribution and regulation of synthetic activity was not understood.

Cabib and coworkers (Cabib, 1975, 1976) have studied the specialised synthesis of chitin during the formation of the yeast primary septum. In this model system, chitin synthesis is regulated both temporally and spatially. Cabib's hypothetical model for chitin synthesis, previously discussed in section IV A, involves the activation of a plasma membrane-bound, zymogenic synthase by a vacuolar protease which, in turn, can be inactivated by a cytoplasmic inhibitor. However, it has been shown recently that chitin synthase is also present on internal vesicular membranes, termed chitosomes, in S. cerevisiae and other diverse fungi (Bartnicki-Garcia et al, 1978). Biosynthesis of the other cell-wall components, glucan and mannan, may occur by two complementary mechanisms (Linnemans et al, 1977; Santos et al, 1978; Rodriguez et al, 1979). These are the granulocrine mode, for apical growth, involving reverse pinocytosis by

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vesicles and the ecrine mode, or random cell-wall deposition without vesicles; they are discussed in relation to mannan synthesis in section IV C, ii.

Farkaš (1979) has recently proposed a further model in which some enzymes involved in the formation of cell-wall components exist in a cryptic form in the plasma membrane. He suggested that their activity might be inhibited by contact with the cell wall or by some extracytoplasmic component. Delocalisation of cell-wall synthesis would result from either a disturbance of the contact between the cell wall and plasma membrane or a depletion of a metabolically unstable, extracellular, proteinaceous inhibitor of the synthase. Farkaš again proposes that cytoplasmic vesicles take enzymes, precursors, inhibitors and wall matrix material (synthesised on the rough and smooth endoplasmic reticulum) to the growth region, fuse with the plasmalemma and discharge their contents into the periplasmic space.

Activation and inactivation of polysaccharide synthases is undoubtedly an important mechanism in the regulation of cell-wall formation. Chitin synthase can exist in active or inactive states. The rate and site of cell-wall synthesis, and hence the shape of the cell will be determined by the distribution pattern of the active synthases. Synthases may be active when they reach the plasma membrane (Bartnicki-Garcia et al, 1978), be activated at the plasma membrane by specific proteases (Cabib and Ulane, 1973), be activated on association with lipophilic membrane components (Durán and Cabib, 1978) or become active after localised dissolution of the contact between the cell wall and the plasma membrane (Farkaš, 1979). The synthases may be inactivated either by prolonged action of proteases on the zymogen (Hasilik, 1974) or the presence of synthase inhibitors at

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the cell surface (Farkaš, 1979).   There is no evidence for the existence

of the latter in <u>S. cerevisiae</u> although cytoplasmic inhibitory proteins

have been reported to be active against chitin synthase in <u>Mucor rouxii</u>

yeast and mycelial forms (McMurrough and Bartnicki-Garcia, 1973; Ruiz-

Herrera and Bartnicki-Garcia, 1976; López-Romero <u>et al</u>, 1978) and

against glucan synthase in the filamentous fungus, <u>Saprolegnia monoica</u>

(Fèvre and Dumas, 1977).

Although most of the evidence for the above models has been

derived from studies on chitin synthesis, the exact molecular mechanisms

for activation and inhibition of chitin synthase in yeast are still not

fully understood.   Furthermore evidence for such processes occurring

in glucan and mannan synthesis in <u>S. cerevisiae</u> has not been forthcoming.

B.   <u>Involvement of vesicles in cell-wall synthesis</u>

All of the models proposed for wall growth in fungi postulate

the involvement of vesicles to explain the spatial distribution of the

components of the cell-wall synthesising machinery.   There are two

types of vesicle (large, 0.62 μm and small, 0.43 μm) present in

<u>S. cerevisiae</u> (Cartledge <u>et al</u>, 1977).   These have been considered, by

Moor (1967), to be derived from the endoplasmic reticulum while

Sentandreu and Northcote (1969a) have shown that the large vesicle (or

vacuole) undergoes fission during the cell-cycle and that progeny of

this fragmentation (small vesicles or sphaerosomes) become concentrated

in the neck of the bud suggesting that they are involved in envelope

growth.   Vesicles have been isolated and found to contain β-glucanase,

mannanase and mannan synthase activity (Cortat <u>et al</u>, 1973) as well as

the activating factor of chitin synthase in <u>S. cerevisiae</u> (Cabib <u>et al</u>,

1973).   Chitosome-like microvesicles can also be isolated from

<u>S. cerevisiae</u> and range from 40-70 nm in diameter (Bartnicki-Garcia

et al, 1978).   They are of two types.   Firstly, there are proctoid

chitosomes, which show little internal structure with negative staining.

Secondly, there are cycloid chitosomes, which have a thin membrane

shell and an interior with a grainy texture.   Chitosomes are all

converted to the latter type when incubated with UDP-N-acetylglucosamine

and trypsin, in vitro, and chitin microfibrils are then produced.

Sterol esters and triacylglycerols are two classes of yeast lipid

located almost exclusively in intracellular low-density vesicles

(Cartledge and Rose, 1973;   Clausen et al, 1974).   Evidence for the role

of these vesicles in wall biogenesis has recently been provided by

two studies.   Ramsay and Douglas (1979) found that phosphate-limited

S. cerevisiae contained decreased contents of glucan, sterol esters and

triacylglycerols.   If small vesicles are indeed involved in cell-

envelope biogenesis, this would explain changes observed in wall

composition, notably in glucan content, caused by phosphate limitation

of growth (Ramsay and Douglas, 1979).   Novick and Schekman (1979)

have isolated a temperature-sensitive mutant of S. cerevisiae that is

unable to secrete acid phosphatase at 37°C.   At the restrictive

temperature, cells accumulate large numbers of intracellular, membrane-

bound vesicles containing acid phosphatase.   In addition, intracellular

chitin synthase activity increases when cells are grown at 37°C whereas

the activity associated with the plasma-membrane fraction decreases.

The authors proposed that membrane proteins and lipids of accumulated

vesicles are precursors of the plasma membrane while the soluble

contents of the vesicles are precursors of the secreted glycoproteins.

Extranuclear microtubules are seen to project into vesicle-

filled buds of S. cerevisiae and it was suggested that these structures

might be involved in controlling bud emergence (Byers and Goetsch,

1974, 1975). α- and β-tubulin proteins have also been isolated from S. cerevisiae (Water and Kleinsmith, 1976; Baum et al, 1978; Clayton et al, 1979). However, it is not known whether these microtubules play a role in guiding vesicles to growing regions. In addition, they may be involved in nuclear division (Byers and Goetsch, 1974, 1975) and may act as a cytoskeleton for the maintenance of shape in yeast protoplasts (Santos et al, 1978).

An ordered helical ring of 10 nm filaments is found in intimate association with the plasma membrane following bud emergence but disappears when cytokinesis begins (Byers and Goetsch, 1976). Byers and Goetsch (1976) suggested that this ring might inhibit activating factor-carrying vesicles in Cabib's model of chitin synthesis (section IV A) from fusing with the plasma membrane until the appropriate phase of cell division. It is possible that these filaments, whose composition is unknown, might be made of actin. Actin-like protein has recently been isolated from S. cerevisiae and can polymerise, in vitro, to form 7 nm filaments (Koteliansky et al, 1979).

OBJECT OF RESEARCH

Although some of the enzymatic reactions involved in yeast mannan synthesis have been well characterised, very little information is available on regulation of the overall process. By contrast, regulation of chitin synthesis is better understood. The single enzyme involved, chitin synthase, is subject to regulation by effectors such as UDP and GlcNAc, which inhibit and activate respectively. In addition, chitin synthesis is controlled by an activation-inactivation process requiring compartmentalisation of different components. The aims of this study were twofold:

1) to screen potential low molecular-weight effectors for their ability to regulate mannosyltransferase activity.

2) to determine whether mannan synthesis is subject to activation-inactivation processes analogous to those demonstrated for chitin synthesis.

Such studies might lead to a better understanding of the control mechanisms involved in mannan biosynthesis and of those for yeast cell-wall biogenesis in general.

MATERIALS AND METHODS

Organism and growth conditions

The strains of S. cerevisiae used in this study were all provided by Dr. C.E. Ballou, University of California, Berkeley. The haploid wild-type, Saccharomyces cerevisiae X2180-1A a and the mutant strains LB1-3B mnn 2-1a and LB1-10B mnn 1-2a were maintained on slopes of glucose-peptone-yeast extract medium (see below) containing 1.5% (w/v) agar. The strains were subcultured on these slopes at monthly intervals as well as being preserved, freeze-dried, in a sterile mixture of horse serum and 30% (w/v) glucose (10:3, v/v).

The yeasts were grown aerobically in a medium of the following composition (per litre) : glucose, 20 g; bactopeptone, 20 g; yeast extract, 10 g. Batches of medium (500 ml, in Erlenmeyer flasks) were inoculated with overnight yeast cultures (50 ml) and incubated at 30°C in an orbital shaker at 150 oscillations per min. Cells were harvested, during the mid-exponential growth phase, by centrifugation at 2,075 x g for 45 min in an MSE Mistral 6L refrigerated centrifuge and washed once with cold 0.85% (w/v) NaCl.

Preparation and lysis of sphaeroplasts

Sphaeroplasts were prepared by digestion of the yeast with Zymolyase 5000. Cells (approximately 1.6 g dry weight) were suspended in 50 mM Tris-HCl buffer, pH 7.2 (100 ml) containing 10 mM $MgCl_2$ and 1 M sorbitol. Zymolyase 5000 (70-500 mg) was added and the suspension incubated at 30°C in an orbital shaker at 100 oscillations per min. Formation of osmotically-sensitive structures was monitored by adding 0.1 ml of suspension to 2.9 ml of water; the diluted suspension was shaken gently, left at room temperature for 10 min and its absorbance (A_{600}) then measured in a Unicam SP600 spectrophotometer (Alterthum and Rose, 1973). The cell suspension,

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when diluted in 1 M sorbitol, never showed more than a 7% drop in absorbance during the period of sphaeroplast formation which varied from 30-60 min. Completion of sphaeroplast formation was confirmed by phase-contrast microscopy; sphaeroplasts were perfectly round and surrounded by a phase-bright halo. Sphaeroplasts were recovered by centrifugation at 1,500 x g for 10 min in an MSE High Speed 18 centrifuge and washed twice with buffered sorbitol. The pellet was finally resuspended in 50 mM Tris-HCl buffer (pH 7.2) and subjected to twenty strokes in a Teflon-glass hand homogeniser, care being taken to avoid cavitation. Microscopic examination revealed that more than 95% of sphaeroplasts were lysed by this procedure. The lysate contained sphaeroplast 'ghosts', which retained their original shape although had lost their bright halos; there were also vesicles and some aggregates of lysed material in the preparation. The sphaeroplast lysate was dispensed in 1 ml aliquots containing 20-40 mg protein and stored at -20°C.

Preparation of membrane and supernatant fractions

Sphaeroplast lysate was centrifuged at 100,000 x g for 2 h at 4°C in an MSE Superspeed 65 Ultracentrifuge. The supernatant fluid was translucent with a small, milky-white layer at the surface, which contained many small vesicles, presumably lipid in nature. Supernatant fluid was gently mixed, using a Pasteur pipette, and stored at -20°C at a protein concentration of 2.8-8.2 mg/ml. This material is referred to as the supernatant fraction.

The pellet was washed once with 50 mM Tris-HCl buffer (pH 7.2), recovered by centrifugation at 100,000 x g for 30 min, and resuspended in the same buffer to give a protein concentration of 20-40 mg/ml. This washed membrane fraction was stored at -20°C.

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A 'clean' membrane fraction was prepared by washing the washed membrane fraction a further four times with 50 mM Tris-HCl buffer (pH 7.2). The clean membrane fraction was stored at -20°C at a protein concentration of 9.2 mg/ml.

Mannosyltransferase assay

Mannosyltransferase activity was assayed by following the incorporation of $[^{14}C]$mannose from GDP-$[^{14}C]$mannose into perchloric acid-precipitable material. The standard incubation mixture (0.1 ml) contained 40 mM Tris-HCl buffer (pH 7.2), 7.5 mM $MnCl_2$, GDP-$[^{14}C]$mannose (110-185 pmol; 20 nCi) and sphaeroplast lysate or washed membrane fraction (0.1-1.0 mg protein). Following incubation at 30°C for 20 min, reactions were terminated by the addition of ice-cold 0.3 M $HClO_4$ (1 ml) and the mixtures kept on ice for a further 10 min at least. They were then filtered through Oxoid membrane filters (0.45 μm pore size; 25 mm diameter), prewashed with cold 1% (w/v) mannose solution. For determination of optimal conditions for mannosyltransferase activity (Results, section II) and for testing effectors on activity (section III), the precipitate remaining on each filter was washed with a further portion of cold 0.3 M $HClO_4$ (20 ml) followed by more cold 1% (w/v) mannose solution (5 ml). However, for mixtures containing high levels of protein (10 mg/ml) this was impracticable and hence for all results in section IV (whether low or high protein concentrations were used) filters were washed with only a further portion of $HClO_4$ (1 ml). The filters were transferred to scintillation vials, dried under an infra-red lamp and finally counted in 10 ml toluene scintillant. For all independent assays, reactions were done in duplicate. Zero-time control mixtures were inactivated by 0.3 M $HClO_4$ (1 ml) before the addition of GDP-$[^{14}C]$mannose. These

controls had the same background count rate as those in which boiled enzyme (100°C for 3 min) was used. Mannosyltransferase activity is expressed as moles of $[^{14}C]$ mannose incorporated into polymer per hour per milligram of protein.

Preincubation and dialysis of enzyme preparations

Where indicated, sphaeroplast lysate and washed membrane fraction were preincubated at 4°C for 24 h, at a protein concentration of 20 mg/ml prior to assaying for mannosyltransferase activity. Sphaeroplast lysate preparations were dialysed overnight (24 h at 4°C) against 200 volumes of 50 mM Tris-HCl buffer (pH 7.2). Dialysis tubing (Visking size 1-8/32 inch; Medicell International Ltd., London) was cleaned in boiling 0.5 M acetic acid for 5 min then thoroughly washed with distilled water before use.

Polymer synthesis from different precursors

Polymer synthesis was measured using the standard mannosyl-transferase assay mixture with different radioactively-labelled precursors at various concentrations. Either 20 nCi or 100 nCi of UDP-$[^{14}C]$ glucose (306 Ci/mol) or GDP-$[^{14}C]$ glucose (277 Ci/mol) were used in assays; these sugar nucleotides were diluted with unlabelled carrier when preparing precursors with the same specific activity as GDP-$[^{14}C]$ mannose (172 Ci/mol).

Lipid extraction

Lipids were extracted from reaction mixtures either by butan-1-ol or by chloroform-methanol mixtures.

(i) Butan-1-ol extraction. Standard assay mixtures (0.1 ml) were incubated at 30°C for 20 min and the reactions were terminated by the addition of ice-cold water (0.9 ml) and butan-1-ol

(1 ml), followed by vigorous mixing; separation into an upper layer
of butan-1-ol, a lower aqueous layer and a layer of protein at the
interface was aided by centrifugation at 2,000 x g for 10 min in an
MSE Super Minor bench centrifuge. The upper layer was removed with
a Pasteur pipette and the interface was extracted twice with further
portions of butan-1-ol (1 ml each). The butan-1-ol extracts were
combined, backwashed once with water (1 ml), transferred to scintillation
vials and then evaporated to dryness, in vacuo. Total radioactivity
was counted in 10 ml of toluene scintillant. Alternatively, washed
extracts were redissolved in butan-1-ol or water (50 µl), applied to
Whatman No. 1 paper and separated by paper chromatography (p. 77).

(ii) <u>Chloroform-methanol extractions</u>. Standard assay
mixtures (0.1 ml) were incubated at 30°C for 20 min and the reactions
terminated by the addition of 2 ml of ice-cold chloroform-methanol
(2:1, v/v), followed by thorough mixing. After centrifugation at
2,000 x g for 10 min in an MSE Super Minor bench centrifuge, the
precipitates were extracted twice more with the same solvent (2 ml
each). To the combined extracts (6 ml), 1.5 ml of 0.88% (w/v) KCl
was added and the interface was washed twice with the upper phase of
chloroform-methanol-0.88% KCl (8:4:3, by vol.; 1.5 ml) according to
Folch <u>et al</u> (1957). The washed lower phase was transferred to
scintillation vials and evaporated to dryness, <u>in vacuo</u>. The residue
was extracted twice with chloroform-methanol-water (10:10:3, by vol.)
and the combined extracts evaporated to dryness, <u>in vacuo</u>, in
scintillation vials. Radioactive material was either counted in
10 ml of toluene scintillant or redissolved in chloroform-methanol
(2:1, v/v; 50 µl), applied to silica gel G plates and separated by
t.l.c. (p. 78).

Zero-time controls in both extraction procedures had organic solvents added before GT$-$[^{14}C] mannose.

β-Elimination of radioactive mannoproteins

The β-elimination procedure was done on [^{14}C]-labelled polymer formed in standard assay mixtures, where reactions were terminated by heating at 100°C for 3 min. Mixtures were made up to 1 ml with water and centrifuged at 1,000 x g for 5 min in an MSE Super Minor bench centrifuge. The pellets were washed twice with water, resuspended in 0.1 M NaOH (1 ml) and incubated at room temperature (18-22°C) for 18 h. They were then neutralised by the addition of 2 M acetic acid (50 µl), centrifuged and the pellets washed twice with water. The pellets were finally transferred to scintillation vials with methanol (0.5 ml and 2 x 0.5 ml washings). The methanol was removed by evaporation, *in vacuo*, and radioactivity counted in 10 ml of toluene scintillant. Control mixtures were incubated for 18 h with water instead of alkali and zero-time controls were from reaction mixtures containing heat-inactivated (100°C for 3 min) sphaeroplast lysate.

Incorporation of [^{14}C] mannose into mannan polysaccharide

Synthesis of the long mannan polysaccharides was measured by the method of Parodi (1979a). In this procedure mannan is precipitated with 66% (v/v) aqueous methanol and then treated with 2 M LiOH. This liberates the short oligosaccharides attached to serine or threonine residues and the polysaccharide chains from the protein. The oligosaccharides are soluble in 66% methanol whereas the polysaccharide part of mannan remains insoluble in that solution. Standard assay mixtures (0.1 ml) were incubated at 30°C for 20 min and reactions

terminated by 0.4 ml of 50 mM LiCl and 1 ml of methanol. The

precipitates were dissolved twice in 0.5 ml of 50 mM LiCl and precipi-

tated each time with 1 ml of methanol. They were then heated for

15 min at 100°C in glass-stoppered tubes, with 2 M LiOH (0.5 ml).

Methanol (1 ml) was added and the precipitates twice dissolved in LiCl

and reprecipitated as above. The precipitates were transferred to

scintillation vials with methanol (0.5 ml and 2 x 0.5 ml washings).

The methanol was removed by evaporation and radioactivity counted in

10 ml toluene scintillant.

Effect of supernatant fraction on mannosyltransferase activity

To test the effect of supernatant fraction on mannosyl-

transferase activity, 10-30 µl of this fraction was included in standard

incubation mixtures. In control mixtures, heat-treated (60°C for 10

min) supernatant fraction was added.

Treatment of sphaeroplast lysate and supernatant fraction with
proteases and protease inhibitors

Standard incubation mixtures containing high concentrations

of sphaeroplast lysate protein (10 mg/ml) were assayed for mannosyl-

transferase activity in the presence of different proteases. The

proteases used included trypsin, subtilisin BPN', pronase, pepsin,

bromelain and Aspergillus acid protease; they were added at final

concentrations ranging from 1-4 mg/ml.

Supernatant fraction was also treated with proteases at

30°C for 20 min and kept on ice briefly before assaying its effect on

mannosyltransferase activity. In some cases, protease action was

terminated by the addition of specific inhibitors. Supernatant

fraction (20 µl) was incubated at 30°C for 20 min with 10 µl of trypsin

or pepsin (16.7 mg/ml) or proteinase A (0.67 mg/ml); trypsin action was then stopped by the addition of a solution of trypsin inhibitor (10 μl, containing half the weight of trypsin used) and that of pepsin and proteinase A by 10 μl of pepstatin A (10 mM, dissolved in 10%, v/v, DMSO). In control experiments, protease inhibitor was added to supernatant fraction before the protease.

The action of protease inhibitors was tested on sphaeroplast lysate and supernatant fraction during preincubation of these preparations at low temperature. Sphaeroplast lysate at high protein concentrations (20 mg/ml) was incubated at 4°C for 24 h with trypsin inhibitor (200 μg/ml) or quinacrine, PMSF or o-phenanthroline (all at 1 mM) prior to assaying mannosyltransferase activity. The last two inhibitors were dissolved in 10% (v/v) ethanol. Similarly, supernatant fraction was incubated at 4°C for 24 h with trypsin inhibitor (0.2-4.0 mg/ml) or 3.3 mM TAME (both soluble in water) or the water-insoluble inhibitors, PMSF (1-20 mM in 10%, v/v, ethanol), pepstatin A, TPCK or TLCK (all at 3.3 mM in 33%, v/v, DMSO). After incubation of the supernatant fraction, its effect on mannosyltransferase activity was measured. Control mixtures contained supernatant fraction which had been incubated with solvent but no inhibitor.

Degradation of sugar nucleotides

Degradation of $\begin{bmatrix} 14 \\ C \end{bmatrix}$-labelled sugar nucleotides was assessed by separating reaction products by paper chromatography. Standard incubation mixtures (0.1 ml) contained Tris-HCl buffer (40 mM; pH 7.2), 7.5 mM $MnCl_2$, $\begin{bmatrix} 14 \\ C \end{bmatrix}$-labelled sugar nucleotide (72-578 pmol; 20 nCi or 100 nCi) and either sphaeroplast lysate or washed membrane fraction (0.1-1.0 mg protein) or 10-30 μl of supernatant fraction. After incubation at 30°C for 20 min, reactions were terminated by

heating at 100°C for 3 min.   Each mixture was applied, as a 1.0

inch-long streak, to Whatman 3MM paper and subjected to descending

chromatography (p. 77).   After development, the chromatograms were

left to dry in a fume-cupboard, then cut into longitudinal strips,

1.5 inches wide, for each reaction.   These strips were divided into

0.5 cm or 1.0 cm segments and counted in 5 ml toluene scintillant.

Radioactive material which did not co-migrate with sugar

nucleotides or remain at the origin was regarded as products of sugar-

nucleotide hydrolysis.   Percentage degradation was therefore determined

by counting the radioactivity in these products and expressing the

counts as a fraction of the total radioactive material on the chromato-

gram.   Degradative activity was also expressed quantitatively as moles

of GDP-$[^{14}C]$ mannose hydrolysed per hour per mg of protein.

Degradation of $[^{14}C]$ mannan

Degradation of $[^{14}C]$-labelled mannan by either supernatant

fraction or high concentrations of sphaeroplast lysate protein was

measured as follows.

(i) Standard incubation mixtures, containing 40 mM Tris-HCl

buffer (pH 7.2), GDP-$[^{14}C]$mannose (120 pmol;   20 nCi) and sphaeroplast

lysate protein (10 mg/ml) were incubated at 30°C for 20 min.   Reactions

were terminated by the addition of ice-cold $HClO_4$ (0.3 M; 4 ml).

These mixtures were centrifuged at 1,000 x g for 10 min in an MSE

Super Minor bench centrifuge at room temperature.   The pellets were

washed twice with 50 mM Tris-HCl buffer (pH 7.2).   The pellets were

finally reincubated with Tris-HCl (40 mM;   pH 7.2), 7.5 mM $MnCl_2$ and

either high concentrations of sphaeroplast lysate protein (10 mg/ml)

or the same amount of heat-treated (100°C for 3 min) sphaeroplast

lysate at 30°C for 20 min.   The reactions were terminated by the

addition of ice-cold 0.3 M $HClO_4$ (1 ml).

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(ii) Standard incubation mixtures, containing sphaeroplast
lysate protein (1 mg/ml), Tris-HCl (40 mM; pH 7.2), 7.5 mM $MnCl_2$ and
GDP-$[^{14}C]$mannose (120 pmol; 20 nCi), were incubated at $30^{o}C$ for 20 min.
Reactions were terminated by heating at $100^{o}C$ for 3 min. Tris-HCl
buffer (50 mM; pH 7.2; 4 ml) was added to the mixtures, which were
then centrifuged and washed as in (i) above. The pellets were
incubated at $30^{o}C$ for 20 min with Tris-HCl (40 mM; pH 7.2), 7.5 mM
$MnCl_2$, and 30 μl of either supernatant fraction or heat-treated ($60^{o}C$
for 10 min) supernatant fraction. The reactions were terminated by
0.3 M $HClO_4$ (1 ml).

With both methods, the resultant mixtures were filtered
and counted as described for the standard mannosyltransferase assay.
The amount of $[^{14}C]$mannan hydrolysed by either preparation was calculated
by subtracting the radioactivity remaining, after treatment of the
$[^{14}C]$-labelled polymer with supernatant fraction or sphaeroplast
lysate, from that remaining after treatment with heat-inactivated
preparations.

EDTA treatment of the supernatant fraction

The ability of the supernatant fraction to inhibit mannosyl-
transferases and to degrade GDP-mannose was examined following pre-
treatment with EDTA. Supernatant fraction (20 μl) was incubated at
$0^{o}C$ for 10 min with 60 mM EDTA (10 μl). EDTA-treated supernatant
fraction was included in standard mannosyltransferase assay mixtures
containing 40 mM Tris-HCl buffer (pH 7.2), $MnCl_2$ (at a final concen-
tration, in excess of EDTA, of 7.5 mM), GDP-$[^{14}C]$mannose (137 pmol;
20 nCi) and washed membrane fraction (10 mg protein/ml). For
estimation of GDP-$[^{14}C]$mannose degradation, washed membrane fraction
was omitted and mixtures were separated by paper chromatography as
described previously (p. 72).

Large-scale preparations of supernatant fraction

Two methods were used to prepare large batches of supernatant fraction. Firstly, cells were broken by mechanical disruption with glass beads and supernatant fraction was obtained by differential centrifugation of the cell-free extract. A suspension of cells (about 30% wet wt. per vol. in 50 mM Tris-HCl, pH 7.2) was disrupted in precooled Braun bottles containing 25 ml of cell suspension and 30 ml of No. 10 Ballotini beads. The bottles were shaken at 4,000 oscillations per min in a Braun MSK homogeniser (B. Braun, Melsungen, West Germany) for 2 min, during which time the temperature was maintained at 0-4°C by a constant stream of CO_2. The beads were removed by filtration through sintered glass (porosity grade 0) and cell-wall debris by centrifugation at 2,000 x g for 20 min in a MSE Mistral 6L refrigerated centrifuge. The walls were twice washed with ice-cold Tris-HCl buffer (50 mM; pH 7.2) and the combined supernatant fluid and washes were centrifuged at 100,000 x g for 1 h in an MSE Superspeed 65 Ultracentrifuge. The supernatant fluid thus obtained (250 ml) included a milky-white surface layer; this layer was thoroughly mixed with the rest of the supernatant fluid before treating with ammonium sulphate (see below). A small portion of supernatant fluid was stored at -20°C, at a protein concentration of 3.8 mg/ml.

The second method involved a large-scale (fourfold) preparation of sphaeroplast lysate. Sphaeroplast lysate (34 ml) was centrifuged at 100,000 x g for 2 h in an MSE Superspeed 65 Ultracentrifuge to yield about 22 ml of supernatant fraction (10.8 mg protein/ml) which was stored at -20°C.

Ammonium sulphate precipitation of supernatant fractions

Supernatant fluid, obtained as described above, was

fractionated by ammonium sulphate precipitation. $(NH_4)_2SO_4$ was added slowly, with stirring at $0°C$ to 40% (w/v) saturation (Dawson et al, 1969). Precipitated material was removed by centrifugation at 20,000 x g for 30 min in an MSE High Speed 25 centrifuge and resuspended in 50 mM Tris-HCl buffer (pH 7.2; 5 ml). Further addition of $(NH_4)_2SO_4$ to the second supernatant fluid gave a second precipitate, at 60% saturation, which was again resuspended in buffer (5 ml). A third precipitate was obtained at 80% saturation and this was re-dissolved in buffer (5 ml). The three fractions were dialysed against 3 l of Tris-HCl buffer at $4°C$ for 12 h and against two further changes of buffer for 2 h each. They were all stored at $-20°C$.

Elution and hydrolysis of peak X material

Radioactively-labelled peak X material, identified by paper-chromatographic separation of the products of mannosyltransferase reaction mixtures, was eluted from chromatograms with 70% (v/v) aqueous ethanol. About 0.5 ml of eluant was required to elute all radioactivity from each strip (1 cm x 1.5 inches). Peak X material was treated with mild and strong acid. Approximately 6,000 cpm of the peak X eluate was evaporated to dryness, in vacuo. This was treated with either 0.01 M HCl (2 ml) at $100°C$ for 30 min, in glass-stoppered tubes or 2 M HCl at $110°C$ for 4 h in ampoules sealed under vacuum. The hydrolysates were concentrated to dryness by rotary evaporation, dissolved in 70% (v/v) ethanol (50 μl), then applied to t.l.c. cellulose plates and subjected to ascending chromatography (p. 78). Following development, 0.5 cm sections of cellulose were scraped off plates into scintillation vials and counted in 10 ml toluene scintillant.

Extraction and isolation of yeast mannans

Mannan was prepared by two methods. Method A. Mannan was

extracted from stationary-phase cells of S. cerevisiae X2180-1A a

(137 g wet weight) by autoclaving them in neutral citrate buffer

(20 mM; pH 7.2); the mannan was twice precipitated with Fehling's

solution (Appendix IIA, p. 182) as described by Kocourek and Ballou

(1969). The yield of mannan by this method was 870 mg dry wt.

Method B. Mannan (500 mg), prepared by Method A, was dissolved in

2 M LiOH (10 ml), heated at $100^{\circ}C$ for 2 h, and precipitated with two

volumes of methanol. It was then dissolved three times in 50 mM LiCl

and reprecipitated each time with two volumes of methanol. This

procedure ensures that only the polysaccharide part of yeast mannan is

obtained (Parodi, 1979a). The yield of mannan by this method was 185 mg

dry wt.

Protein determinations

Protein was estimated by the method of Lowry et al (1951)

using bovine plasma albumin as a standard (see Appendix IIB, p. 182).

Prior to analysis, samples were digested in 0.5 M NaOH at $100^{\circ}C$ for

15 min, in glass-stoppered tubes.

Paper chromatography

Descending chromatography on Whatman No. 1 or 3 MM paper

(23 cm x 56 cm) was done at room temperature for 12-18 h in one of the

following solvents:

A) ethyl acetate–water–butan-1-ol–acetic acid (3:4:4:2.5, by vol.;
Tanner, 1969)

B) iso-butyric acid – M aqueous ammonia (5:3, v/v; Krebs and
Hems, 1953)

C) butan-1-ol – acetic acid – water (4:1:1, by vol.; Partridge,
1948).

Products were identified by co-chromatography with radio-active or non-radioactive authentic standards. The latter were detected by the following methods:

1) alkaline silver nitrate dip reagent for sugars (Trevelyan et al, 1950; Appendix IIC, p. 182)

2) perchloric acid – molybdate spray reagent for phosphate esters (Hanes and Isherwood, 1949; Appendix IID, p. 183)

3) exposure to ultraviolet light (Hanovia Lamps, Slough, England) which shows up sugar nucleotides as dark-blue spots on a faintly fluorescing paper background (Holiday and Johnson, 1949).

Thin-layer chromatography

Glass plates (5 cm x 20 cm) were coated with a layer (0.25 mm thick) of either silica gel G, for separation of lipids, or cellulose (Sigmacell) for separation of peak X material. The plates were prepared using a Shandon Unoplan Leveller model SA (Shandon Scientific Co. Ltd., Willesden, London). Only the silica plates were activated before use at 110°C for 30 min. Separation of radio-active material was accomplished by developing the plates in solvent system B (cellulose plates) or in chloroform-methanol-water (65:25:4, by vol.; solvent system D, for silica plates). Products were identified by comparison with either radioactive or non-radioactive authentic standards. The latter were detected as charred spots after spraying with 50% (v/v) sulphuric acid and heating at 180°C for 10-15 min.

Radioactive counting

All samples were counted for 10 min in a Nuclear Enterprises scintillation spectrometer (NE 8320) in toluene scintillant

(5 ml or 10 ml).   The composition of the scintillant was:

toluene, 1 l; 2,5-diphenyloxazole, 4 g; 1,4-bis-(4-methyl-5-phenyl-

oxazole-2-yl)benzene, 0.1 g .

Glassware

All glassware used in biochemical work was cleaned by

steeping in a 1% (w/v) solution of Alconox (Alconox Inc., New York).

Cleaning fluid was removed by exhaustive rinsing in tap water followed

by two rinses in distilled water prior to drying in an oven at $105^{\circ}C$.

Chemicals

All radioactive material was supplied by the Radiochemical

Centre, Amersham, England.   Bactopeptone, yeast extract and agar were

from Difco Laboratories (Detroit, Michigan, U.S.A.) and bovine plasma

albumin was obtained from Armour Pharmaceutical, Eastbourne, England.

Ficoll 400 was purchased from Pharmacia (Fine Chemicals) Ltd., London,

Zymolyase 5000 from Kirin Brewery Co. Ltd., Takasaka, Gumma Pref.,

Japan, Silica gel G from E. Merck, A.G., Darmstadt, Germany

and mannonic acid lactone from Koch-Light Laboratories, Colnbrook,

England.   All solvents and other chemicals were obtained from BDH

Chemicals Ltd., Poole, England or from Sigma London Chemical Co. Ltd.,

and were of AnalaR grade.   All other biochemicals and enzymes were

purchased from Sigma London Chemical Co. Ltd.

RESULTS

I.   GROWTH OF ORGANISMS AND PREPARATION OF ENZYMES

A.   Growth of Saccharomyces cerevisiae strains

A growth curve for S. cerevisiae X2180-1Aa is shown in figure 4. The yeast grew exponentially for up to 5 hours before entering the stationary phase. Cells were always harvested after 2-4 hours incubation, during the mid-exponential phase. The growth of S. cerevisiae LB1-3B and LB1-10B was identical.

B.   Sphaeroplast formation

Sphaeroplasts were prepared by resuspending cells in 1M sorbitol buffer and incubating at 30°C with Zymolyase 5000, a commercial preparation of β-(1→3)glucanase. Cells were readily converted to sphaeroplasts with Zymolyase 5000, as determined by microscopic examination. The osmotic sensitivity of cells during incubation with the enzyme was monitored by dilution of samples in water; typical results are shown in figure 5. The rate at which the yeast became osmotically sensitive was dependent on the concentration of Zymolyase 5000 used; complete lysis in water occurred after 30, 40 and 70 minutes incubation at 30°C with Zymolyase 5000 concentrations of 5.0, 2.0 and 0.7 mg/ml respectively. A Zymolyase 5000 concentration of 5.0 mg/ml tended to cause lysis of the sphaeroplasts in incubation buffer containing 1M sorbitol. The extent of lysis was decreased by using buffer containing 1.2M or 1.4M sorbitol but, at both these concentrations, cells began to plasmolyse. For assays testing the optimal conditions and effectors of mannosyltransferase activity (sections II and III), enzyme was prepared from cells treated with a Zymolyase 5000 concentration of 0.7 mg/ml; for other work, a concentration of 2.0 mg/ml was used.

Figure 4.   Growth curve for S. cerevisiae X2180-1A a

Values shown represent the mean absorbance for eight
independent cultures.
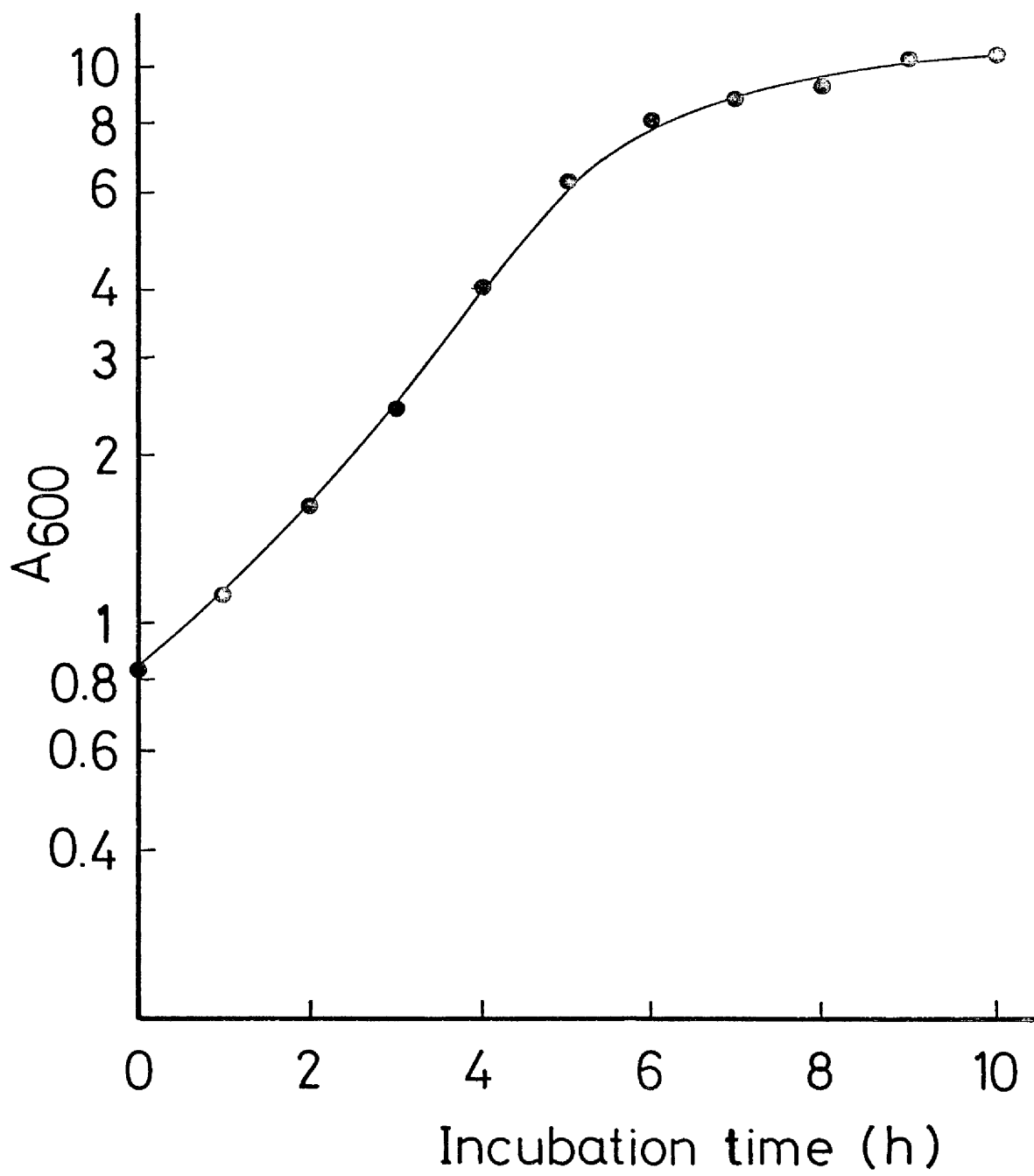
Figure 5.   Sphaeroplast formation in S. cerevisiae X2180-1A a

Suspensions of yeast in 1 M sorbitol buffer were incubated at 30°C with Zymolyase 5000 at concentrations of 0.7 (●), 2.0 (○) or 5.0 mg/ml (■). Sphaeroplast formation was monitored as described in Materials and Methods and sphaeroplasts were harvested at times indicated by the arrows.
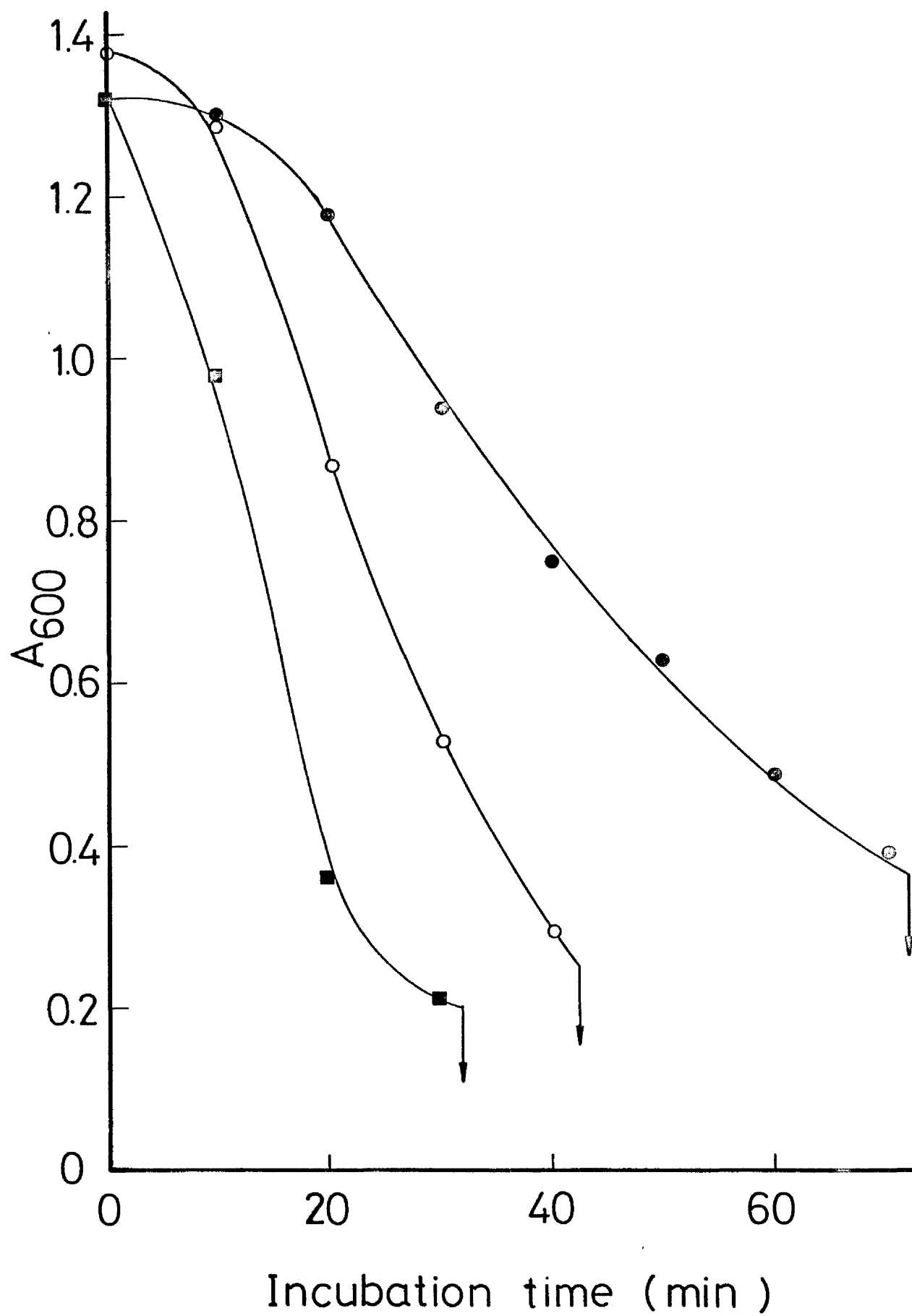
Sphaeroplast formation with the mutant strains, LB1-3B and LB1-10B, was similar but less rapid. A suspension of S. cerevisiae LB1-3B required 70 min incubation with Zymolyase 5000 (2 mg/ml) before sphaero-plast formation (indicated by change in A_{600}) was identical to that obtained with S. cerevisiae X2180-1Aa similarly incubated for 45 min.

II. OPTIMAL CONDITIONS FOR MANNOSYLTRANSFERASE ACTIVITY

A. Temperature

Mannosyltransferase activity of sphaeroplast lysate prepara-tions was optimal at 30°C (figure 6). 75% of this activity was observed at 15°C and 43°C, and 22% at 0°C. Mannosyltransferase activity was destroyed after 10 min at 60°C and 3 min at 100°C.

B. pH

Mannosyltransferase activity of sphaeroplast lysate prepara-tions was optimal between pH values of 6.5 and 7.5 using Tris-HCl or imidazole-HCl buffers (figure 7). Since enzyme was prepared in Tris-HCl, this buffer at pH 7.2 was used in the standard assay mixture. The concentration of Tris-HCl in the assay did not significantly affect mannosyltransferase activity over the range of 25-40 mM (100 ± 2.9%).

C. Incubation time

Incorporation of $[^{14}C]$mannose from GDP-$[^{14}C]$mannose into polymer by both sphaeroplast lysate and washed membrane preparations was almost linear for the first 30 min incubation at 30°C (figure 8). Incorporation was variable with different batches of enzyme but was usually greater with sphaeroplast lysates than with washed membrane fractions.

Figure 6.   Effect of varying incubation temperature on

mannosyltransferase activity of a sphaeroplast lysate

preparation from S. cerevisiae.

Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2),

$MnCl_2$ (7.5 mM), sphaeroplast lysate protein (1 mg/ml) and

GDP-$[^{14}C]$mannose (185 pmol ; 20 nCi).   They were incubated, at the

temperature indicated, for 20 min.
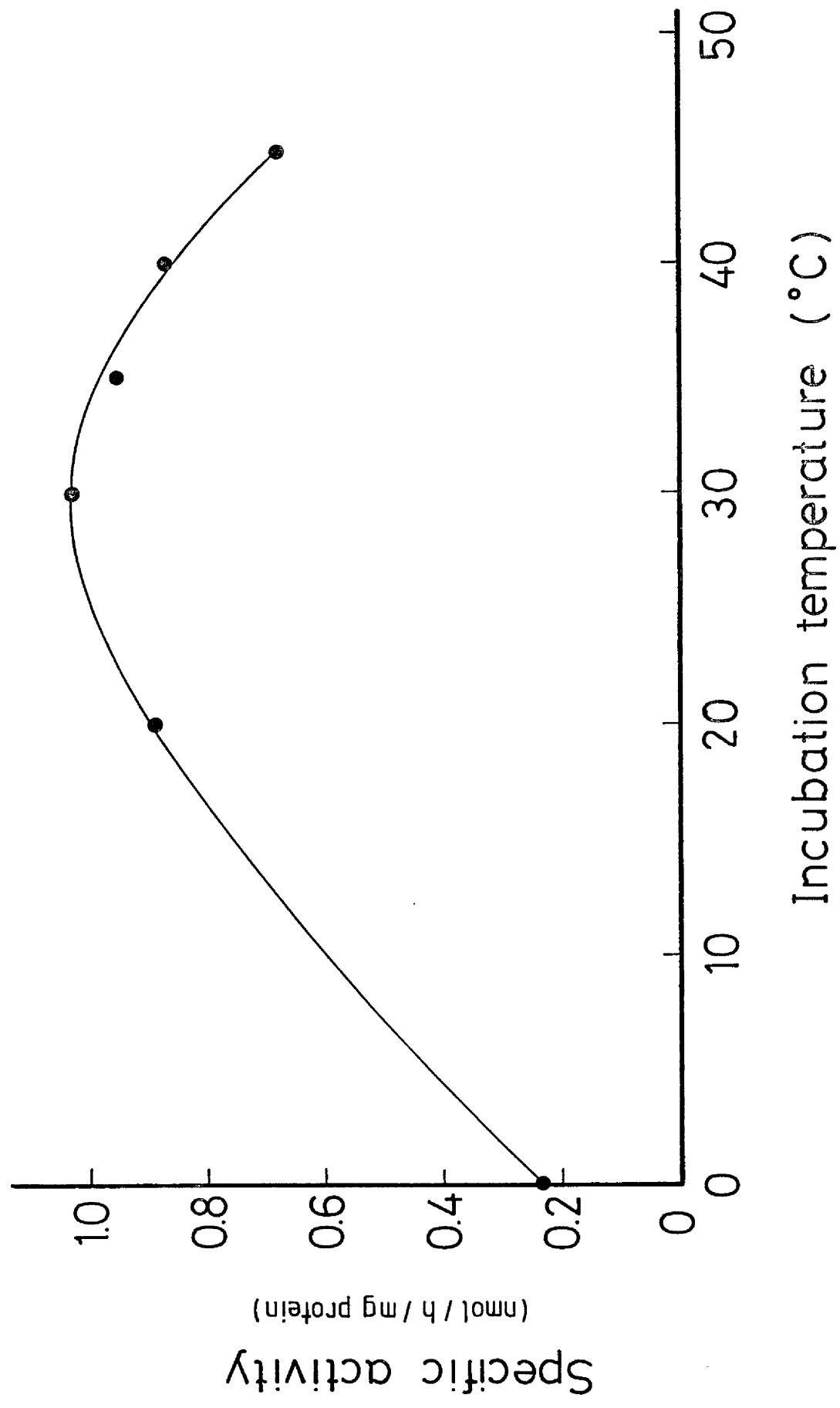
Figure 7.   Effect of pH on mannosyltransferase activity of a

sphaeroplast lysate preparation from S. cerevisiae.

Reaction mixtures contained $MnCl_2$ (7.5 mM), sphaeroplast

lysate protein (1 mg/ml), GDP-$[^{14}C]$mannose (185 pmol ; 20 nCi)

and different buffers, as indicated (all at 50 mM).   They were

incubated at $30^{o}C$ for 20 min.

■ Na_3 citrate-citric acid   ● Tris-HCl

△ NaH maleate-NaOH   □ Borate-HCl

○ Imidazole-HCl
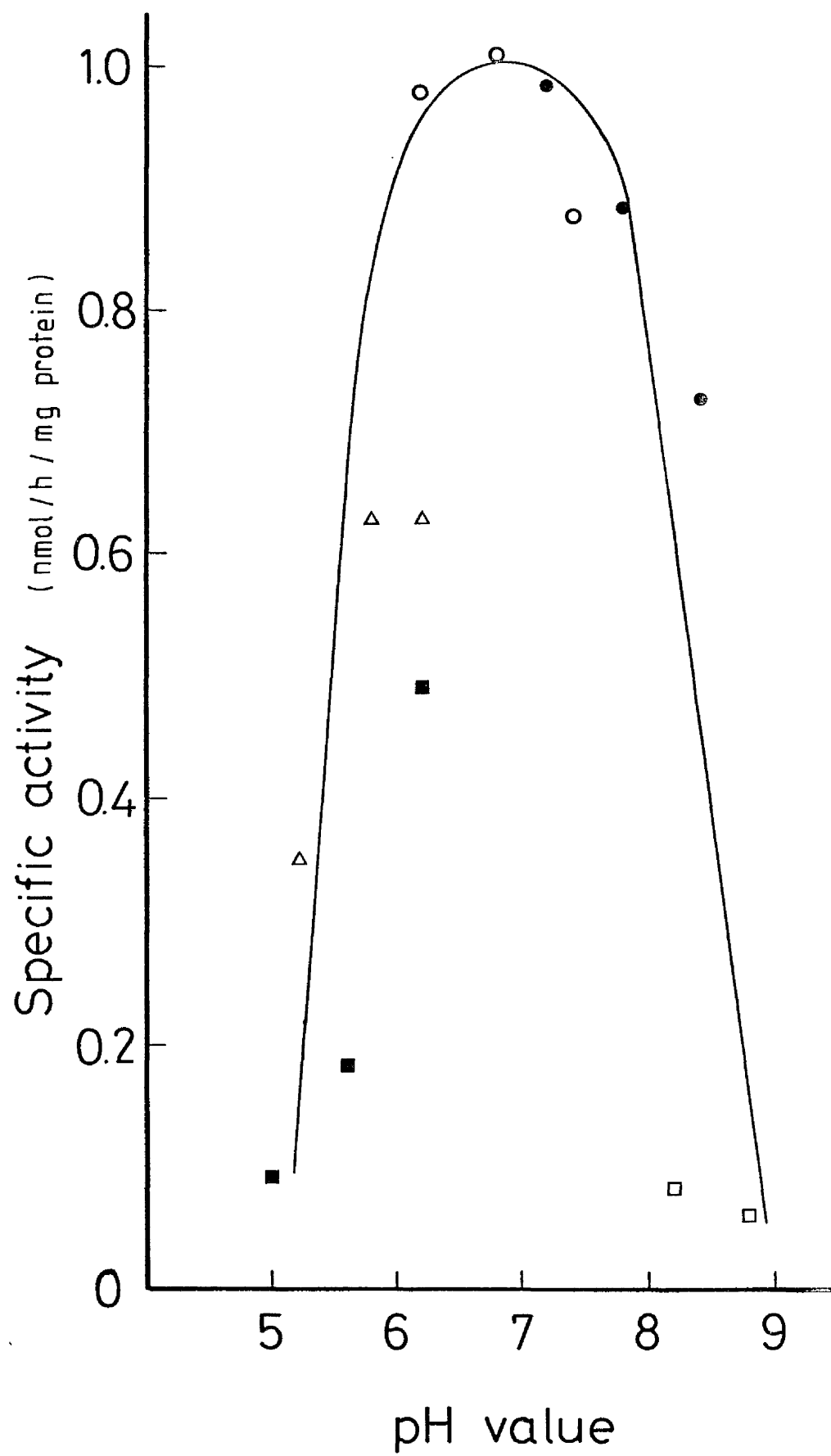
Figure 8.   Time course for incorporation of $[^{14}C]$mannose into

polymer by sphaeroplast lysate and washed membrane

preparations from S. cerevisiae.

Reaction mixtures contained Tris-HCl (40 mM ; pH 7.2),

MnCl$_2$ (7.5 mM), GDP-$[^{14}C]$mannose (116 pmol ; 20 nCi) and sphaeroplast

lysate protein (1 mg/ml ; ●) or washed membrane fraction (1.0 mg

protein per ml ; O) and were incubated at 30$^{\text{o}}$C, for the times

indicated.
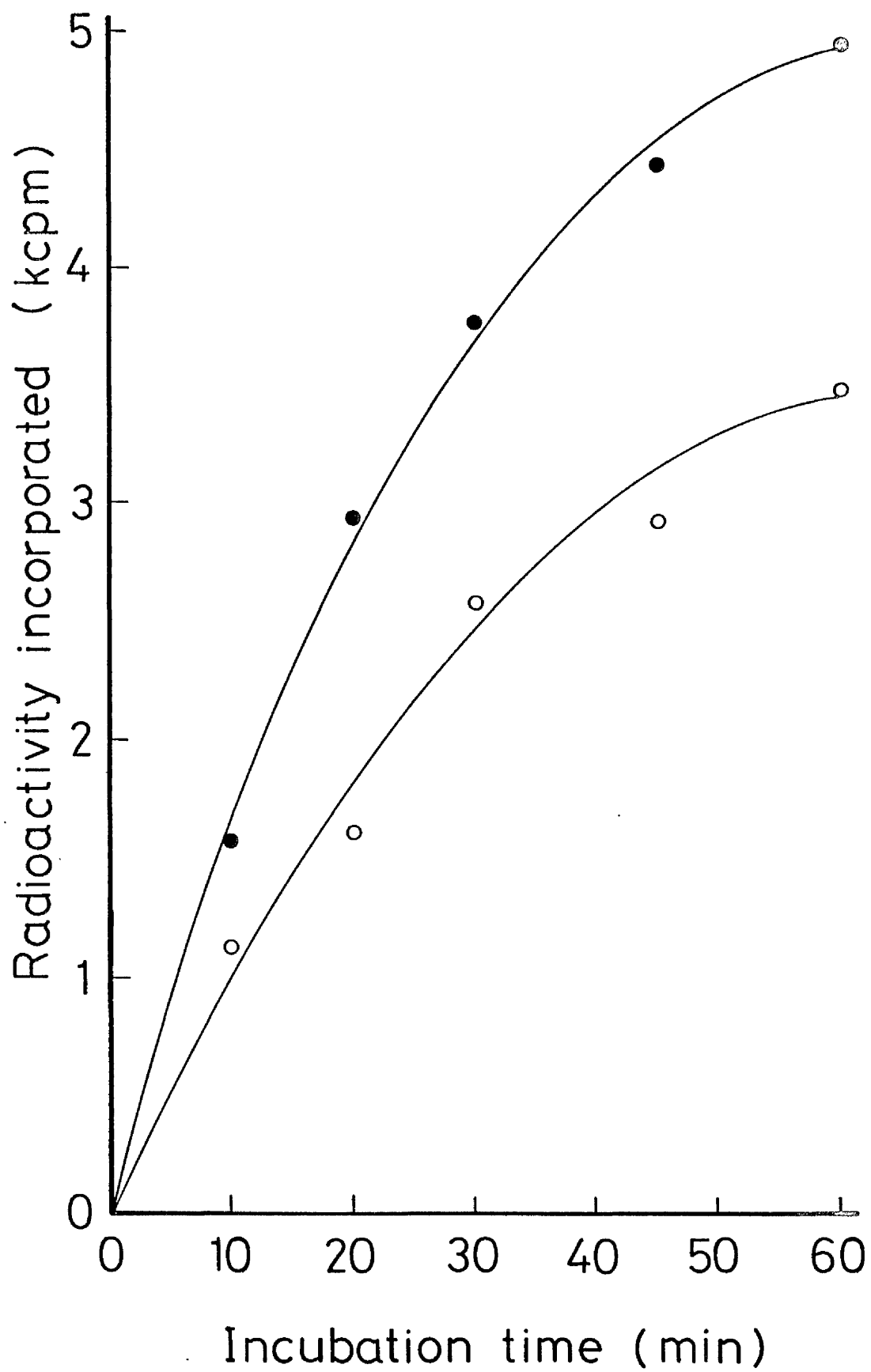
D. Enzyme protein and exogenous acceptor concentration

Incorporation of radioactivity into polymer as a function of enzyme concentration is shown in figure 9. It was similar for both sphaeroplast lysate and washed membrane preparations and appeared to be activated when protein concentrations of 0.5-1.0 mg/ml were used. If concentrations greater than 1.0 mg/ml were used, the filtration of mixtures was slowed down considerably. Results obtained using higher protein concentrations are presented, in detail, in section IV.

When sphaeroplast lysate protein (1 mg/ml) was assayed for activity, no stimulation of incorporation occurred if heat-inactivated washed membrane fraction ($100^{\circ}C$ for 5 min; 0.2-1.0 mg protein per ml) or mannan (prepared by methods A and B in Materials and Methods; 1-10 mg dry wt per ml) was included in assay mixtures.

E. $MnCl_2$ concentration

Mannosyltransferase activity of both sphaeroplast lysate and washed membrane preparations was optimal with $MnCl_2$ concentrations ranging from 2.5 mM to 10 mM (figure 10). A concentration of 7.5 mM $MnCl_2$ was used for future assays, unless otherwise stated. Activity, with no exogenous ions added, varied from 40-60% of the activity with 7.5 mM $MnCl_2$. The stimulation of activity by 5 mM $MgCl_2$ was only 50% of that obtained with 7.5 mM $MnCl_2$ and activity was not enhanced further with $MgCl_2$ concentrations up to 20 mM. The effect of some other cations on mannosyltransferase activity is described in section IVB (table 13, p. 130).

F. Stability of enzyme preparations

Mannosyltransferase activity of sphaeroplast lysate prepara-

Figure 9.   Effect of varying protein concentration on mannosyl-
             transferase activity of sphaeroplast lysate and washed
             membrane preparations from S. cerevisiae.

     Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2),
MnCl$_2$ (7.5 mM), GDP-$\left[^{14}C\right]$mannose (116 pmol ; 20 nCi) and different
concentrations of sphaeroplast lysate (●) or washed membrane (O)
preparations.   They were incubated at 30°C for 20 min.
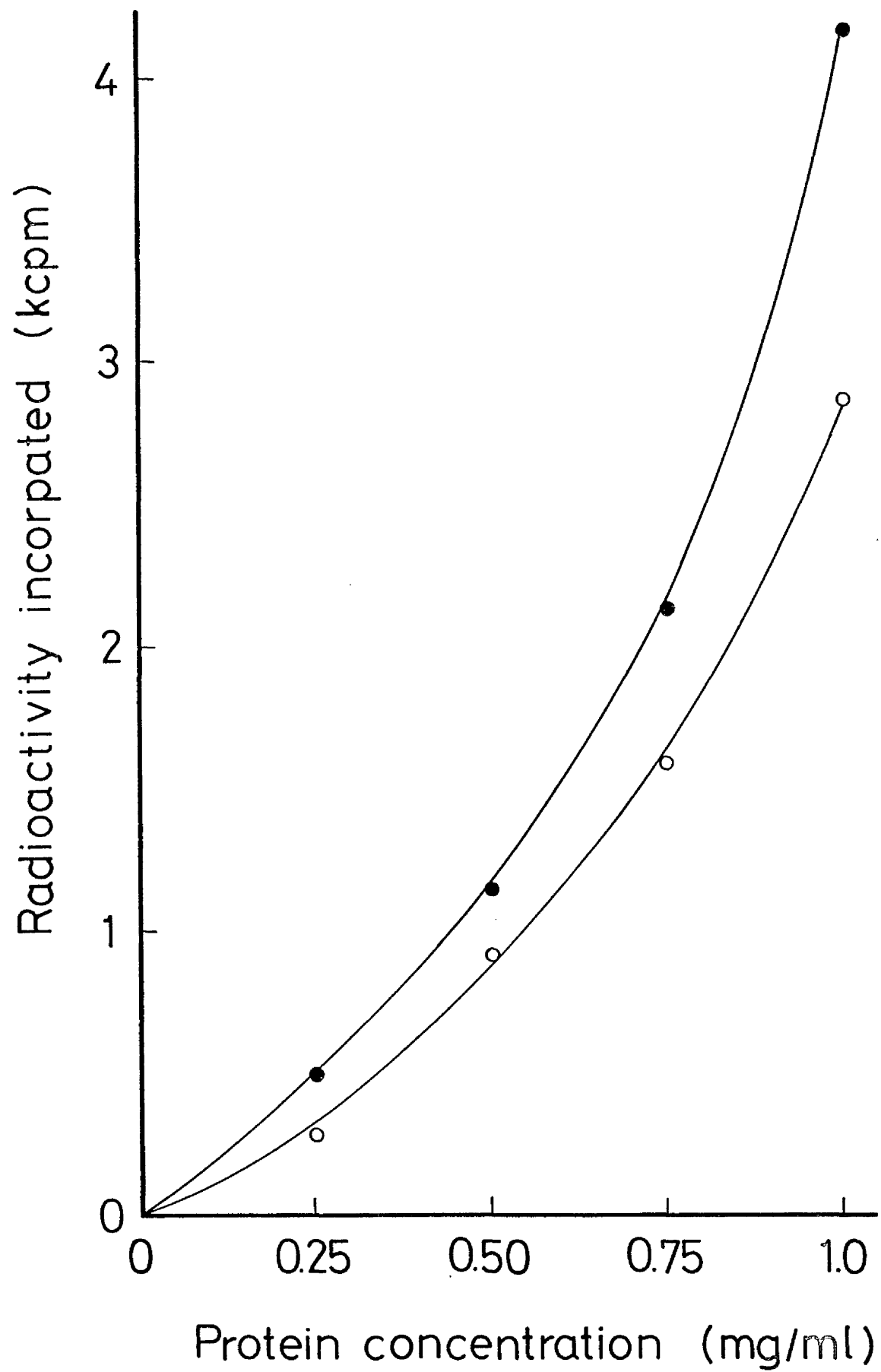
Figure 10.   Effect of varying MnCl$_2$ concentration on mannosyl-

transferase activity of sphaeroplast lysate and

washed membrane preparations from <u>S. cerevisiae</u>.

Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2),
sphaeroplast lysate (1 mg protein per ml ; ●) or washed membrane
fraction (1 mg protein per ml ; O), GDP-[^{14}C]mannose (116 pmol ;
20 nCi) and different concentrations of MnCl$_2$.   They were
incubated at 30°C for 20 min.
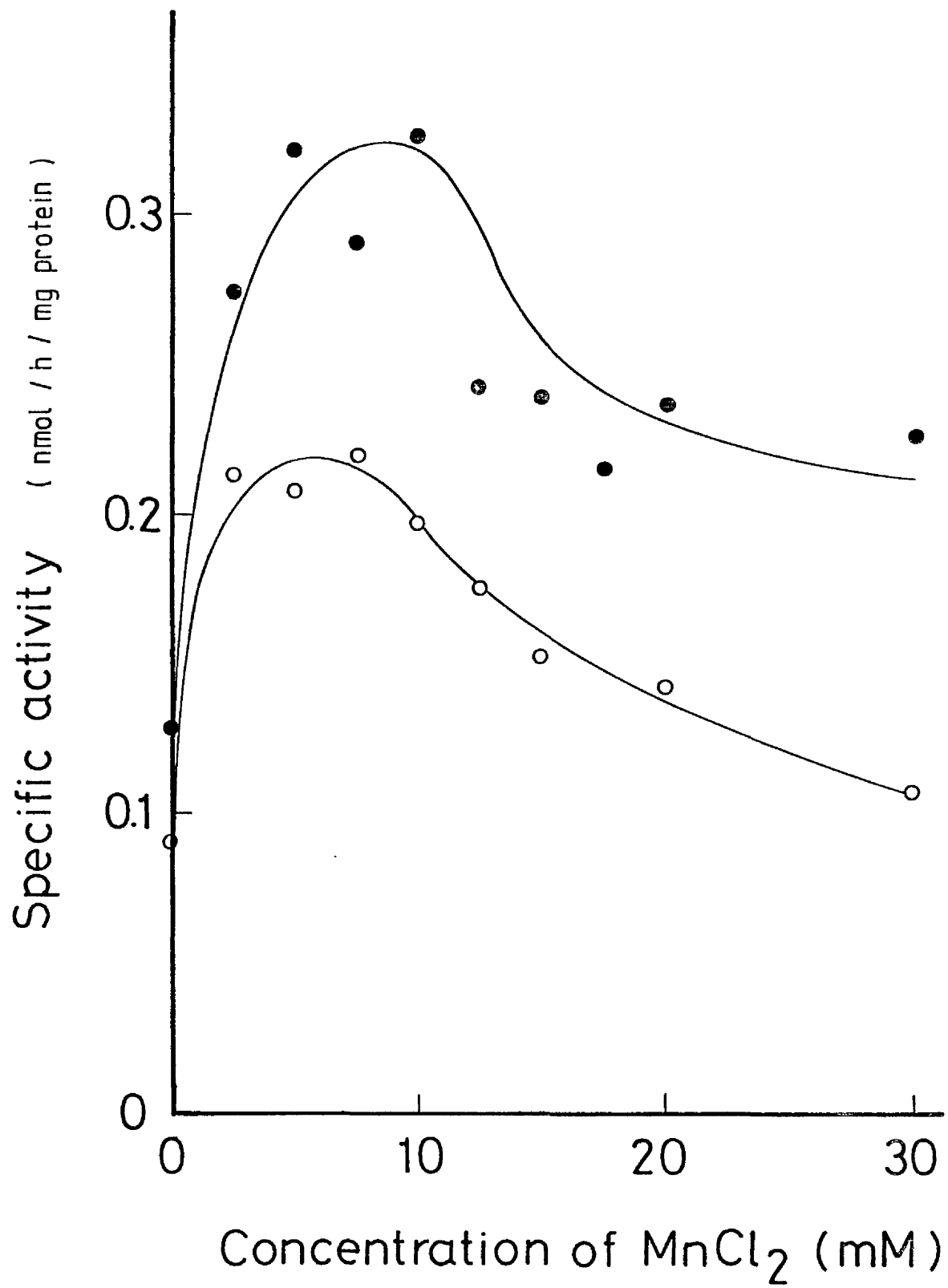
tions was rapidly lost at 4°C; about 70% of the activity was lost after 36 h (figure 11). The washed membrane fraction, however, only lost about 10% of its activity during storage for 64 h at 4°C (figure 11). Enzyme activity was similarly lost by both preparations when stored at -20°C. Figure 11 shows that most of this loss occurred during the first month's storage, with little change during the subsequent three month's storage. Slightly less activity was lost after storage of both enzyme preparations at -70°C. The decrease in activity of the sphaeroplast lysate during storage at -20°C for six weeks is shown, in more detail, in figure 12. Enzyme kept at -20°C for short periods (12 h to 7 days) at a protein concentration of 2 mg/ml, consistently showed an initial activation followed by a loss of mannosyltransferase activity. Freezing and thawing of enzyme preparations resulted in a gradual loss of activity. After 8-12 daily freezing and thawing events, 83% and 65% of the activity remained for the washed membrane and sphaeroplast lysate preparations, respectively. Although Ficoll 400, at a concentration (7%, w/v) which did not interfere with mannosyltransferase activity, slightly stabilised the loss of enzyme activity (figure 12), Ficoll 400 appeared to prevent the initial activation of mannosyltransferases that occurred when the enzyme was stored in Tris-HCl buffer alone. Behrens and Cabib (1968) stored similar enzyme preparations at -5°C, in 33% (v/v) glycerol, to stabilise mannosyltransferase activity. However, these authors also reported that glycerol was inhibitory in the enzyme assay. Storage of sphaeroplast lysate at -20°C with glycerol (30%, v/v) in 50 mM Tris-HCl buffer (pH 7.2) therefore required that the sphaeroplast lysate preparation be centrifuged and washed before assaying enzyme activity. Thus, the preparation was no longer a sphaeroplast lysate; furthermore, the specific

Figure 11.   Stability of mannosyltransferase activity at different

temperatures.

Enzyme preparations were stored at a protein concentration

of 2 mg/ml at different temperatures before assaying mannosyl-

transferase activity using the standard assay.

☐ Washed membrane fraction at 4°C		● Sphaeroplast lysate preparation at -20°C
■ Sphaeroplast lysate preparation at 4°C		○ Sphaeroplast lysate preparation at -70°C

The standard assay contained Tris-HCl buffer (40 mM ; pH 7.2),

$MnCl_2$ (7.5 mM), GDP-$[^{14}C]$mannose (120 pmol ; 20 nCi) and enzyme

as indicated (1 mg protein/ml).   Mixtures were incubated at 30°C

for 20 min.
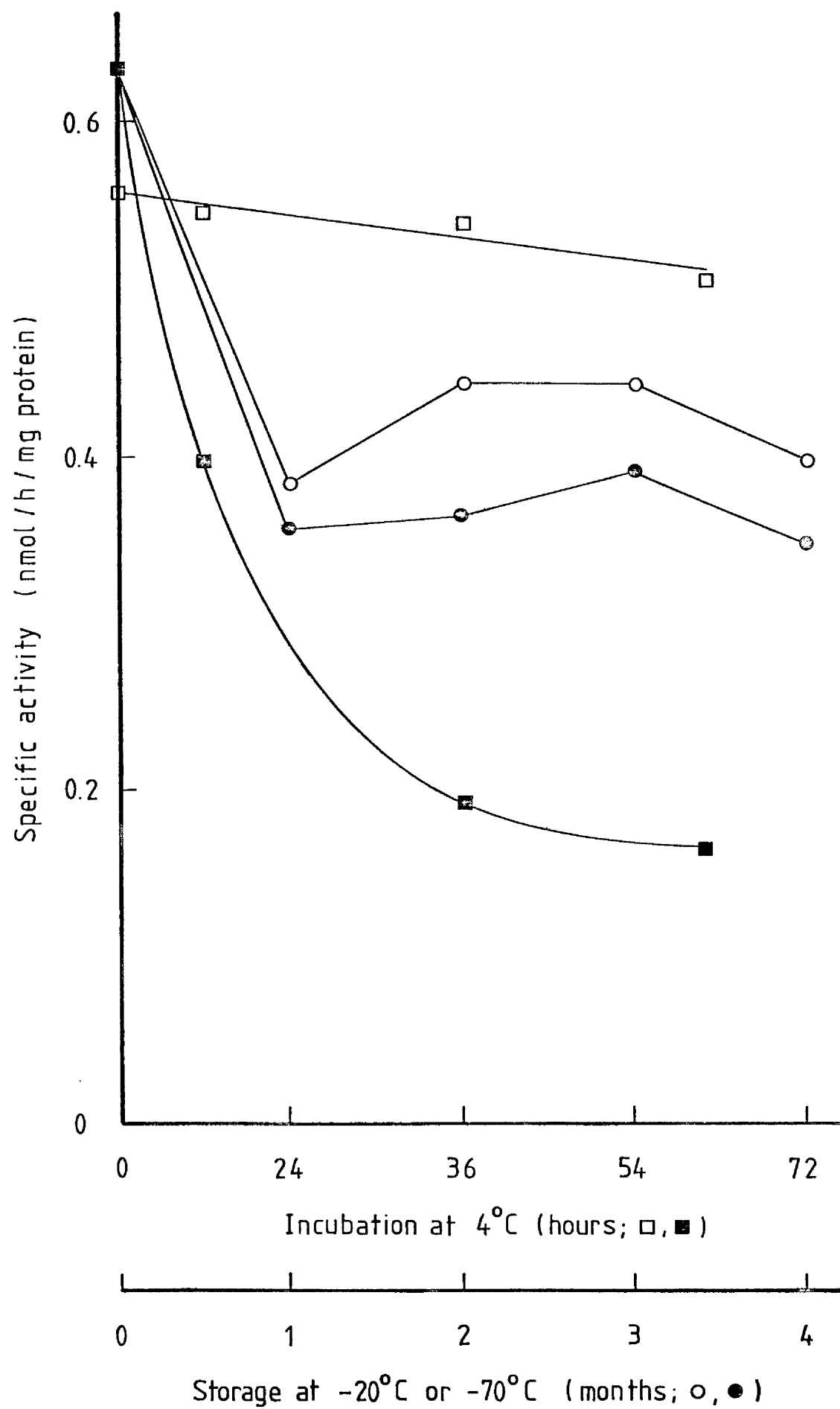
Figure 12.   Stability of mannosyltransferase activity in Tris-HCl
and in Ficoll 400

Sphaeroplast lysate preparation was stored at $-20^{\circ}C$ at a

protein concentration of 2 mg/ml in either Tris-HCl (50 mM, pH 7.2; ●)

or the same buffer containing Ficoll 400 (7%, w/v; O).   After thawing,

the enzyme activity was assayed using the standard assay.

The standard assay contained Tris-HCl buffer (40 mM ; pH 7.2),

$MnCl_2$ (7.5 mM), GDP-$\left[^{14}C\right]$mannose (120 pmol ; 20 nCi) and sphaeroplast

lysate protein (1 mg/ml).   Mixtures were incubated at $30^{\circ}C$ for 20 min.
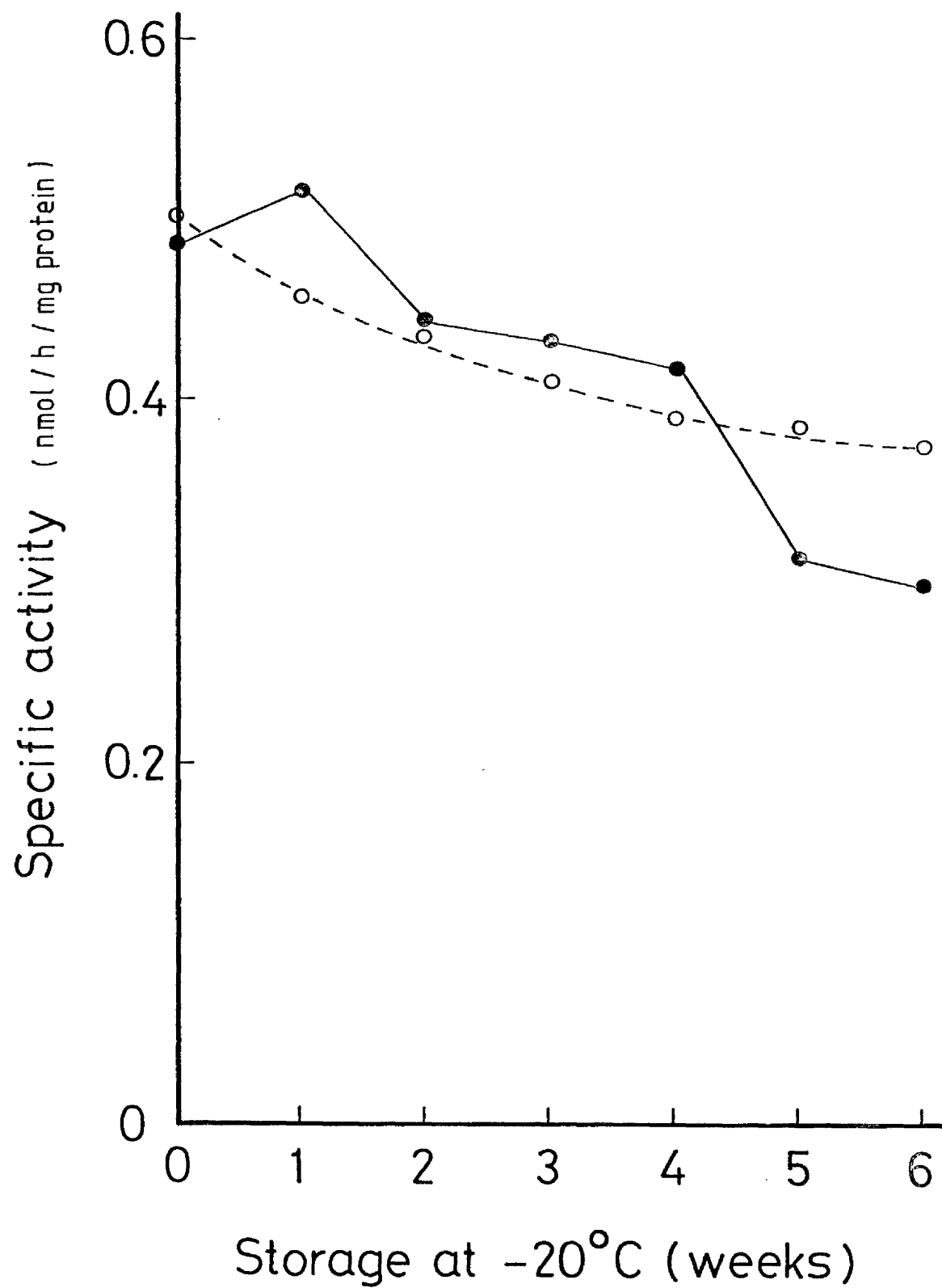
activity of enzyme stored in this manner was very irregular when tested

over a six-month period.

III. EFFECTORS OF MANNOSYLTRANSFERASE ACTIVITY

A.   Survey of effectors

        Mannosyltransferase activity was assayed at an enzyme protein

concentration of 1 mg/ml with a variety of effectors, all at a final

concentration of 1 mM.   For these experiments, enzyme was prepared from

cells treated with Zymolyase 5000 at a concentration of 0.7 mg/ml;

the results are listed in tables 1 and 2.   Of the sugars tested,

including glucose, mannose and N-acetylglucosamine, none had any dramatic

effect although, with the washed membrane preparation, mannose caused

10% inhibition of activity (table 1).   Cyclic AMP and the 5'-monophos-

phate nucleosides, AMP, UMP and GMP, similarly had little effect except

that UMP caused 11% inhibition of activity of the sphaeroplast lysate.

ADP and ATP, especially, stimulated mannosyltransferase activity in

both preparations while GTP and, more obviously, GDP inhibited activity.

Finally, of the nucleotides, UDP had no effect while UTP had a different

effect on activity of the two preparations.   UTP caused 27% inhibition

of activity of the sphaeroplast lysate preparation and 20% stimulation

of that of the washed membrane fraction.   Both inorganic phosphate and

pyrophosphate gave a similar 20% stimulation of activity of the latter

fraction while having no effect on mannosyltransferase activity of the

sphaeroplast lysate preparation.   Glutamine, required in the synthesis

of N-acetylglucosamine, had no effect on activity with either preparation.

        Table 2 shows the effect of 8 sugar nucleotides on mannosyl-

transferase activity.   UDP-N-acetylglucosamine, ADP-, CDP-, TDP- and

Table 1.   Effect of sugars, nucleotides and other compounds on

mannosyltransferase activity of sphaeroplast lysate and washed membrane

preparations from S. cerevisiae

Addition to incubation mixture (1 mM)	Relative enzyme activity (%)	
	Sphaeroplast lysate preparation	Washed membrane fraction
NONE	100.0	100.0
Glucose	101.2 ± 1.8 (3)	102.5 ± 1.0 (3)
Mannose	96.3 ± 5.7 (4)	89.4 ± 3.4 (3)
Mannose-1-phosphate	96.6 ± 0.8 (2)	ND
N-Acetylglucosamine	97.2 ± 1.0 (3)	99.4 ± 0.5 (3)
Glucosamine	100.4 ± 6.0 (4)	101.8 ± 3.6 (3)
2-Deoxyglucose	103.8 (1)	98.1 (1)
AMP	97.7 ± 0.7 (3)	97.1 ± 1.4 (3)
ADP	119.3 ± 5.3 (3)	114.1 ± 2.1 (3)
ATP	242.4 ± 6.3 (3)	232.4 ± 7.2 (4)
GMP	94.9 ± 3.1 (3)	100.0 ± 4.4 (3)
GDP	38.9 ± 3.2 (3)	40.3 ± 0.3 (3)
GTP	65.0 ± 3.4 (3)	66.3 ± 2.6 (3)
UMP	88.9 ± 4.2 (3)	105.1 ± 3.3 (3)
UDP	101.1 ± 3.8 (3)	97.1 ± 1.4 (3)
UTP	73.3 ± 5.5 (3)	120.5 ± 2.3 (4)
Pi	106.3 ± 6.0 (3)	120.3 ± 3.4 (3)
PPi	100.0 ± 5.4 (3)	120.9 ± 5.4 (4)
Cyclic AMP	98.6 ± 3.9 (3)	96.0 ± 2.2 (3)
Glutamine	100.0 ± 7.6 (3)	96.7 ± 5.4 (3)

Values shown are means ± the standard deviation.   The number of

independent assays done is indicated in parentheses.

ND, Not determined.

Table 2.   Effect of sugar nucleotides on mannosyltransferase activity

of sphaeroplast lysate and washed membrane preparations

from S. cerevisiae

Addition to incubation mixture (1 mM)	Relative activity (%)	
	Sphaeroplast lysate preparation	Washed membrane fraction
NONE	100.0	100.0
UDP-N-acetylglucosamine	101.0 ± 1.9	101.7 ± 3.0
ADP-glucose	112.1 ± 1.5	100.2 ± 1.3
CDP-glucose	103.4 ± 4.8	91.5 ± 1.6
dTDP-glucose	99.5 ± 3.1	94.1 ± 2.4
UDP-glucose	95.2 ± 5.6	104.1 ± 4.6
GDP-glucose	19.4 ± 5.0	23.8 ± 7.0
UDP-glucose and GDP-glucose	19.4 ± 4.7	24.9 ± 7.0
UDP-mannose	80.1 ± 5.9	79.4 ± 4.9
ADP-mannose	75.7 ± 4.1	89.0 ± 3.6

Values shown are the means of three independent assays ± standard deviation.

UDP-glucose all affected mannosyltransferase activity by no more than 12%. ADP- and UDP-mannose inhibited activity of both enzyme preparations by 11-24%. GDP-glucose, on the other hand caused about 81% and 76% inhibition of activity of the sphaeroplast lysate and washed membrane preparations respectively. When GDP- and UDP-glucose were both included in reaction mixtures, at concentrations of 1 mM, there was no change in the inhibition compared with that caused by GDP-glucose alone. Four sugar nucleotides were tested over a range of concentrations up to 2 mM (figure 13). GDP-glucose was a potent inhibitor of mannosyltransferase activity. It caused 30% inhibition of activity when the concentrations of GDP-glucose and GDP-$[^{14}C]$mannose were 10 μM and 1 μM respectively. Inhibition by ADP- and UDP-mannose was optimal at approximately 1 mM, with little extra effect at 2 mM. UDP-N-acetylglucosamine, however, which had little effect on mannosyltransferase activity, caused its maximum inhibition (8%) at a concentration of 0.5 mM.

B. Mannosyltransferase activity of enzymes prepared from mutant strains

The mannosyltransferase activity of sphaeroplast lysate and washed membrane preparations of S. cerevisiae X2180-1A was compared with that of washed membrane fractions from two mutant strains of S. cerevisiae, mnn 1 and mnn 2 (table 3). Activity was lower in the washed membrane fraction than in the sphaeroplast lysate from the wild-type, and activity was lower still in washed membrane fractions prepared from the mutants. The effect of ATP and GDP-glucose on the activities of the different enzymes was compared (table 3). ATP stimulated mannosyltransferase activity in preparations from the mutants, but to a lesser extent than with the wild-type preparations. GDP-glucose caused the same inhibition (74-80%) of activity with washed membrane fractions from the mutant strains and the wild-type.

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Figure 13.   Effect of sugar nucleotides on mannosyltransferase

activity of a sphaeroplast lysate preparation

● UDP-N-acetylglucosamine

□ ADP-mannose

■ UDP-mannose

○ GDP-glucose

Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2), MnCl$_2$ (7.5 mM), GDP-$[^{14}C]$mannose (116 pmol ; 20 nCi), sphaeroplast lysate protein (1 mg/ml) and sugar nucleotides as indicated.   They were incubated at 30°C for 20 min.
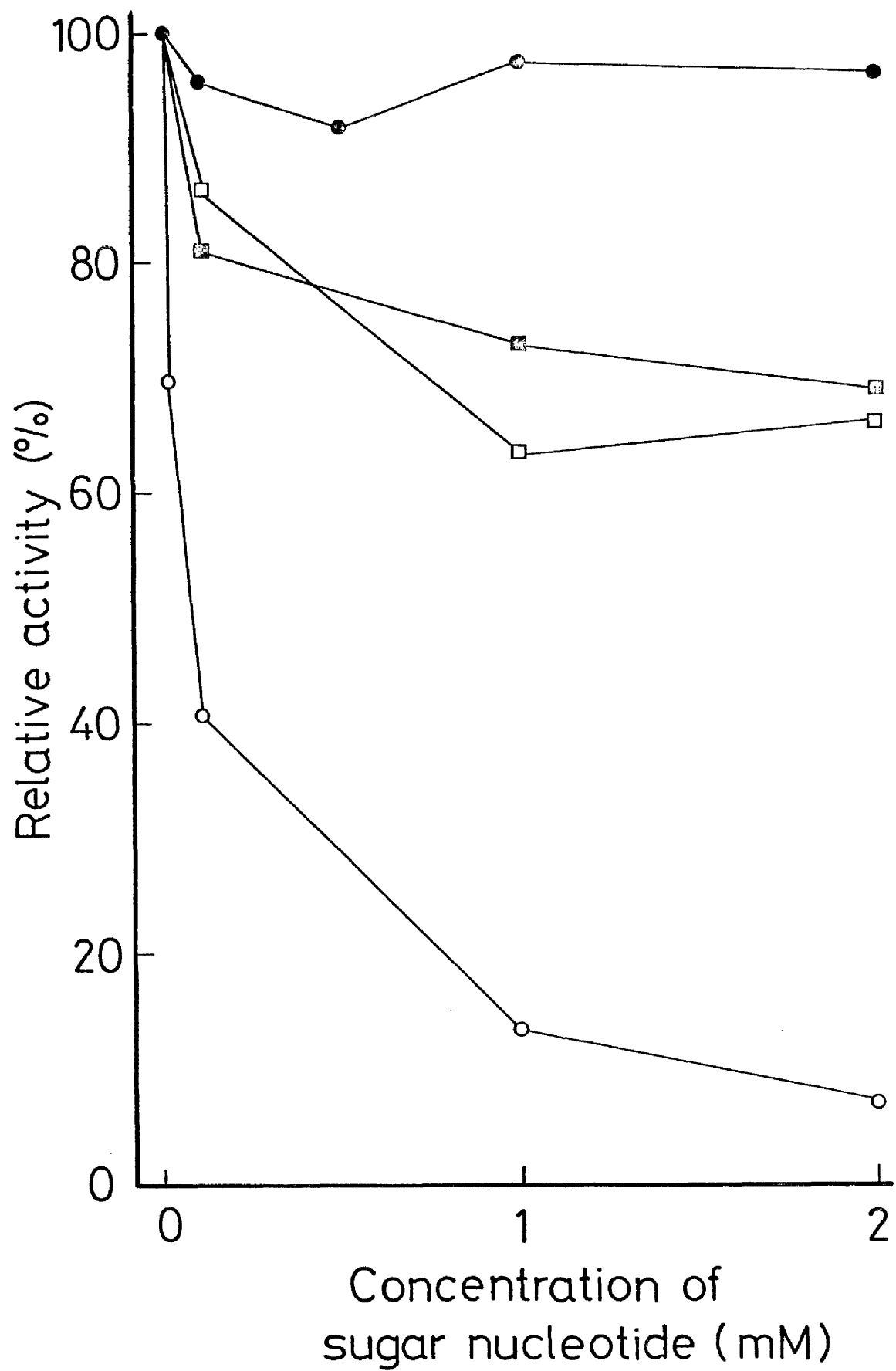
Table 3. Comparison of mannosyltransferase activities in enzyme preparations from

different strains of S. cerevisiae

Strain of S. cerevisiae	Enzyme preparation	Specific activity (pmol/h/ mg protein)	Relative enzyme activity (%)[a] with:	
			ATP (1 mM)	GDP-glucose (1 mM)
X2180-1A	Sphaeroplast lysate	398	232	19
X2180-1A	Washed membrane fraction	264	242	24
LB1-3B mnn2	Washed membrane fraction	256	151	22
LB1-10B mhn1	Washed membrane fraction	226	185	26

[a] activity expressed as a percentage of activity without effectors.

98

C.   Effect of ATP and UTP

Stimulation of mannosyltransferase activity was greatest with
ATP (table 1, p. 94).   However, in replicate experiments, using fresh
enzyme preparations, the stimulatory effect of ATP was lost. This was
considered to be due to slight changes in the method used for enzyme
preparation. Previously, addition of 1 mM ATP gave a relative enzyme
activity of 244% with an enzyme prepared from cells harvested after 3.5 h
growth at 30°C (table 1). Cells had been treated with a Zymolyase 5000
concentration of 0.7 mg/ml for 1 h at 30°C to convert them to sphaeroplasts.
Further enzyme preparations from cells either (a) harvested after 2.25 h and
incubated at 30°C for 48 min with Zymolyase 5000 (2 mg/ml), or (b) harvested
after 2 h and incubated under identical conditions with 5 mg/ml of Zymolyase
5000, were not activated by ATP.   Both of these preparations had higher
mannosyltransferase activity than previous ones but, while enzyme (a) was
activated 37% by 1 mM ATP, enzyme (b) most actually inhibited by 1 mM ATP
(9%).

In light of these results, the effect of ATP and UTP on enzyme
activity were reinvestigated using higher nucleotide concentrations and
different enzyme preparations (table 4).   Late log-phase cells, which
required longer periods of incubation for sphaeroplast formation, gave rise
to washed membrane fractions that were activated by both ATP and UTP at
concentrations of 1 mM and 5 mM.   However sphaeroplast lysate and washed
membrane preparations, derived from cells harvested earlier and incubated
for only 45 min with higher concentrations of Zymolyase 5000, possessed
mannosyltransferase activity that was marginally inhibited by UTP and ATP
at 1 mM.   It was again found that 1 mM UTP inhibited mannosyltransferase
activity of the sphaeroplast lysate while enhancing activity of the
washed membrane fraction to the same extent as previously (table 1).
At higher nucleotide concentrations, activity was further

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Table 4. Effect of ATP and UTP on mannosyltransferase activity from different preparations

| | Relative activity (%)[b] in the presence of: | | | |
| | ATP | | UTP | |
Enzyme preparation[a]	1 mM	5 mM	1 mM	5 mM
Sphaeroplast lysate 1	137.2	ND	78.6	ND
Washed membrane fraction 1	135.4	216.1	121.7	144.5
Sphaeroplast lysate 2	97.5	93.6	95.5	88.3
Washed membrane fraction 2	98.8	115.3	99.2	98.4

[a]Reaction mixtures contained an enzyme protein concentration of 1 mg/ml. Enzymes were obtained following lysis of sphaeroplasts prepared by one of two methods:
1. Cells harvested after 3.5 h; treated at 30°C for 60 min with 0.7 mg Zymolyase 5000 per ml.
2. Cells harvested after 3 h; treated at 30°C for 45 min with 2.0 mg Zymolyase 5000 per ml.

[b]Mannosyltransferase activity expressed as the percentage activity in each preparation without nucleotide.

ND, Not determined.

100

inhibited in all but one case. Mannosyltransferase activity of the
washed membrane fraction was enhanced by 15.3% with 5 mM ATP. With
the two exceptions above, there appeared to be a general trend in the
results. Enzymes prepared from cells harvested early and incubated
for short periods with Zymolyase 5000, were inhibited by ATP and UTP.
On the other hand, enzymes prepared from cells harvested later and
incubated longer with Zymolyase 5000 were activated by these nucleotides.

Stimulation of mannosyltransferase activity by ATP is not due
to the protection of GDP-$\left[^{14}C\right]$mannose from pyrophosphatase degradation.
Less than 1% of the substrate was hydrolysed to mannose-1-phosphate or
mannose as determined by paper chromatographic separation of reaction
products in solvent A from mixtures containing sphaeroplast lysate
protein (1 mg/ml). The small amount of $\left[^{14}C\right]$-labelled mannose and
mannose-1-phosphate was converted to material with the same chromato-
graphic mobility as ADP-mannose when 5 mM ATP was included in the
reaction mixtures.

D. <u>Inhibition of mannosyltransferase activity by GDP-glucose</u>

(i) <u>Effect of GDP-glucose on total mannosyltransferase activity</u>

Mannosyltransferase activity of the sphaeroplast lysate
preparation was assayed over a range of GDP-mannose concentrations
(0.2-2.0 mM) and the results, expressed in the form of a Lineweaver-
Burk plot, are shown in figure 14. The reaction exhibited biphasic
kinetics, suggesting that the enzyme preparation contained more than
one mannosyltransferase activity. Two Km values (0.13 mM and 0.53 mM)
and two V values (6.94 and 11.43 nmol/h/mg protein), for low and high
substrate concentrations respectively, were calculated. In the presence
of 1 mM GDP-glucose, the V values remained unaltered, while the apparent

Figure 14.   Lineweaver-Burk plot for mannosyltransferase activity

with and without GDP-glucose

         Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2),

$MnCl_2$ (7.5 mM), sphaeroplast lysate protein (1 mg/ml), GDP-$\left[^{14}C\right]$mannose

(578 pmol ; 100 nCi) and carrier GDP-mannose (0-2 mM).   Mixtures

contained either 1 mM GDP-glucose (O) or no GDP-glucose (●).   They were

incubated at $30^{\circ}C$ for 20 min.
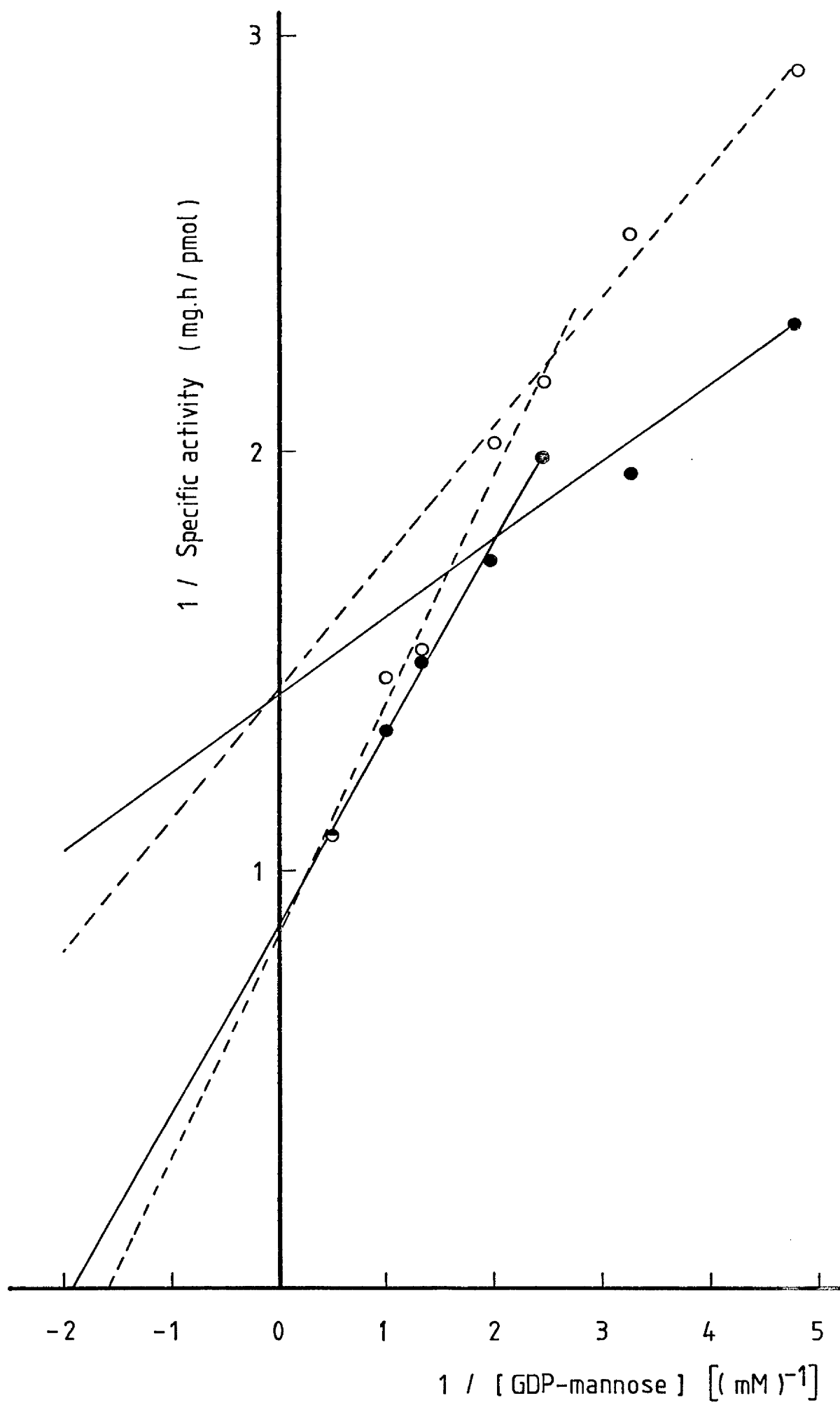
Km values were increased to 0.23 mM and 0.63 mM. Inhibition of mannan synthesis by GDP-glucose became markedly noticeable only at GDP-glucose : GDP-mannose molar ratios of 2 : 1, or greater (figure 15). These results indicated that GDP-glucose was a competitive inhibitor of mannosyl-transferase.

The inhibition of mannan synthesis by GDP-glucose was not the result of enhancing GDP-$[^{14}C]$mannose degradation. Chromatographic separation of the reaction products in solvent A showed that there was only 1% degradation of GDP-$[^{14}C]$mannose during 20 min incubation at $30^{o}C$ without GDP-glucose. When reaction mixtures containing 1 mM GDP-glucose were applied to paper chromatograms and the products separated, the decrease in labelled polymer remaining at the origin (6,009 cpm) was reflected in an increase in radioactivity that co-migrated with the GDP-mannose standard (7,038 cpm) with no additional breakdown of the labelled substrate.

As would be predicted for a competitive inhibitor, GDP-glucose does not inhibit mannosyltransferase activity by binding irreversibly to the enzymes. Sphaeroplast lysate was incubated at $0^{o}C$ for 30 min with 1 mM GDP-glucose and then dialysed for 24 h against 200 volumes of Tris-HCl (50 mM ; pH 7.2). Mannosyltransferase activity of the enzyme was then assayed with or without 1 mM GDP-glucose. Although 56% of enzyme activity was lost during dialysis at $4^{o}C$, GDP-glucose still caused the same inhibition (76%) of the remaining activity as it did for an enzyme that had been similarly dialysed at $4^{o}C$ without the pre-incubation with GDP-glucose (74.6%).

UDP-glucose is a precursor for β-glucans (Sentandreu et al, 1975; Bálint et al, 1976; López-Romero and Ruiz-Herrera, 1977; Shematek et al, 1980) and can also act as a glucosyl donor in the complex

Figure 15.   Inhibition of mannosyltransferase activity by GDP-glucose

with different concentrations of GDP-mannose

Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2), MnCl$_2$ (7.5 mM), sphaeroplast lysate protein (1 mg/ml), GDP-$\left[^{14}\text{C}\right]$mannose (578 pmol ; 100 nCi), carrier GDP-mannose (0-2.0 mM) and GDP-glucose (1 mM). Mixtures were incubated at 30$^\text{o}$C for 20 min.
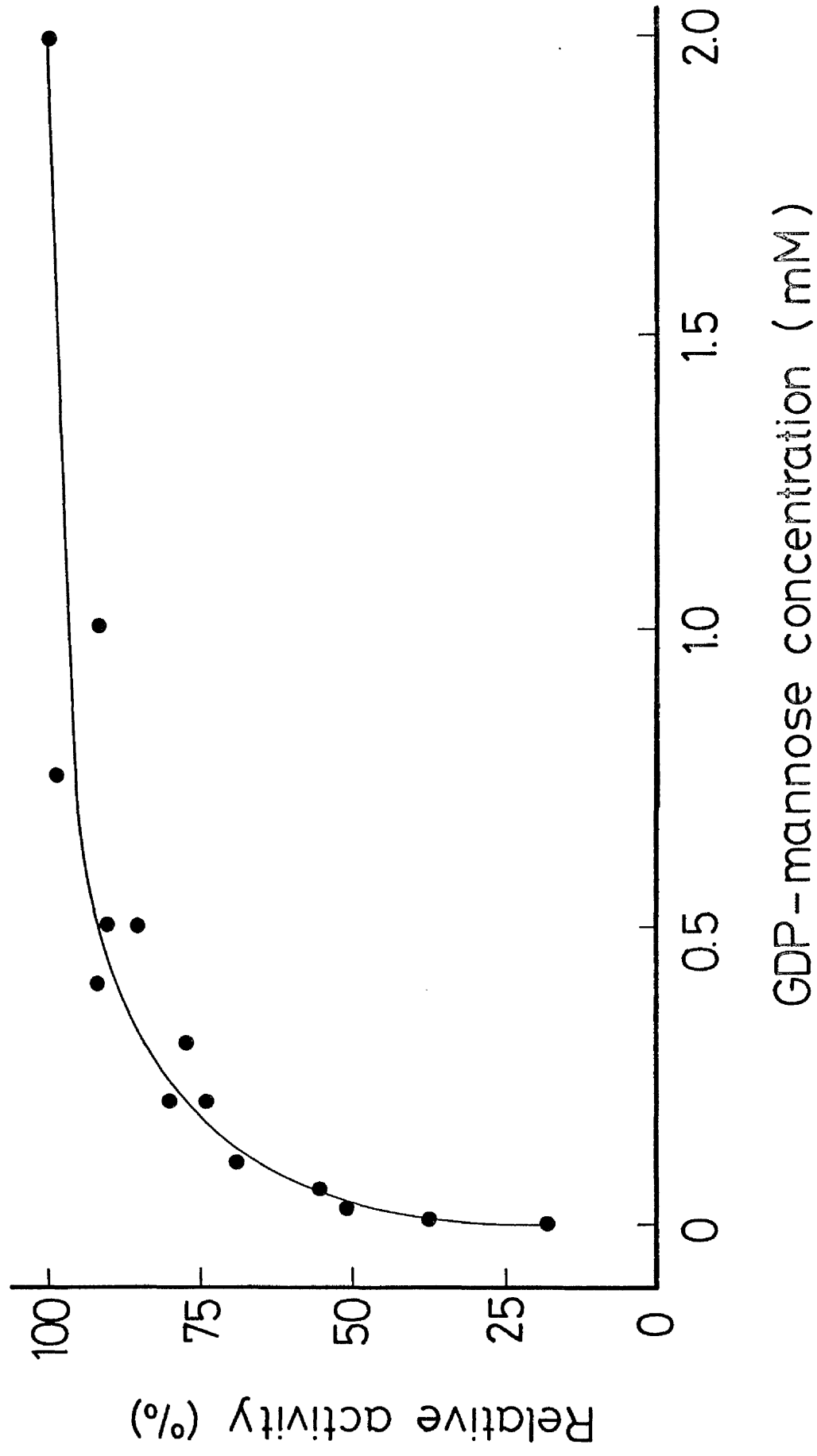
synthesis of the mannan inner core (Parodi, 1979b). GDP-glucose has also been proposed as a precursor in β-(1$\rightarrow$6)glucan synthesis (Bálint et al, 1976). Polymer synthesis from both these sugar nucleotides was investigated using the present assay system. Incorporation of radio-activity into perchloric acid-precipitable material from either UDP- or GDP-$[^{14}C]$glucose was negligible compared with that from equivalent concentrations of GDP-$[^{14}C]$mannose (table 5, experiments 1 and 3). The small amounts of polymer formed precluded their further characterisation. However, it was apparent that very little glucan was synthesised under the standard mannosyltransferase assay conditions. This indicated that glucosyltransferase activity is very low in sphaeroplast lysates and also that mannosyltransferases are quite specific in their requirement for GDP-mannose as a substrate. Nevertheless, GDP-glucose (1 mM) still inhibited mannosyltransferase activity as well as polymer formation from UDP-$[^{14}C]$glucose (about 78% inhibition in each case; experiment 2, table 5). Synthesis of the latter was also completely blocked by 1 mM GDP-mannose.

(ii) Effect of GDP-glucose on the lipid pathway of mannan synthesis

It is possible that GDP-glucose inhibits mannosyltransferase activity by competing with GDP-mannose for dolichol required in the lipid pathway of mannan synthesis. Mannose-containing lipids can be extracted from reaction mixtures by organic solvents; the more polar the solvent, the more hydrophilic are the lipid intermediates extracted. When sphaer-oplast lysate protein (1 mg/ml) was incubated with GDP-$[^{14}C]$mannose and then extracted with two chloroform-methanol mixtures (chloroform-methanol, 2:1, v/v, then chloroform-methanol-water, 10:10:3, by vol.) only 27 pmol $[^{14}C]$mannose per hour per mg protein was incorporated in the combined

Table 5.   Polymer synthesis from different sugar nucleotides

Radioactive sugar nucleotide in assay[a]	Incorporation of [14C] sugar into polymer (pmol/h/mg protein)	Percentage incorporation of total radioactivity added	Relative enzyme activity (%)[b] with :		
			GDP-Mannose[c]	GDP-glucose	UDP-glucose
Expt. 1					
GDP-[14C]mannose (116 pmol; 20 nCi)	471.32	13.96			
GDP-[14C]mannose (578 pmol;100 nCi)	1959.60	19.37			
UDP-[14C]glucose (327 pmol;100 nCi)	26.92	0.29			
GDP-[14C]glucose (361 pmol;100 nCi)	27.73	0.26			
Expt. 2					
GDP-[14C]mannose (116 pmol; 20 nCi)	342.62	9.87	-	21.6	96.0
UDP-[14C]glucose (327 pmol;100 nCi)	19.96	0.22	0.0	22.5	-
Expt. 3					
GDP-[14C]mannose (116 pmol; 20 nCi)	470.30	14.05			
UDP-[14C]glucose (116 pmol; 20 nCi)	0.0[d]	0.0			
GDP-[14C]glucose (116 pmol; 20 nCi)	0.0	0.0			

[a]All reaction mixtures contained 10 mg/ml of sphaeroplast lysate protein.

[b]Relative enzyme activity expressed as a percentage of activity without unlabelled sugar nucleotides.

[c]Non-radioactive sugar nucleotides added to give a final concentration of 1 mM.

[d]Limit for detection of incorporation was 3.0 pmol/h/mg protein.

extracts. Separation of the radioactively-labelled material in extracts by t.l.c. revealed that there were several $[^{14}C]$mannolipids (figure 16). These were only detected in small quantities that were insufficient to allow their characterisation. However, the fastest migrating component extracted by chloroform-methanol (2:1, v/v) is probably dolichol monophosphate mannose (Tanner, 1969; Sentandreu and Lampen, 1971) while the other radioactive components are probably of short dolichol diphosphate-linked oligosaccharides (Lehle and Tanner, 1975; 1978a). Material extracted by the more polar chloroform-methanol-water (10:10:3, by vol.) solvent is likely to comprise a mixture of larger dolichol diphosphate-bound oligosaccharides (Lehle and Tanner, 1978a; Parodi, 1978). Incorporation of radioactivity from GDP-$[^{14}C]$mannose into chloroform-methanol extractable material (combined extracts) was inhibited by 1 mM GDP-glucose. Incorporation was 27 pmol $[^{14}C]$mannose per hour per mg protein without GDP-glucose, where there was no detectable incorporation in its presence, i.e. less than 3 pmol/h/mg protein.

When GDP-$[^{14}C]$glucose was substituted for GDP-mannose as the substrate, no radioactive material could be extracted with either of the two chloroform-methanol mixtures. Therefore, although GDP-glucose inhibits the formation of mannose-containing lipid intermediates, it seems unlikely that GDP-glucose inhibits mannan synthesis by competing with GDP-mannose for the available dolichol phosphate.

An alternative lipid-extraction procedure using butan-1-ol was tried and it indicated, initially, that some lipids might be labelled using GDP-$[^{14}C]$glucose as the substrate (about 40-45 pmol $[^{14}C]$glucose were extracted into butan-1-ol per hour per mg protein). However, when the butan-1-ol-extracted material was analysed by paper chromatography in solvent A it was found to be $[^{14}C]$glucose. This was

Figure 16.   Separation of $[^{14}C]$-labelled lipids by t.l.c.

Reaction mixtures contained Tris-HCl (40 mM ; pH 7.2),
MnCl$_2$ (7.5 mM), sphaeroplast lysate protein (1 mg/ml) and either
GDP-$[^{14}C]$mannose (116 pmol ; 20 nCi) or GDP-$[^{14}C]$glucose (361 pmol ;
100 nCi).   After incubation at 30°C for 20 min the mixtures were
extracted with chloroform-methanol (2:1, v/v) and the residue
extracted with chloroform-methanol-water (10:10:3, by vol.).
Concentrated extracts were applied to silica gel G plates and
separated in solvent D.

———————————   GDP-$[^{14}C]$mannose

- - - - - - - - - -   GDP-$[^{14}C]$glucose

Arrows and S.F. indicate the positions of the origin and
solvent front respectively.
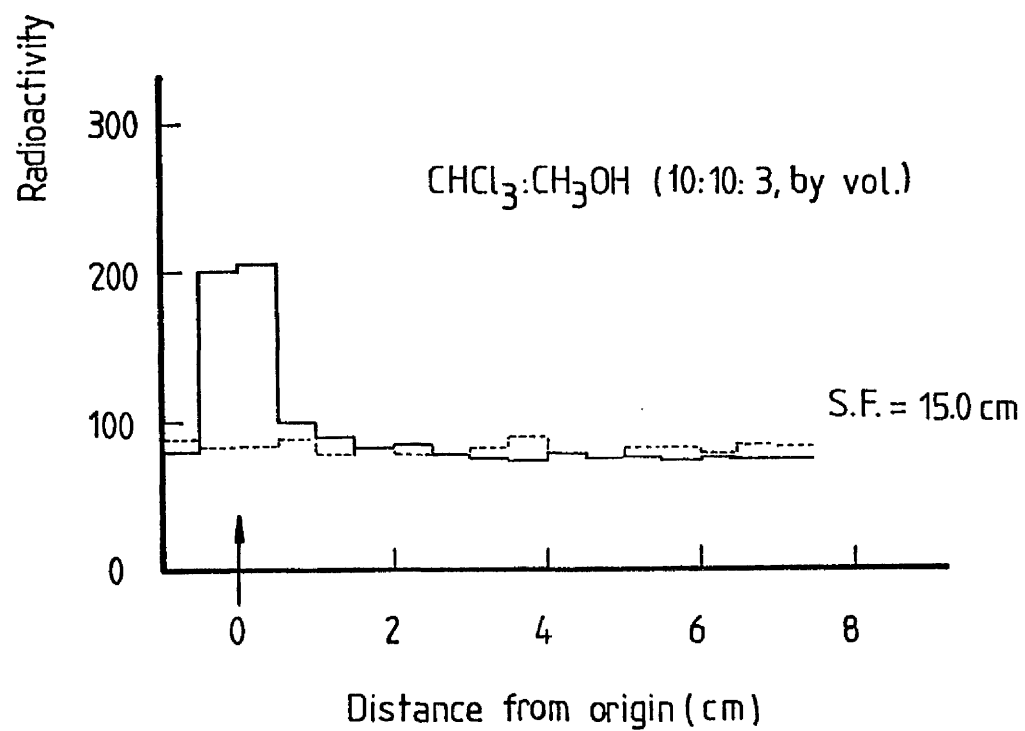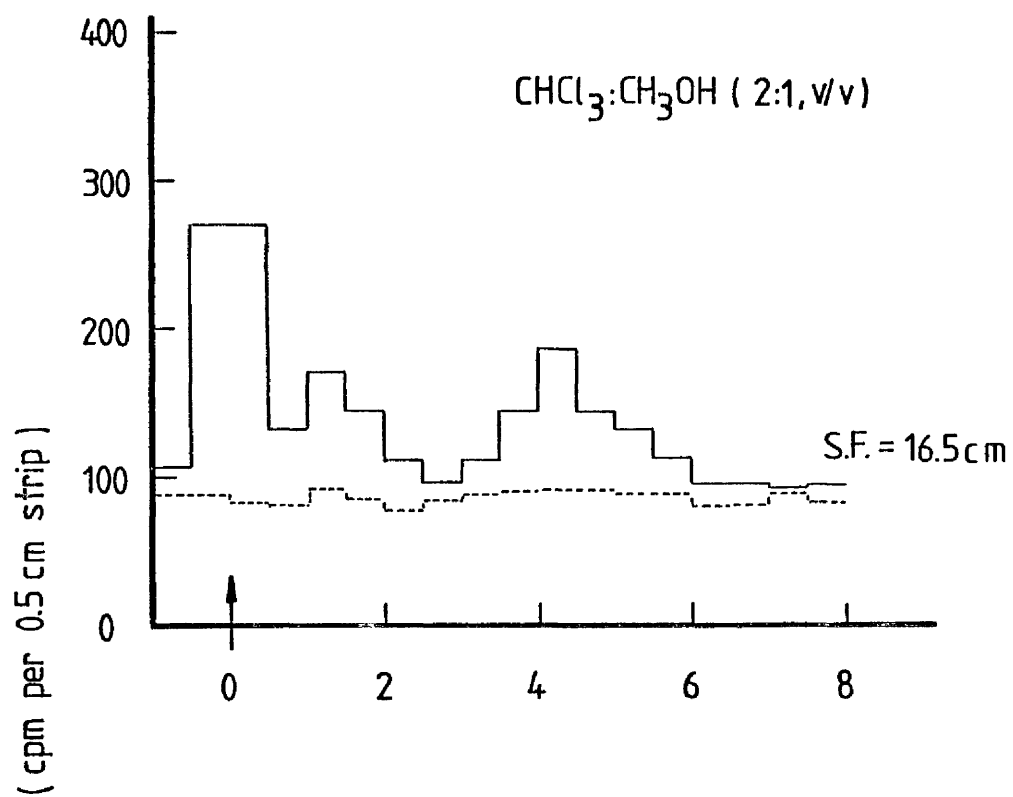
produced by hydrolysis of GDP-$[^{14}C]$glucose during incubation of reaction mixtures (see section (iii) below). When $[^{14}C]$glucose (0.1 ml; 20 nCi) was treated similarly with butan-1-ol, 1.25% of the radioactivity was extracted into the organic phase, even after washing with water. This percentage would account for the amount of radioactive material extracted with butan-1-ol from reaction mixtures containing GDP-$[^{14}C]$glucose.

(iii) <u>GDP-glucose glucohydrolase activity</u>

Sonnino <u>et al</u> (1966) reported that <u>S. cerevisiae</u> contains a soluble GDP-glucose glucohydrolase which catalyses the hydrolysis of GDP-glucose to GDP and glucose and that the enzyme is highly specific for GDP-glucose. When reaction mixtures, containing GDP-$[^{14}C]$glucose (361 pmol; 100 nCi) and sphaeroplast lysate protein (1 mg/ml), were incubated at $30^{\circ}C$ for 20 min and the products separated by paper chromatography in solvent A, it was found that the sugar nucleotide was hydrolysed to $[^{14}C]$glucose. Incubation for 2 min with sphaeroplast lysate protein (5.4 mg/ml) converted 83% of the sugar nucleotide to $[^{14}C]$glucose. The activity was diminished, although never completely removed, by washing the membranes. GDP-$[^{14}C]$glucose (361 pmol; 100 nCi) was incubated for 20 min at $30^{\circ}C$ with 170–183 µg of enzyme protein (in 50 µl) and the extent of hydrolysis was measured by paper chromatographic separation of the reaction products (figure 17). The amount of GDP-$[^{14}C]$glucose converted to $[^{14}C]$glucose by sphaeroplast lysate or membranes that were washed once (washed membrane fraction) or five times (clean membrane fraction) was 93%, 57% and 17.4%, respectively. Heat-inactivated enzyme ($100^{\circ}C$ for 3 min) controls were used in which less than 0.5% of the radioactivity was converted to $[^{14}C]$glucose. Incorporation of radioactivity into polymer from GDP-$[^{14}C]$glucose increased

Figure 17   GDP-glucose glucohydrolase activity in different

enzyme preparations

Reaction mixtures (50 μl) containing Tris-HCl buffer (40 mM ;
pH 7.2), 7.5 mM $MnCl_2$ and GDP-$[^{14}C]$glucose (361 pmol ; 100 nCi) were
incubated at $30^{\circ}C$ for 20 min with:

I   170 μg sphaeroplast lysate protein

II   174 μg washed membrane fraction

III   183 μg clean membrane fraction.

Reactions were stopped by boiling at $100^{\circ}C$ for 3 min and
separated on Whatman 3MM paper in solvent A.   Arrows indicate positions
of the origins and solvent fronts (S.F.) and hatched boxes, the positions
of non-radioactive standards: 1) GDP-glucose, 2) glucose-1-phosphate and
3) glucose.
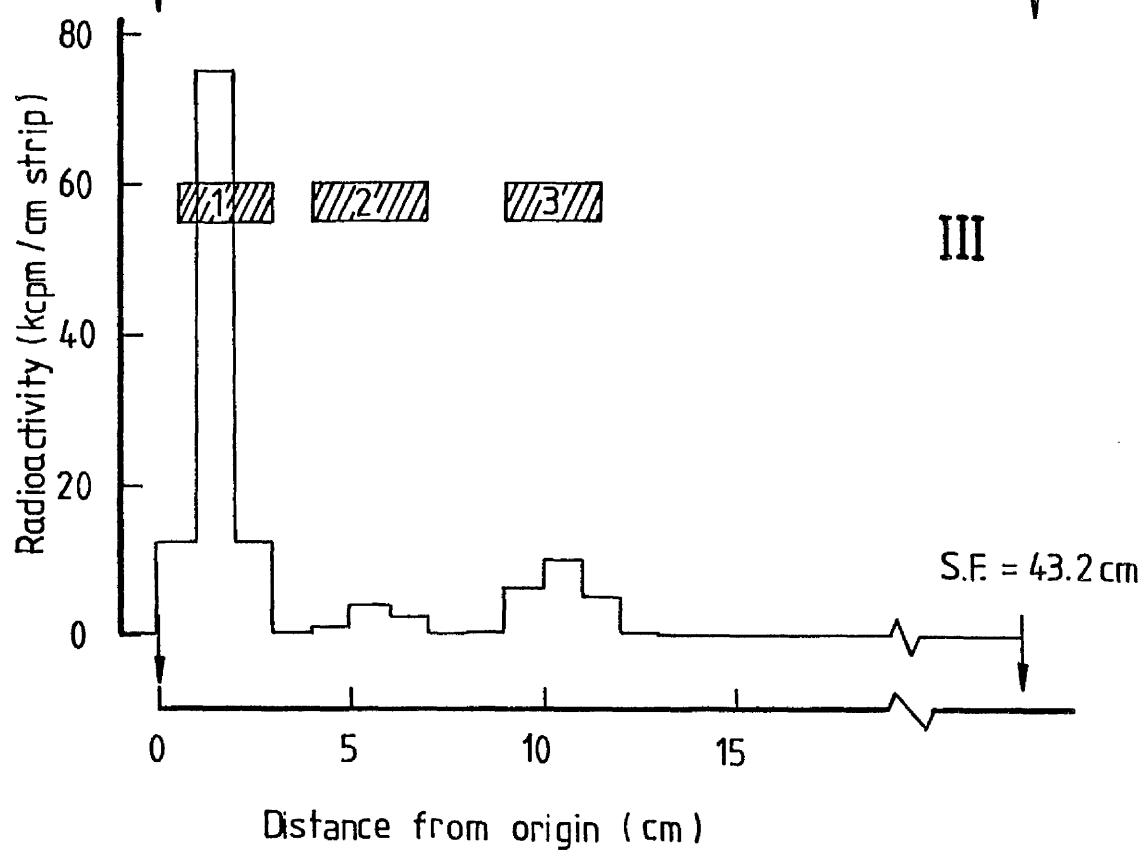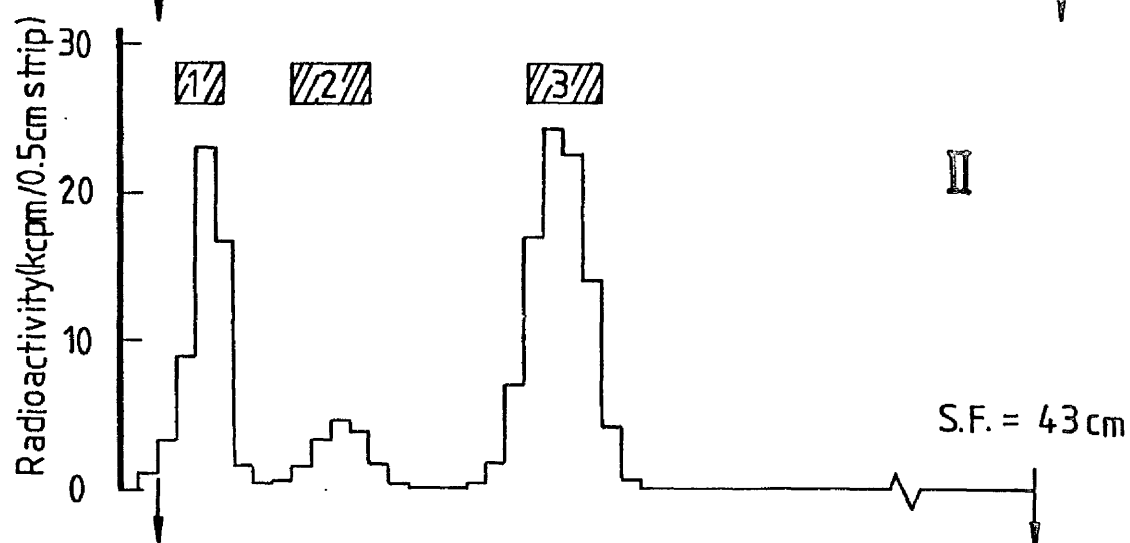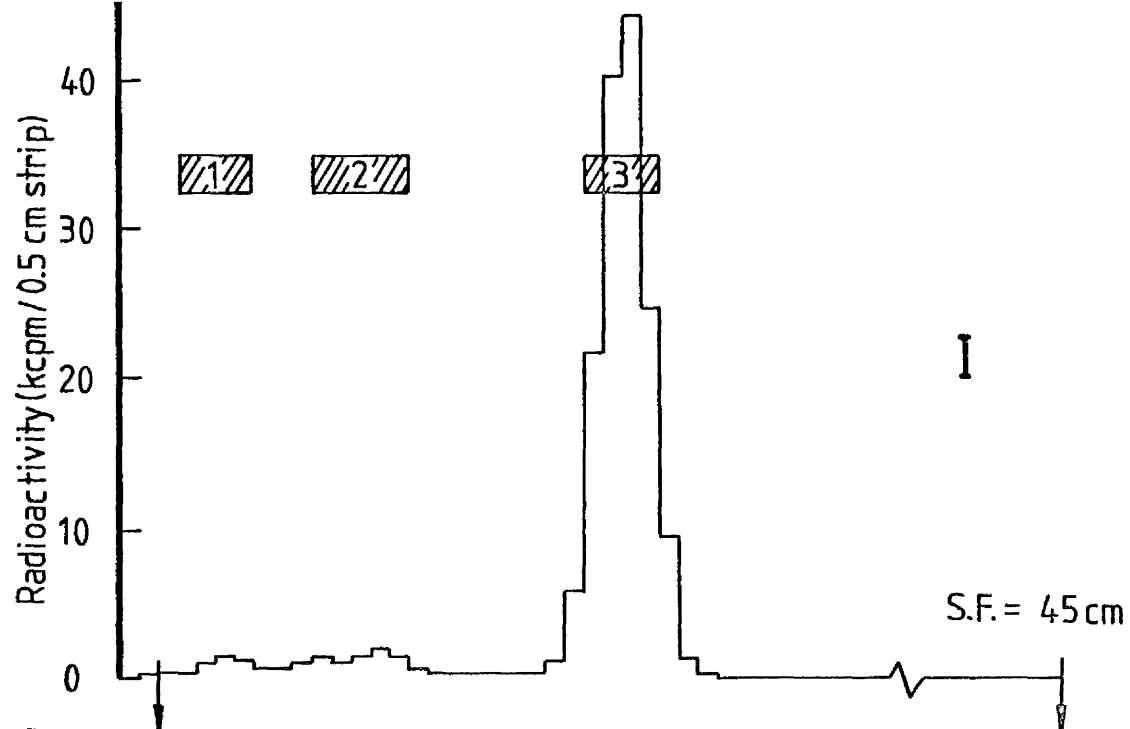
more than tenfold (35.7 pmol/h/mg protein) with membranes that had been

thoroughly washed (clean membrane fraction) compared with that with the

sphaeroplast lysate preparation (less than 3 pmol/h/mg protein).

Although GDP-$[^{14}C]$glucose (361 pmol; 100 nCi) was rapidly

hydrolysed by sphaeroplast lysate preparation, less than 1% of the sugar

nucleotide was converted to glucose at the concentration (1 mM) used to

test its action on mannosyltransferase activity. The enzyme was highly

specific for GDP-glucose. Only 1% breakdown of GDP-$[^{14}C]$mannose

(116 pmol; 20 nCi) occurred when the sugar nucleotide was incubated

with sphaeroplast lysate protein (2 mg/ml) for 20 min at 30°C. Sphaero-

plast lysate (4 mg/ml) incubated similarly with GDP- and UDP-$[^{14}C]$glucose

(116 pmol; 20 nCi) resulted in 85.7% and 9.5% of the respective sugar

nucleotides being degraded.

IV. INHIBITION AND ACTIVATION OF MANNAN SYNTHESIS IN SPHAEROPLAST
     LYSATES

A. Mannosyltransferase activity at high enzyme protein concentrations

When the mannosyltransferase activity of sphaeroplast lysate

preparations was measured in the standard assay, there was a severe

inhibition of enzyme activity at high concentrations of sphaeroplast

lysate protein (figure 18). The possibility that this effect was due

to low molecular-weight inhibitors was tested by dialysing the enzyme

preparation against 50 mM Tris-HCl buffer (pH 7.2). While dialysis

of the sphaeroplast lysate at 4°C for 24 h did relieve this inhibition,

incubation of the preparation at 4°C for 24 h without dialysis was

equally effective. This treatment resulted in a marked increase in

enzyme specific activity when assayed at high (10 mg/ml) protein

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Figure 18. Mannosyltransferase activity of sphaeroplast lysate

at different protein concentrations

Sphaeroplast lysate protein was diluted in 50 mM Tris-HCl

buffer (pH 7.2) and mannosyltransferase activity measured using the

standard assay.

Reaction mixtures contained Tris-HCl buffer (40 mM ; pH 7.2),

$MnCl_2$ (7.5 mM), GDP-$\left[^{14}C\right]$ mannose (120 pmol ; 20 nCi) and sphaeroplast

lysate protein as indicated. They were incubated at $30^{o}C$ for 20 min.
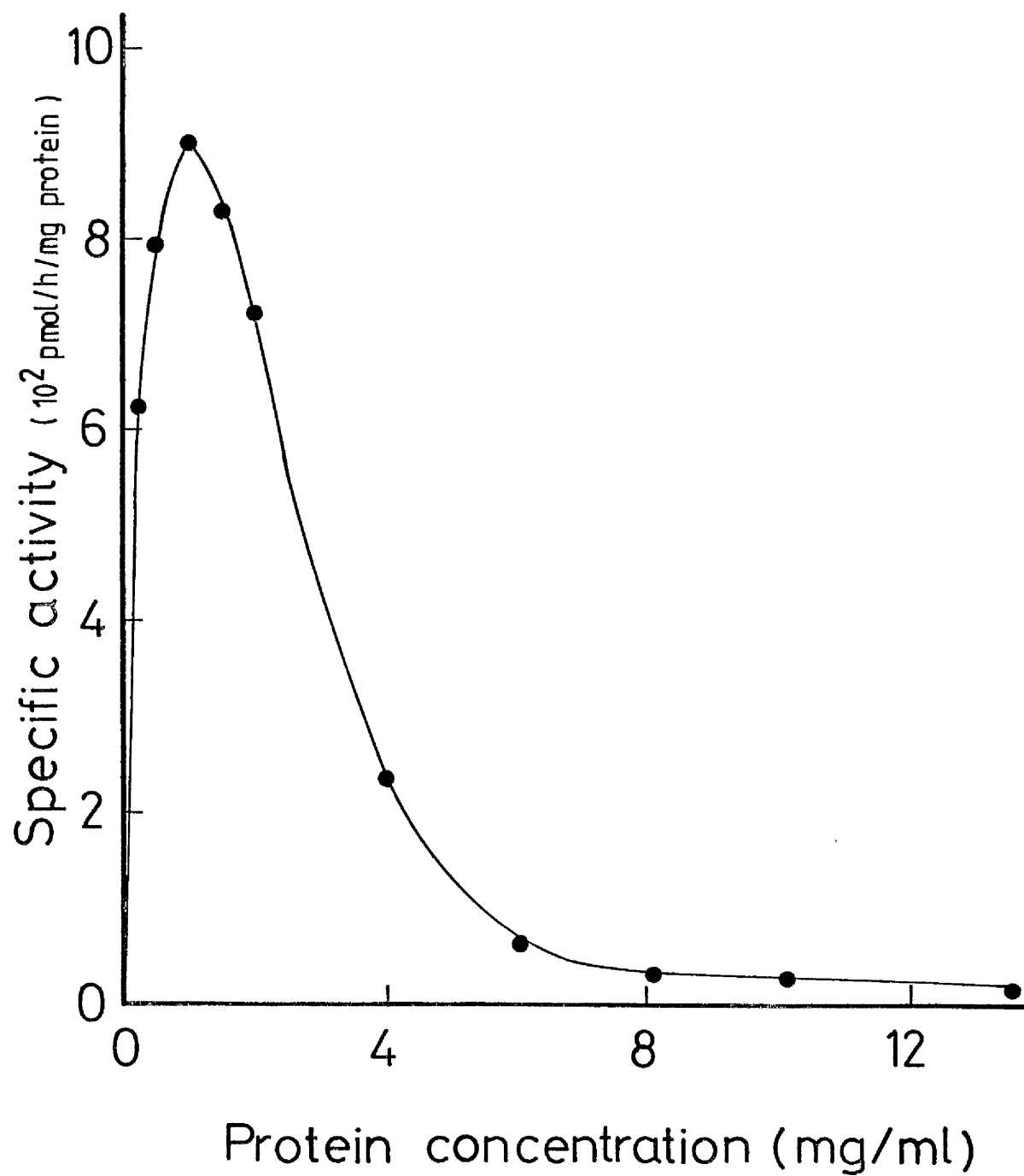
concentrations (table 6). There was a decrease in activity, however, when low protein concentrations (1 mg/ml) were used in the assay. Activation was not observed when a washed membrane fraction was substituted for the sphaeroplast lysate as the source of enzyme; on the contrary, the preparation lost activity during preincubation at $4^{o}C$ (table 6). The apparent inhibition when washed membranes were assayed at 10 mg protein/ml as compared with 1 mg/ml, was due to substrate limitation at this very high protein concentration. The rate of mannan synthesis is linear during the standard 20 min incubation period at low protein concentrations (1 mg/ml) but not at high protein concentrations (10 mg/ml; figure 19). In the latter case, enzyme specific activities calculated from incorporation during 3 min and 5 min (1.52 and 1.11 nmol/h/mg protein) compare well with the figure for 1 mg protein per ml during 20 min (1.34 nmol/h/mg protein). The high activity recorded in this experiment is simply a reflection of the variability found between different batches of enzyme. Inhibition observed at high concentrations of sphaeroplast lysate protein, however, can not be explained by substrate limitation following activation.

Synthesis of perchloric acid-precipitable material by enzyme preparations with GDP-$\left[^{14}C\right]$glucose as substrate was tested under the same set of conditions (table 7). Although polymer synthesis was inhibited by high concentrations of sphaeroplast lysate protein (10 mg/ml), activity was lost after preincubating the enzyme preparation at $4^{o}C$ for 24 h. No activation of glucosyltransferases, analagous to that of mannosyltransferases, was observed. It should be stressed that the percentage incorporation of $\left[^{14}C\right]$glucose into polymeric material was much less than that for $\left[^{14}C\right]$mannose from their respective GDP-derivatives due, in part, to the hydrolysis of GDP-$\left[^{14}C\right]$glucose by enzyme preparations

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Table 6.   Effect of preincubation of enzyme preparations at $4^\circ C$

for 24 h on mannosyltransferase activity

Enzyme preparation[a]	Specific activity (pmol/h/mg protein)[b]	
	before preincubation	after preincubation
Sphaeroplast lysate (1 mg/ml)	668.7	415.5
Sphaeroplast lysate (10 mg/ml)	48.8	195.1
Washed membrane fraction (1 mg/ml)	625.3	406.5
Washed membrane fraction (10 mg/ml)	226.7	178.6

[a]Figures in parentheses indicate protein concentrations in standard incubation mixtures.

[b]Standard incubation mixtures contained Tris-HCl buffer (40 mM ; pH 7.2); $MnCl_2$ (7.5 mM), GDP-$[^{14}C]$ mannose (120 pmol ; 20 nCi) and enzyme as indicated.   They were incubated at $30^\circ C$ for 20 min.

Figure 19.   Time course for incorporation of $[^{14}C]$ mannose into

polymer by a washed membrane fraction from <u>S</u>. <u>cerevisiae</u>

Reaction mixtures contained Tris-HCl (40 mM ; pH 7.2), $MnCl_2$ (7.5 mM), GDP-$[^{14}C]$ mannose (185 pmol ; 20 nCi) and washed membrane fraction (10 mg protein per ml, ● ;   or 1 mg protein per ml, O).   They were incubated at $30^{o}C$.
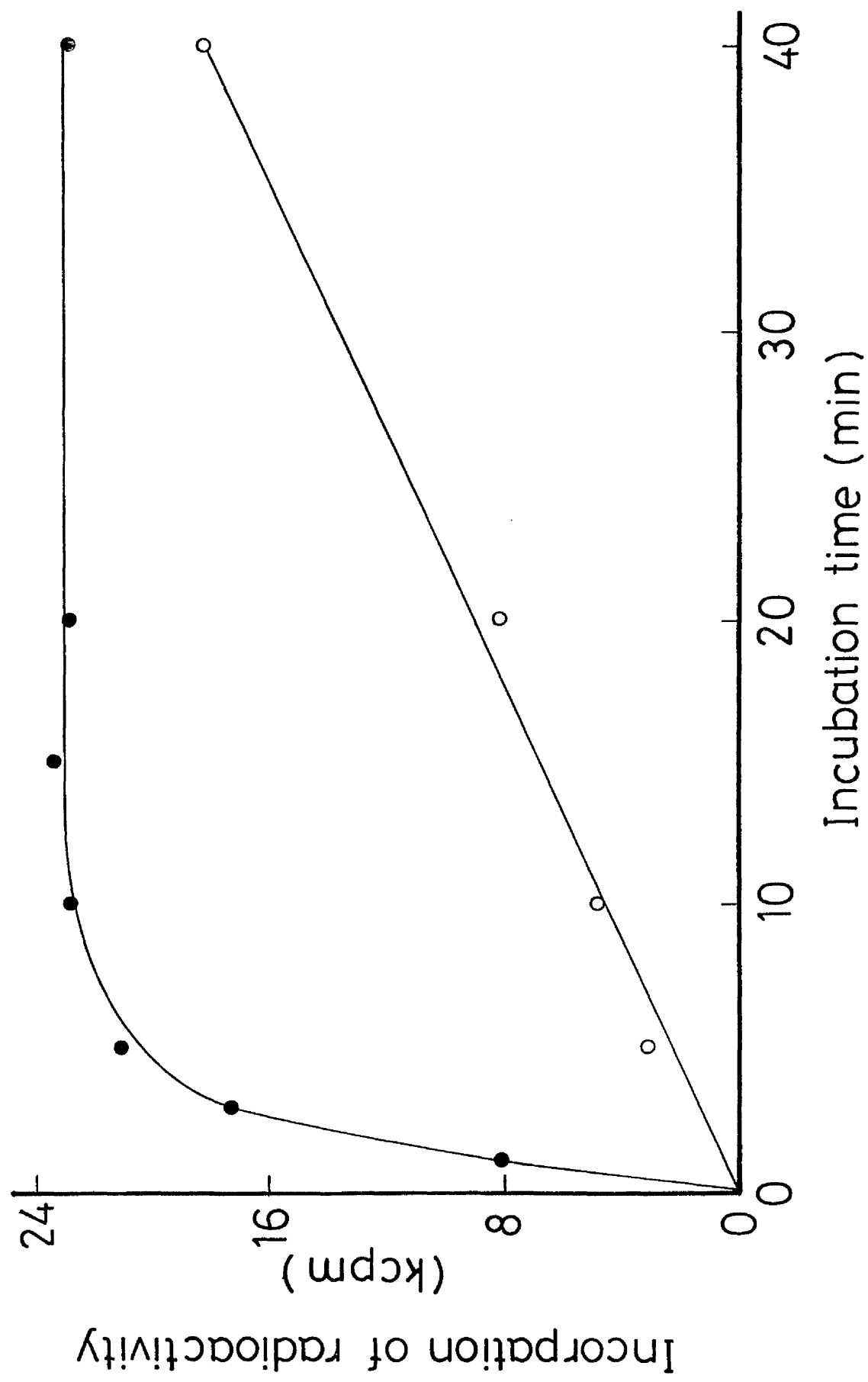
Table 7.   Effect of preincubation of enzyme preparations at 4°C

for 24 h on polymer synthesis using GDP-$\left[^{14}C\right]$glucose

as a substrate

Enzyme preparation[a]	Specific activity (pmol/h/mg protein)[b]	
	before preincubation	after preincubation
Sphaeroplast lysate (1 mg/ml)	16.4	9.5
Sphaeroplast lysate (10 mg/ml)	4.8	1.9
Washed membrane fraction (1 mg/ml)	12.6	14.0
Washed membrane fraction (10 mg/ml)	10.0	6.0

[a]Figures in parentheses indicate protein concentrations in standard incubation mixtures.

[b]Standard incubation mixtures contained Tris-HCl buffer (40 mM ; pH 7.2), MnCl$_2$ (7.5 mM), GDP-$\left[^{14}C\right]$glucose (72 pmol ; 20 nCi) and enzyme as indicated.   They were incubated at 30°C for 20 min.

(section III D, iii; pp 109-111). The data in tables 6 and 7 show

that the incorporation of $[^{14}C]$ mannose varied from 11-66%, while the

corresponding range for $[^{14}C]$ glucose was 0.5-5.0%.

The effect of preincubation at $4^{\circ}C$ on the mannosyltransferase

activity of a typical sphaeroplast lysate preparation is shown in more

detail in figure 20. Following preincubation, there was a sixfold

increase in specific activity when high concentrations of protein (10

mg/ml) were used in assay mixtures. The optimum time period for

enzyme activation at $4^{\circ}C$ was 16-32 h. Activation occurred more rapidly

but less effectively at higher temperatures (figure 21). Freezing and

thawing of the sphaeroplast lysate also caused an increase in enzyme

activity. Relative enzyme activity after 2, 4, 8 and 12 successive,

daily freezing and thawing events was 148, 216, 335 and 355%, respect-

ively while that of washed membranes decreased by 17% after 12 such

events. The extent of activation achieved by any of these methods

varied from 200-800% with different sphaeroplast lysate preparations

(where percentage activation is [relative activity -100]). Differences

were due largely to variations in the initial specific activity.

One possible explanation of these results would be proteolytic

activation of one or more mannosyltransferases. This would be

analagous to the proteolytic activation of chitin synthase which has

been proposed as a mechanism for the regulation of chitin synthesis in

yeast (Cabib and Farkaš, 1971). A variety of proteases from different

sources are known to activate the chitin synthase zymogen, in vitro

(Ulane and Cabib, 1976). Several different proteases (subtilisin BPN',

pronase, trypsin, bromelain or Aspergillus acid protease, at concen-

trations of 1-4 mg/ml, and yeast proteinase A at 0.3-0.5 mg/ml) were

added to incubation mixtures containing 10 mg/ml of sphaeroplast lysate

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Figure 20.   Mannosyltransferase activity of a sphaeroplast lysate

following preincubation at 4°C for 24 h

Sphaeroplast lysate was stored at 4°C for 24 h at a protein

concentration of 20 mg/ml, diluted in 50 mM Tris-HCl buffer (pH 7.2)

and activity assayed in standard incubation mixtures as detailed in

figure 18.   Enzyme activity is expressed as a percentage of the initial

activity (i.e. before preincubation at 4°C).
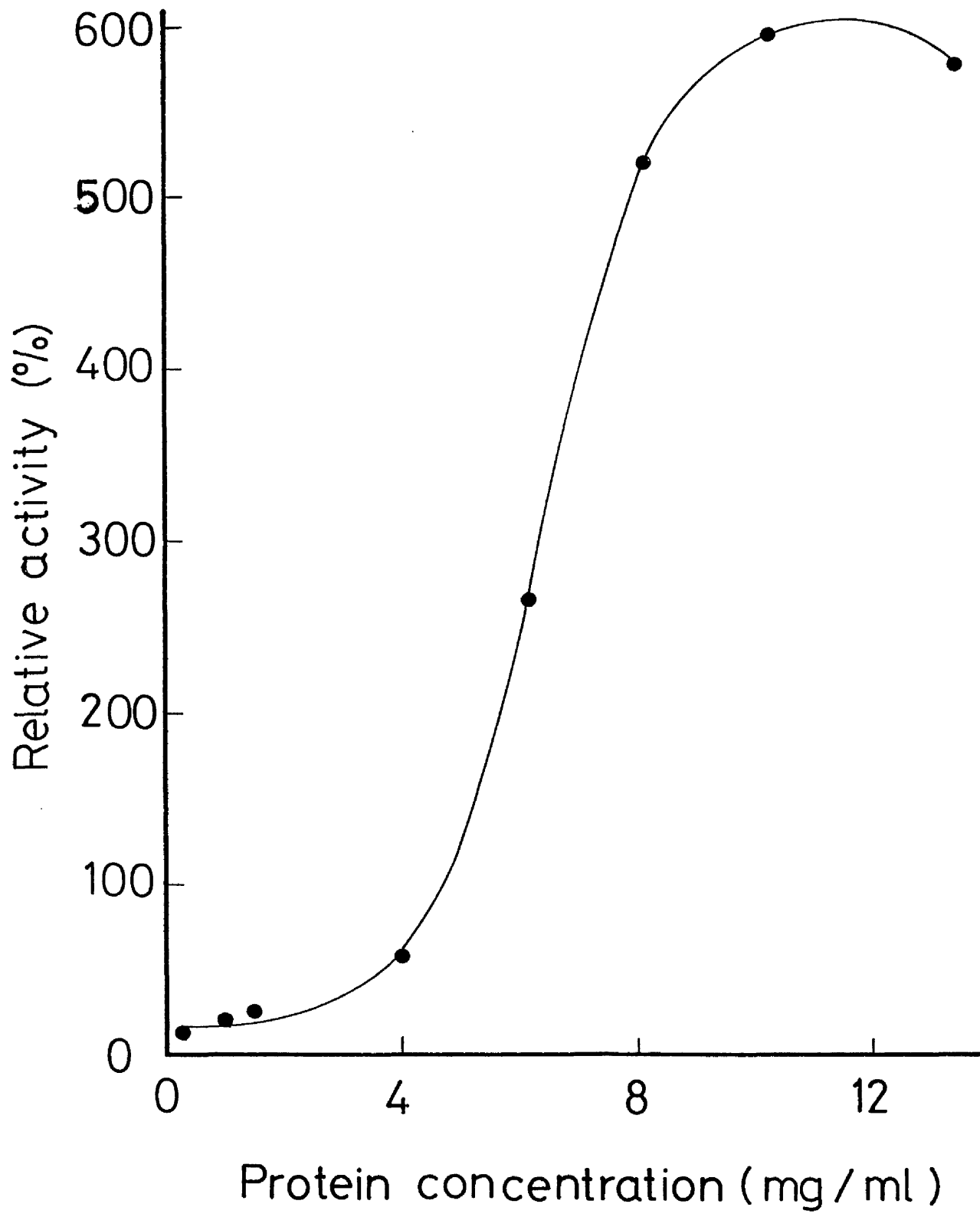
Figure 21.　　Activation of mannosyltransferase activity in a

sphaeroplast lysate preparation following preincubation

at different temperatures

　　　　　Sphaeroplast lysate was incubated at $0^{\circ}C$ (O), $4^{\circ}C$ (●), $20^{\circ}C$ (□)

and $30^{\circ}C$ (■), at a protein concentration of 20 mg/ml.　Aliquots were

removed at intervals, as indicated, and mannosyltransferase activity

determined in standard incubation mixtures containing 10 mg/ml of

sphaeroplast lysate protein.　Enzyme activation (%) is equivalent to

$\left[\text{relative enzyme activity } -100\right]$ (%).
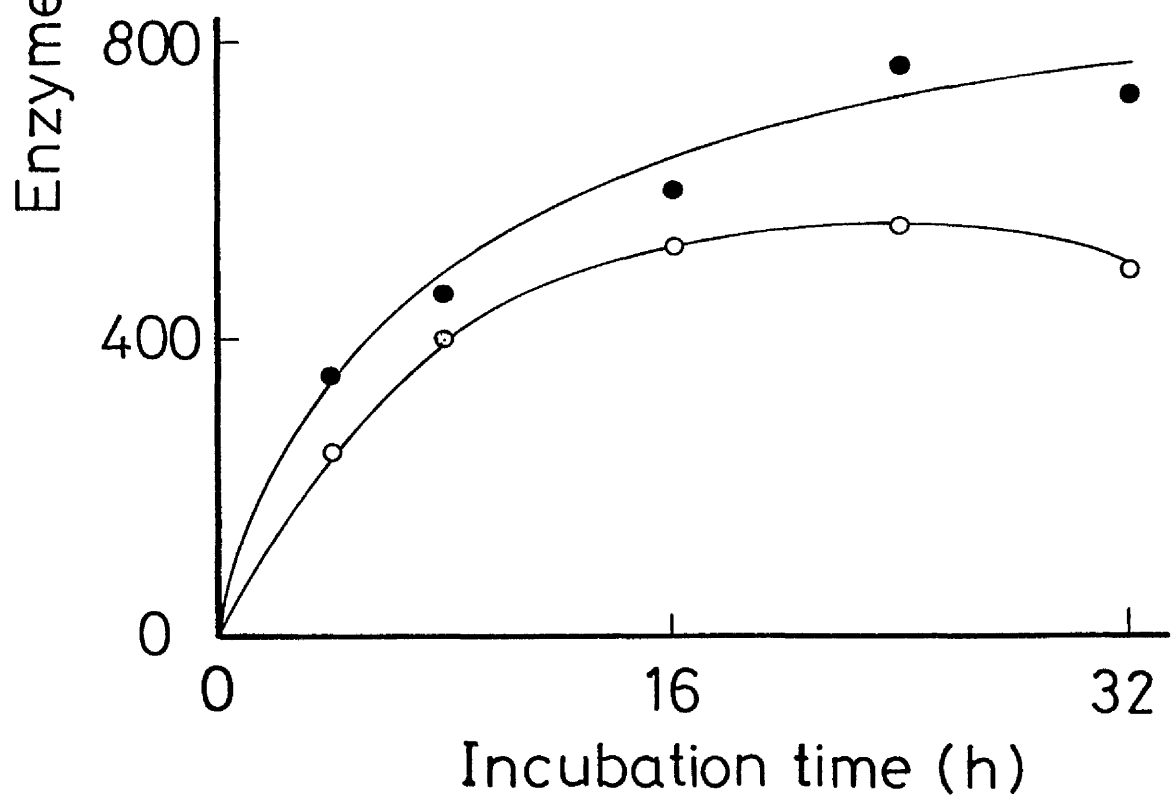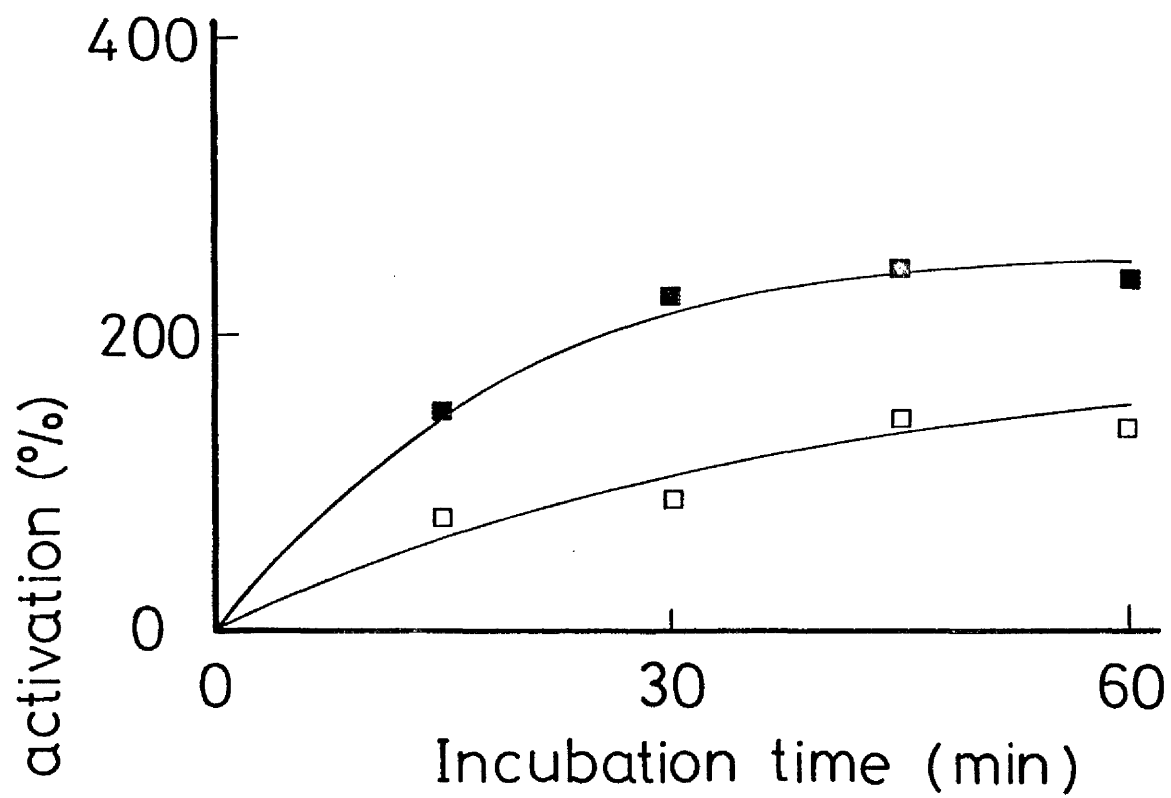
protein, but none of them had any appreciable activating effect. For example, the most noticeable changes in activity were caused by pepsin (4 mg/ml), where the increase in specific activity was only 1.9 pmol/h/mg protein, and <u>Aspergillus</u> acid protease (4 mg/ml), where there was a decrease of 2.2 pmol/h/mg protein. Furthermore, protease inhibitors (trypsin inhibitor at 200 µg/ml; quinacrine, PMSF and o-phenanthroline all at 1 mM) when added to the sphaeroplast lysate before preincubation at 4°C, had little or no effect on the activation of mannosyltransferases.

B. <u>Ionic and pH requirements for inhibition</u>

When low concentrations of sphaeroplast lysate protein (1 mg/ml) were used in the standard assay, 7.5 mM $MnCl_2$ gave optimal mannosyltransferase activity (figure 10, p. 89 ; figure 22). At high protein levels (10 mg/ml), this concentration of Mn^{2+} was inhibitory; under these conditions maximum enzyme activity was observed in the absence of exogenous Mn^{2+} (figure 22). After preincubation of the sphaeroplast lysate at 4°C for 24 h, however, there was no inhibition of mannosyltransferase activity at $MnCl_2$ concentrations up to 25 mM. The inhibition was also relieved by an excess of EDTA in reaction mixtures (table 8) or by the presence of monovalent cations at high ionic strength (table 9). Inhibition appeared to be relatively specific for Mn^{2+}, $MgCl_2$ having much less effect. Inhibition with 7.5 mM $MnCl_2$, 7.5 mM $MgCl_2$ and 20 mM $MgCl_2$ was 82.1 $\pm$ 12.9% (9), 28.1 $\pm$ 6.2% (3) and 41.1 $\pm$ 7.1% (3), respectively (data represent percentage inhibition of mannosyltransferase activity $\pm$ SD, for the number of independent determinations indicated in parentheses). Further different cations were tested (table 14, p.137), but these results are described in section IV C. The washed membrane fraction, which was not subject to activation at high protein levels by

Figure 22.   Mannosyltransferase activity of a sphaeroplast lysate

preparation at different concentrations of $MnCl_2$

Activity was measured using the standard assay with varying

concentrations of $MnCl_2$.   Incubation mixtures contained 1 mg/ml of

sphaeroplast lysate protein (O), 10 mg/ml of sphaeroplast lysate

protein (●) or 10 mg/ml of sphaeroplast lysate protein which had been

preincubated, at a concentration of 20 mg/ml, at $4^{O}C$ for 24 h (■).
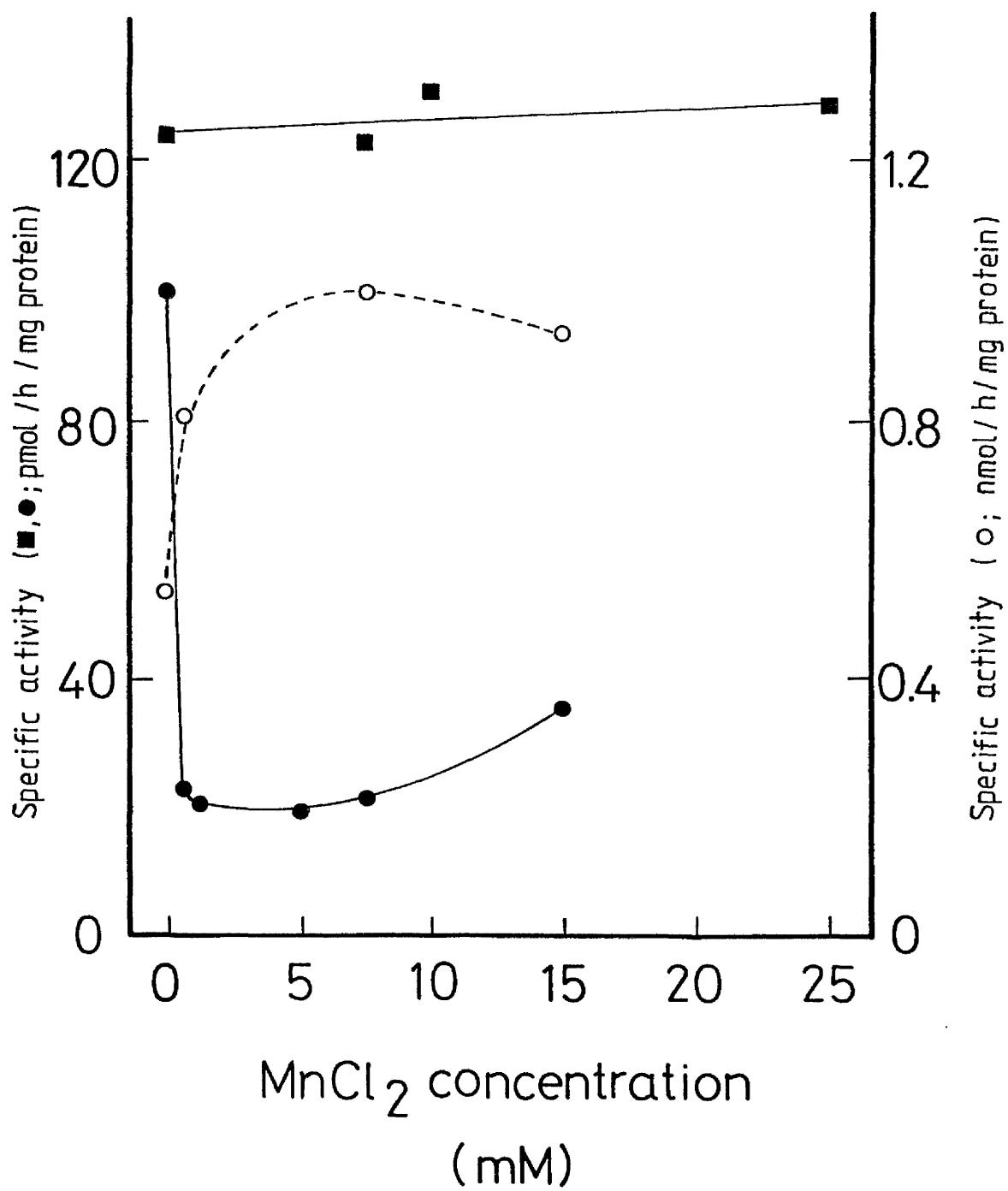
Table 8.   Effect of EDTA on mannosyltransferase activity

| EDTA concentration (mM) | Specific activity (pmol/h/mg protein) | |
	with $MnCl_2$[a]	without $MnCl_2$[b]
0.0	39.4	153.9
1.0	40.7	171.0
2.5	34.2	216.3
5.0	29.6	191.5
10.0	210.6	94.0

[a]Activity was assayed in standard incubation mixtures containing

10 mg/ml of sphaeroplast lysate protein, EDTA as indicated and 7.5 mM

$MnCl_2$.

[b]Activity was assayed in standard incubation mixtures containing

10 mg/ml of sphaeroplast lysate protein and EDTA as indicated, but

no $MnCl_2$.

Table 9.   Effect of high ionic strength on mannosyltransferase activity

Addition	Specific activity[a] (pmol/h/mg protein)		Relative activity (%)	
	Expt 1[b]	Expt 2[b]	Expt 1	Expt 2
None	83.9	26.5	100.0	100.0
KCl	159.4	164.3	190.0	618.5
NaCl	176.7	181.7	210.6	684.8
$(NH_4)_2SO_4$	238.3	257.9	284.0	972.1

[a]Activity was assayed in standard incubation mixtures containing 10 mg/ml of sphaeroplast lysate protein, 7.5 mM $MnCl_2$ and additions, as indicated at an ionic strength of 1.35 mol/l.

[b]For each experiment, different preparations of sphaeroplast lysate were used.

incubation at 4°C, had much the same $MnCl_2$ requirement for mannosyl-transferase activity at both low (figure 10, p. 89) and high protein concentrations. Relative activity at high concentrations of washed membrane protein at 0, 7.5 mM and 20 mM $MnCl_2$ was 100, 113 and 114% respectively.

pH does not appear to be a critical factor in the inhibition of mannosyltransferase activity at high levels of sphaeroplast lysate protein. With 50 mM Tris-HCl buffer, there was no significant difference in activity over the pH range 7.1-7.8 (with or without 7.5 mM $MnCl_2$), and there was always a sixfold increase in specific activity by omission of $MnCl_2$. When 50 mM Hepes-NaOH buffer was substituted for Tris-HCl, the increase in activity caused by the omission of $MnCl_2$ was reduced from 600% at pH 6.8 to 360% at pH 7.8.

C.   Inhibition by a supernatant fraction

The supernatant fluid, obtained by centrifuging sphaeroplast lysate at 100,000 x g, caused inhibition of mannosyltransferase activity when added to assay mixtures containing high concentrations of pre-incubated (4°C for 24 h) sphaeroplast lysate protein (table 10). The extent of inhibition was variable due to the different activities of preincubated sphaeroplast lysate but was always greater with $MnCl_2$ than in its absence. The highest inhibition observed, on addition of super-natant fraction, was 86.4%; this corresponds to an enzyme specific activity of 14.8 pmol/h/mg protein. In figure 18, the specific activity of sphaeroplast lysate at a protein concentration of 10 mg/ml was 28.6 pmol/h/mg protein. Thus it was possible to reach comparably high inhibi-tion of mannosyltransferase activity by addition of supernatant fraction. Mannosyltransferase activity of washed membrane fractions was also

Table 10.   Effect of supernatant fraction on mannosyltransferase activity

Volume of supernatant fraction[a] added (μl)	Relative activity (%)[b]	
	with $MnCl_2$	without $MnCl_2$
0	100.0	100.0
10	88.6	95.4
20	72.1[c]	93.2[c]
30	53.3[d]	85.8[e]
30 (heat-treated)	104.2	98.4

[a]The supernatant fraction had a protein content of 7.3 mg/ml.

[b]Activity was assayed in standard incubation mixtures containing 10 mg/ml of preincubated sphaeroplast lysate protein, supernatant fraction as indicated and either 7.5 mM $MnCl_2$ or no $MnCl_2$.

[c]Corresponding values for relative activity of the washed membrane fraction (10 mg protein per ml) with supernatant fraction (20 μl) were 66.0 and 86.7 with and without $MnCl_2$ respectively.

[d]The mean value for six separate determinations was 39.1 ($\pm$ 19.7 SD).

[e]The mean value for four separate determinations was 85.1 ($\pm$ 7.7 SD).

inhibited (supernatant fraction, 30 µl, caused 69.9 $\pm$ 15.1% inhibition, for 9 separate determinations).

The inhibitory activity of the supernatant fraction was destroyed by heat treatment (60°C for 10 min). Inhibitory activity was also gradually lost at 4°C and 30°C, and the rate of this loss at 30°C could be increased by protease treatment. Treatment of the supernatant fraction with trypsin at 30°C for 20 min almost completely destroyed its inhibitory activity (table 11) provided that the action of trypsin was terminated by the addition of trypsin inhibitor. The specificity of this proteolytic destruction was tested using five other proteases (table 12). In experiments 1 and 2, supernatant fraction was pretreated with high concentrations of protease and mannosyltransferase activity was then measured in the presence of treated supernatant fraction which still contained active protease. Under these conditions, neutral proteases (trypsin, pronase and subtilisin BPN') all enhanced the extent of inhibition by the supernatant fraction, presumably due to proteolytic destruction of mannosyltransferases. Meanwhile, the acid protease from Aspergillus caused little change in inhibitory activity and pepsin, another acid protease, relieved inhibition completely. In experiment 3 (table 12), the proteolytic action of trypsin, pepsin and yeast proteinase A was prevented during the mannosyltransferase assay by the addition of specific protease inhibitors (trypsin inhibitor or pepstatin A) at the end of the pretreatment period. In this case, the inhibitory action of the supernatant fraction was removed by trypsin or pepsin but not by the much lower concentrations of proteinase A.

Mannosyltransferase activity at low sphaeroplast lysate concentrations could also be inhibited by the supernatant fraction. In this instance, however, inhibition by supernatant fraction was the same

Table 11.   Effect of trypsin-treated supernatant fraction on

mannosyltransferase activity of preincubated sphaeroplast

lysate

Concentration of trypsin added to supernatant fraction (μg/ml)	Specific activity[a] (pmol/h/mg protein) of:	
	sphaeroplast lysate preparation 1	sphaeroplast lysate preparation 2
0	85.5	81.1
200	136.3	94.0
400	132.3	122.6
800	151.5	133.0

[a]Activity was assayed in standard incubation mixtures containing $MnCl_2$

(7.5 mM), sphaeroplast lysate protein (10 mg/ml) and 30 μl of

supernatant fraction (6.1 mg protein per ml) which had been pretreated

with trypsin, followed by trypsin inhibitor, as described in Materials

and Methods.   Control mixtures, containing either heat-treated

supernatant fraction (with sphaeroplast lysate preparation 1) or an

equivalent volume of Tris buffer (with preparation 2), gave enzyme

specific activities of 164.5 and 153.0 pmol/h/mg protein, respectively.

Table 12. Effect of different proteases on inhibition of mannosyl-

transferase activity by the supernatant fraction

Pretreatment of supernatant fraction[a]	Relative activity with 20 μl of supernatant fraction (%)[b]			
	Expt 1[c]	Expt 2[c]	Expt 3[d]	Expt 3[d] (controls)
60°C for 10 min	100.0	100.0	100.0	-
Kept at 0°C for 20 min	79.7	82.0	67.2	-
Kept at 30°C for 20 min	ND	90.4	ND	-
Pepsin	104.3	101.1	88.9	77.6
Aspergillus acid protease	80.4	72.0	ND	-
Trypsin	63.1	62.2	91.7	72.7
Subtilisin BPN'	63.1	43.5	ND	-
Pronase	59.3	ND	ND	-
Proteinase A	ND	ND	72.5	73.2

[a]Supernatant fraction (20 μl; 2.8 mg protein/ml) was pretreated as indicated. Proteases were used at a concentration of 16.7 mg/ml except for proteinase A (0.67 mg/ml), and were incubated with supernatant fraction at 30°C for 20 min.

[b]Mannosyltransferase activity was measured using standard incubation mixtures containing preincubated (4°C for 24 h) sphaeroplast lysate protein (10 mg/ml) and supernatant fraction as indicated; the final concentration of protease was 5 mg/ml, except for proteinase A which was used at a concentration of 0.2 mg/ml.

[c]Following pretreatment, supernatant fraction was kept on ice briefly before being used in the mannosyltransferase assay.

[d]Pretreatment of supernatant fraction by proteases was terminated by the addition of specific protease inhibitors, as described in Materials and Methods. Inhibitors were added first in control reactions.

ND, Not determined.

with 7.5 mM $MnCl_2$ as in its absence (table 13). This is because $MnCl_2$

is required for mannosyltransferase activity at low concentrations of

sphaeroplast lysate protein. A range of different cations was tested

on mannosyltransferase activity with and without supernatant fraction

to determine how specific inhibition of mannan synthesis by the

supernatant fraction was for $MnCl_2$ (table 13). $MnSO_4$ did not greatly

differ in its effect from $MnCl_2$. Inhibition of enzyme activity at

high concentrations of sphaeroplast lysate protein and inhibition of

mannosyltransferase activity by supernatant fraction was much less with

7.5 mM $MgCl_2$. Ca^{2+}, Co^{2+} and Cu^{2+} were all inhibitory for mannosyl-

transferase activity at both high and low protein concentrations.

While Sn^{2+} and Sr^{2+} actually stimulated activity at high levels of

sphaeroplast lysate protein, Fe^{2+} and Fe^{3+} seemed to provide inhibition

of mannosyltransferase activity by the supernatant fraction more

effectively than Mn^{2+}, although the situation is complicated by the

apparent massive stimulation of mannosyltransferase activity, at low

levels of protein, by these ions.

The significance of these results will not be understood

until individual components of the mannan-synthesising system can be

separated and their properties fully characterised. Behrens and

Cabib (1968) showed that mannosyltransferase activity in lysed sphaero-

plasts of S. carlsbergensis was optimal with Mn^{2+}. Mg^{2+} had no effect,

although activity was slightly enhanced by Co^{2+} or Fe^{2+}. Since that

report, some mannosyltransferases in S. cerevisiae have been found to

have a requirement for Mn^{2+} specifically while others are fully active

with either Mn^{2+} or Mg^{2+} (Sharma et al, 1974; Parodi, 1979a); mannosyl-

phosphate transferase requires either Mn^{2+} or Co^{2+} (Karson and Ballou,

1978). The ionic requirement for mannan synthesis, in vitro, is

Table 13. Effect of different cations on mannosyltransferase activity of sphaeroplast lysate preparations

| | Relative activity (%)[b] at protein concentrations of : | | |
| | 10 mg/ml | 1 mg/ml | |
Addition[a]	without supernatant fraction	without supernatant fraction	with 30 μl of supernatant fraction
MnCl$_2$	–	–	100.0 (heat-treated)[c]
None	100.0[d]	100.0[d]	36.7
MnCl$_2$	11.6	191.9	36.6
MnSO$_4$	14.1	192.3	49.2
MgCl$_2$	75.9	189.4	63.6
CaCl$_2$	82.7	28.3	40.9
CoCl$_2$	13.1	34.6	32.5
CuCl$_2$	14.3	7.5	1.7
SnCl$_2$	130.3	115.1	55.6
SrCl$_2$	154.4	101.6	42.3
FeCl$_2$	46.2	819.7	24.2
FeCl$_3$	80.3	329.1	11.8

[a]Final concentration of all cations in incubation mixtures was 7.5 mM.

[b]Activity was assayed in standard incubation mixtures which contained sphaeroplast lysate protein, cations and supernatant fraction as indicated.

[c]Control mixtures contained sphaeroplast lysate protein (1 mg/ml), 7.5 mM MnCl$_2$, and 30 μl of heat-treated supernatant fraction.

[d]Control mixtures contained sphaeroplast lysate protein as indicated and no cations.

therefore very dependent upon the strain of yeast and type of enzyme preparation; both of these parameters will determine which of the various mannosyltransferases are active in a given preparation.

D.   Degradation of mannan and GDP-$[^{14}C]$mannose

The results in the last sub-section indicated that the supernatant fraction contained a protein inhibitor of mannosyltransferase activity but did not preclude the possibility that inhibition was due simply to enzymatic destruction of either the reaction product or the substrate, GDP-mannose.   Mannan degradation was estimated, as described in Materials and Methods, by including $[^{14}C]$-labelled polymer in reaction mixtures containing either 30 μl of supernatant fraction (248 μg protein) or sphaeroplast lysate protein at a concentration of 10 mg/ml. Following incubation at $30^{o}C$ for 20 min, the amount of perchloric acid-precipitable radioactive material had decreased by only 3.62 and 4.79 pmol/h in each case.   Addition of the α-mannosidase inhibitor, mannonic acid lactone (0.5-10 mM) to reaction mixtures containing high concentrations of sphaeroplast lysate protein resulted in only slightly elevated mannan synthesis (by no more than 5 pmol/h/mg protein).   These results indicate that degradation of mannan - either newly-synthesised $[^{14}C]$polymer or endogenous acceptor - is not responsible for the observed inhibition of mannosyltransferase activity.

Two soluble enzymes capable of degrading GDP-mannose have been reported in S. cerevisiae: a nucleotide pyrophosphatase which requires Mg^{2+} for activity (Cabib and Carminatti, 1961) and a sugar nucleotide phosphorylase which has no divalent cation requirement (Cabib et al, 1965).

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$$\text{GDP-mannose} \quad \underset{\longleftarrow}{\overset{\text{nucleotide pyrophosphatase}}{\longrightarrow}} \quad \text{GMP + mannose-1-phosphate}$$

$$\text{GDP-mannose + P}_i \quad \underset{\longleftarrow}{\overset{\substack{\text{sugar nucleotide} \\ \text{phosphorylase}}}{\longrightarrow}} \quad \text{GDP + mannose-1-phosphate}$$

The presence of one or both of these enzymes, and their possible interference in the assay by hydrolysing GDP-$[^{14}C]$mannose, was investigated by paper chromatographic separation of the products of reaction mixtures, containing high levels of sphaeroplast lysate protein (10 mg/ml) or supernatant fraction alone. In both cases, GDP-$[^{14}C]$mannose hydrolysis did indeed occur. Typical patterns of breakdown are shown in figures 23 and 24. Supernatant fraction converted the substrate into mainly mannose-1-phosphate and mannose, with some additional faster-running material (figure 23). This suggested the presence, in the supernatant fraction, of one or both of the above-mentioned enzymes to produce mannose-1-phosphate, which might then be hydrolysed to mannose by phosphatase action. Degradation of GDP-mannose was greater with either $MgCl_2$ or $MnCl_2$ (7.5 mM) than in the absence of divalent cations, and could be destroyed by briefly treating with EDTA (see below), suggesting that degradation was not due to sugar nucleotide phosphorylase action. Breakdown of GDP-mannose could not be prevented by one of the end-products, mannose-1-phosphate, when present at a final concentration of 1 mM. Under these conditions there was an accumulation of $[^{14}C]$mannose-1-phosphate at the expense of $[^{14}C]$mannose. A similar accumulation of $[^{14}C]$mannose-1-phosphate was observed on the addition of NAD (1 mM) which has been reported to inhibit, competitively, GDP-mannose breakdown

Figure 23.   Breakdown of GDP-$\left[^{14}C\right]$mannose by supernatant fraction

Reaction mixtures containing Tris-HCl buffer (pH 7.2), MnCl$_2$ (7.5 mM), and GDP-$\left[^{14}C\right]$mannose (185 pmol ; 20 nCi) were incubated at 30°C for 20 min with 20 μl of supernatant fraction (8.2 mg protein per ml).   Reactions were terminated at 100°C for 3 min and reaction products were separated on Whatman 3MM paper in solvent B.   Arrows indicate positions of origin and solvent front.   Hatched boxes represent positions of non-radioactive standards :   1) GDP-mannose, 2) mannose-1-phosphate, and 3) mannose.
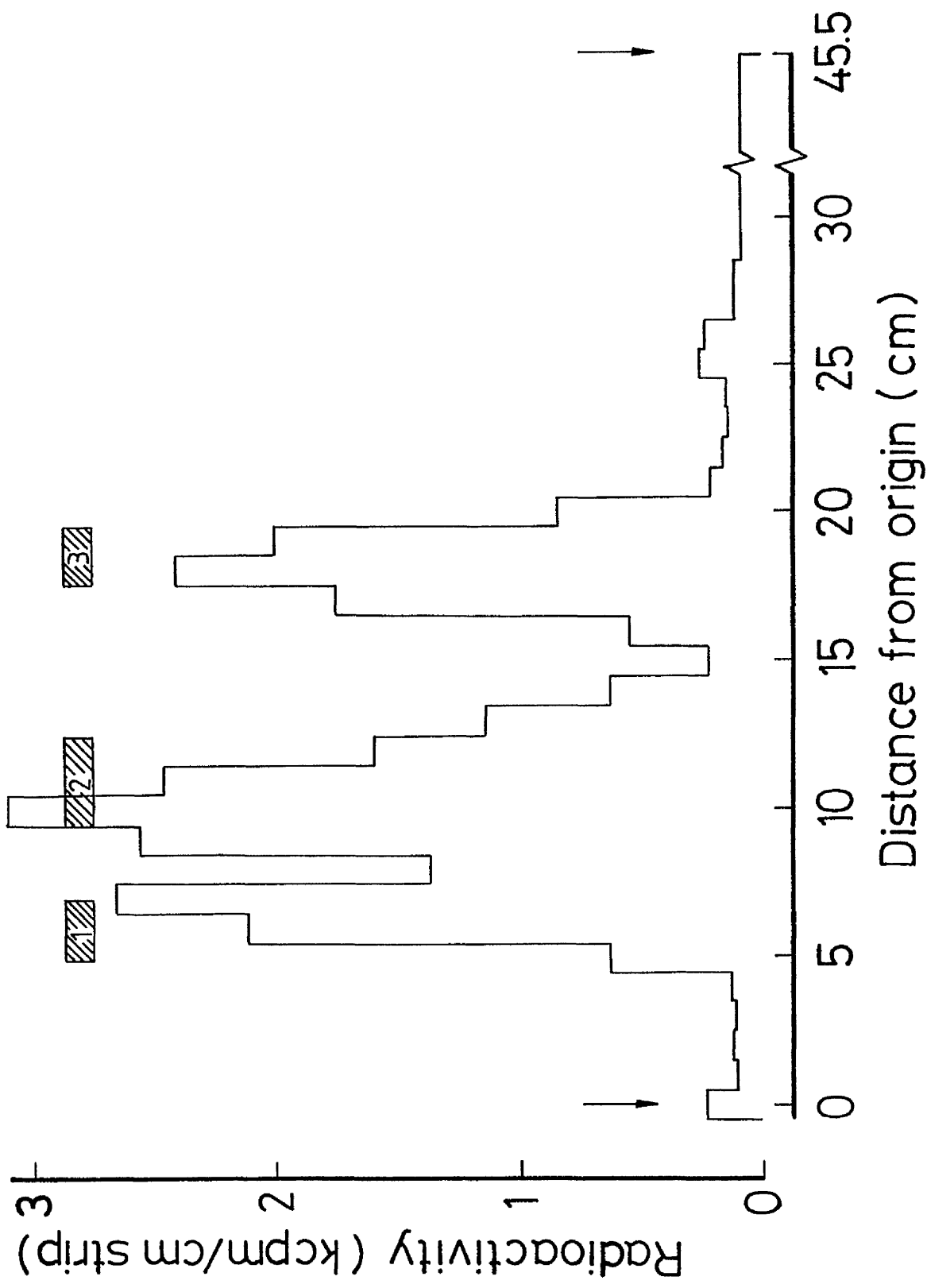
Figure 24. Breakdown of GDP-$[^{14}C]$mannose by sphaeroplast lysate

preparation

Reaction mixtures (0.1 ml final volume), containing Tris-HCl

buffer (40 mM ; pH 7.2), MnCl$_2$ (7.5 mM) and GDP-$[^{14}C]$mannose (578 pmol ;

100 nCi) were incubated at 30°C for 20 min with sphaeroplast lysate

protein (10 mg/ml). Reactions were stopped by boiling for 3 min and

separated on Whatman 3MM paper in solvent B (histogram I) or solvent A

(histogram II). Arrows indicate positions of origins and solvent fronts.

Hatched boxes represent positions of non-radioactive GDP-mannose (1) and

mannose (2) standards. W, X, Y and Z refer to peaks discussed in text.
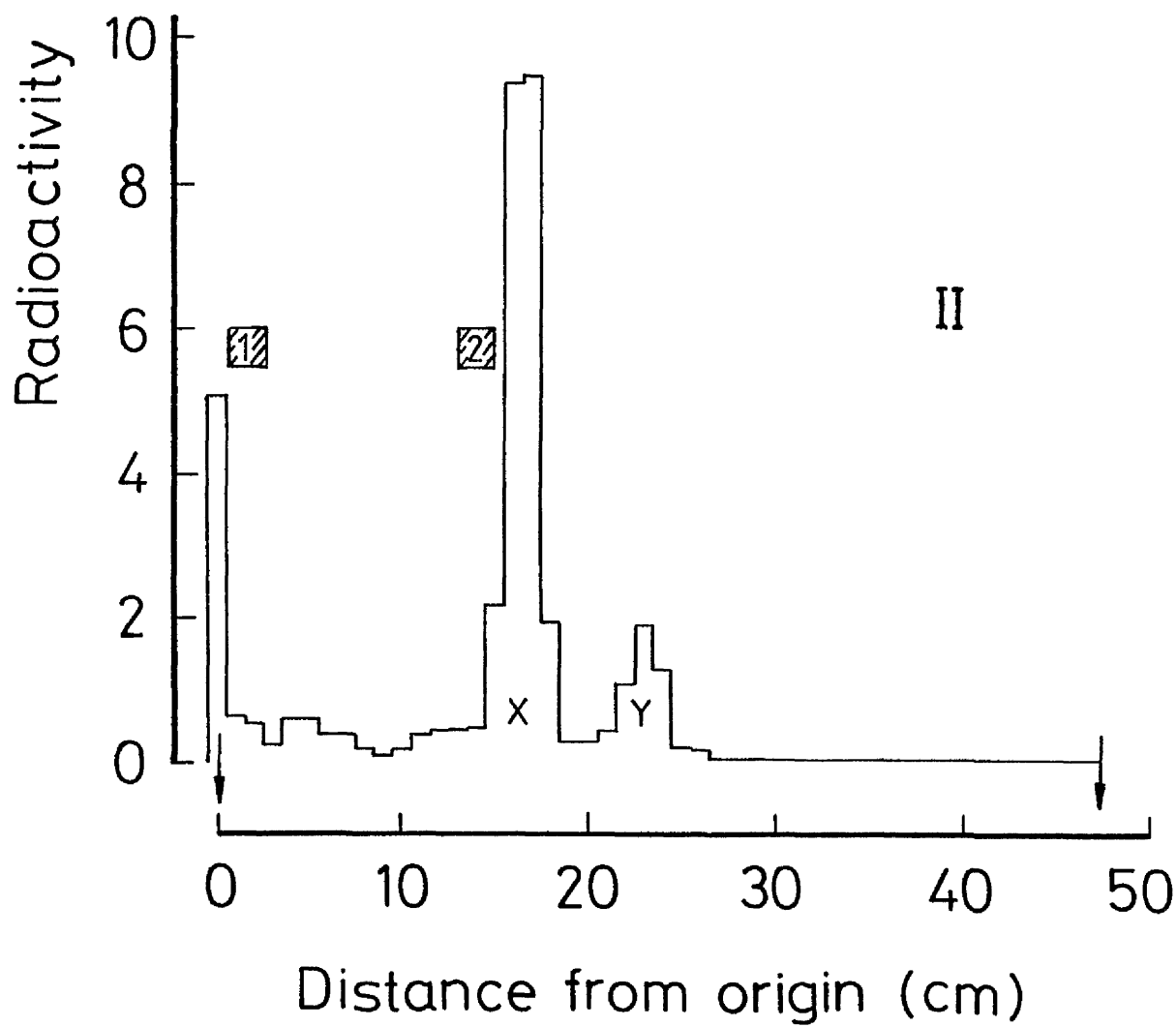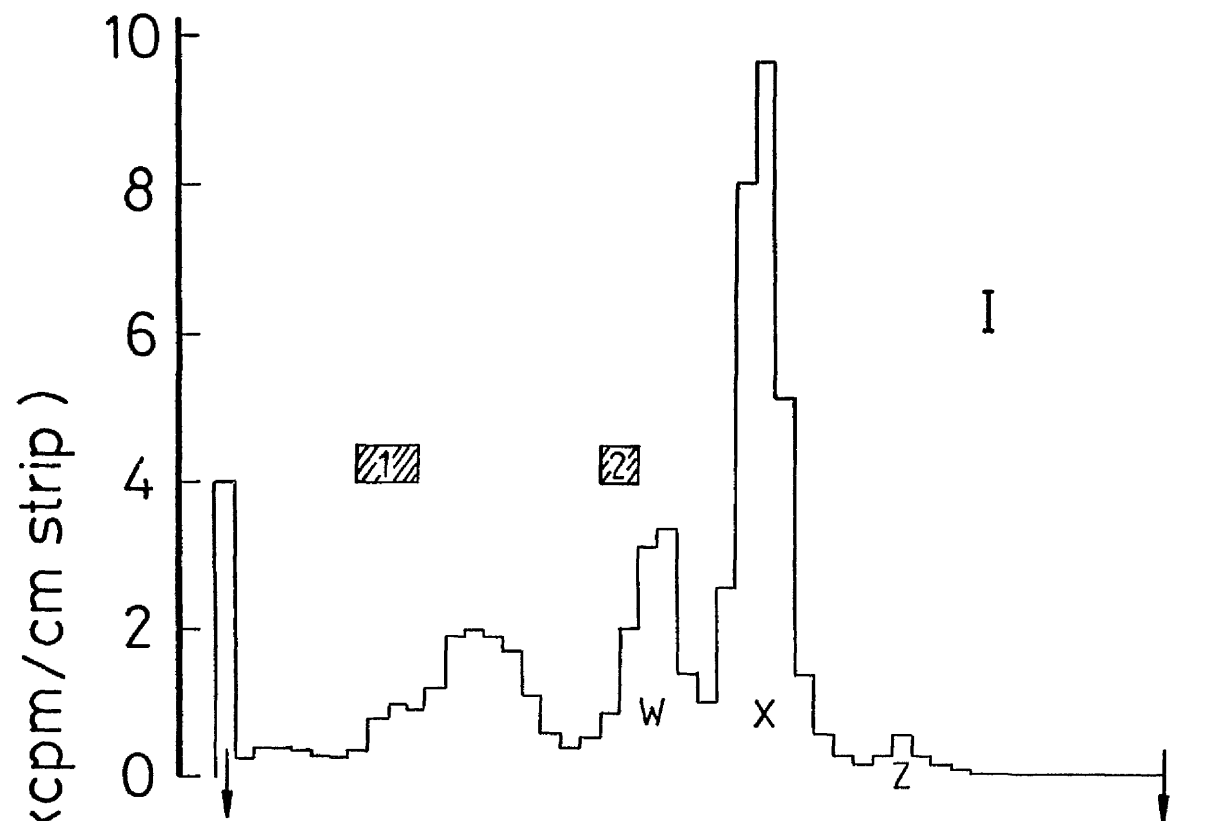
by the nucleotide pyrophosphatase (Cabib and C$_a$rminatti, 1961).

GDP-mannose degradation by reaction mixtures containing sphaeroplast lysate (10 mg/ml) was more complex. Several products were formed, most of which migrated faster than mannose in solvents A and B (figure 24). Since compounds with such high R$_F$ values might be expected to be lipid intermediates, the nature of this material was briefly investigated. The only compounds that appeared to be lipid in nature were peak Y (figure 24) and some even faster-running material which could be separated from reaction mixtures containing preincubated (4°C for 24 h) sphaeroplast lysate protein (10 mg/ml) or washed membrane fraction (10 mg/ml) without preincubation. Evidence that these products were lipids was indirect as they were only obtained in small quantities. The second, fast-running compound had an R$_F$ value of 0.9 in solvent A (Tanner, 1969) and was extracted from reaction mixtures with chloroform-methanol (2:1, v/v; Sentandreu and Lampen, 1971) suggesting that it was dolichol monophosphate mannose. Peak Y could be extracted with chloroform-methanol-water (10:10:3, by vol.) and had an R$_F$ value in solvent A characteristic of a lipid-bound oligosaccharide (Lehle and Tanner, 1978a).

Peak X, on the other hand, was not a mannose-containing lipid since both mild (pH 2; 30 min at 100°C) and strong (2M HCl; 4 h at 110°C, in vacuo) acid hydrolysis had no effect on its chromatographic mobility in solvent B on t.l.c. cellulose plates. Peak X migrated very close to mannose in three solvent systems (A, B and C). However, when internal [^{14}C] mannose standards were included in reaction mixtures, following termination of reactions, it was apparent that peak X was definitely not mannose. Solvent B gave the best separation of peak X from mannose either by paper chromatography on Whatman 3MM paper (figure 24) or by t.l.c. on cellulose plates.

Initial evidence to rule out the possibility of substrate degradation was derived from experiments with end-products of the reactions catalysed by degradative enzymes (mannose-1-phosphate and GMP or GDP) as well as competitive inhibitors of nucleotide pyrophosphatase (NAD or AMP). When included in standard assay mixtures, at a final concentration of 1 mM, none of these compounds relieved the inhibition of mannosyltransferase activity observed at high concentrations of sphaeroplast lysate protein or that caused by addition of supernatant fraction to preincubated sphaeroplast lysate. However, this concentration of NAD or mannose-1-phosphate, as mentioned above, did not prevent GDP-mannose degradation. Further evidence was therefore required to prove that inhibition of mannan synthesis was not due to degradative enzymes. This was obtained by briefly exposing the supernatant fraction to 20 mM EDTA at 0°C (table 14). Degradative activity was almost completely abolished by this procedure and was not restored by the subsequent addition of $MnCl_2$ to a final concentration of 7.5 mM. However, when EDTA-treated supernatant fraction was included in standard assay mixtures, adjusted to 7.5 mM $MnCl_2$, mannosyl-transferase activity of washed membranes was inhibited just as effectively as with untreated supernatant fraction. The absence of substrate degradation in these reactions containing EDTA-treated supernatant fraction was confirmed by paper chromatography. EDTA inhibits nucleotide pyrophosphatase from S. cerevisiae (Cabib and Carminatti, 1961) as well as that from a hybrid Saccharomyces yeast (Twu et al, 1977). The latter enzyme is also inhibited by o-phenanthroline, a metal chelator that is more specific for Fe^{2+} than EDTA. Experiments with o-phenanthroline gave results similar to those shown in table 14 for EDTA. Degradation of GDP-mannose was reduced from 11% to 7% by brief

Table 14. Activities of supernatant fraction following pretreatment with EDTA

Pretreatment of supernatant fraction	Action of supernatant fraction (20 μl)					
	Inhibition of mannosyltransferase activity (%)[a]				Breakdown of GDP-$[^{14}C]$mannose (%)[b]	
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 1	Expt 2
None	34.2	23.4	31.1	30.4	59.0	47.2
10 min at 0°C with EDTA (20 mM)	39.6	30.2	27.2	32.3	1.4	1.3
10 min at 60°C with EDTA (20 mM)	ND	ND	13.8	0.0	0.0	ND
10 min at 60°C without EDTA	0.0	ND	0.0	ND	0.0	ND
24 h at 4°C	ND	16.9	ND	ND	ND	0.0

[a]Activity was assayed in standard incubation mixtures containing washed membrane fraction (10 mg protein per ml), 20 μl of supernatant fraction pretreated as indicated and MnCl$_2$, in excess of EDTA, to a final concentration of 7.5 mM. They were incubated at 30°C for 20 min.

[b]Activity was assayed in standard incubation mixtures as above except that washed membrane fraction was omitted.

exposure of the supernatant fraction to 6.7 mM o-phenanthroline.

However in this case the inhibitory action of the supernatant fraction

was also decreased by ethanol alone, which was the solvent for the

chelator. pHMB, another inhibitor of nucleotide pyrophosphatase

(Cabib et al, 1965) could not be used in similar experiments since it

substantially inhibited mannosyltransferase activity.

Whereas degradation of mannan during the assay procedure is

negligible, GDP-mannose breakdown is significant, although insufficient

to account for either the inhibition of activity at high concentrations

of sphaeroplast lysate protein or inhibition by the supernatant fraction.

The presence of a GDP-mannose degradative enzyme in sphaeroplast lysates

suggests that there must be strict compartmentalisation of this and

mannosyltransferases in vivo. Alternatively, the enzymes may have

different affinities for their substrate, GDP-mannose. Km values

calculated from the data in figure 25 indicate that mannosyltransferases

do have a greater affinity for GDP-mannose than does the hydrolytic

enzyme. However, the conditions used for the two assays were not

directly comparable.

E. Attempts to purify the mannosyltransferase inhibitor

Results in table 14 show that GDP-mannose degradative activity

in the supernatant fraction was more labile than the mannosyltransferase

inhibitor. Nevertheless, the substantial loss of the latter activity

at low temperature has made attempts at its purification very difficult.

Large volumes of supernatant fraction were prepared from batches of cells

disrupted in a Braun MSK homogeniser as a preliminary step. The

supernatant fluid obtained in this way, however, did not show detectable

inhibition of mannan synthesis. A second attempt at purification

Figure 25. <u>Effect of varying GDP-mannose concentration on</u>

<u>mannosyltransferase activity of a washed membrane</u>

<u>fraction and GDP-mannose degradative activity in the</u>

<u>supernatant fraction</u>

Standard incubation mixtures contained Tris-HCl buffer

(40 mM ; pH 7.2), $MnCl_2$ (7.5 mM), varying concentrations of carrier

GDP-mannose together with GDP-$[^{14}C]$ mannose (20 nCi ; 1.85 μM) and

either 10 mg/ml of washed membrane fraction protein (for assay of mannosyl-

transferase activity; O) or 84 μg of supernatant fraction protein

(for assay of GDP-mannose degradative activity; ●).   Mixtures were

incubated at 30°C for 5 min (mannosyltransferase) or 20 min (GDP-mannose

degradation) and processed as described in Materials and Methods.

The Km values, from the graph, were 3.57 and 14.29 μM for

mannosyltransferase and GDP-mannose degradative activities, respectively.
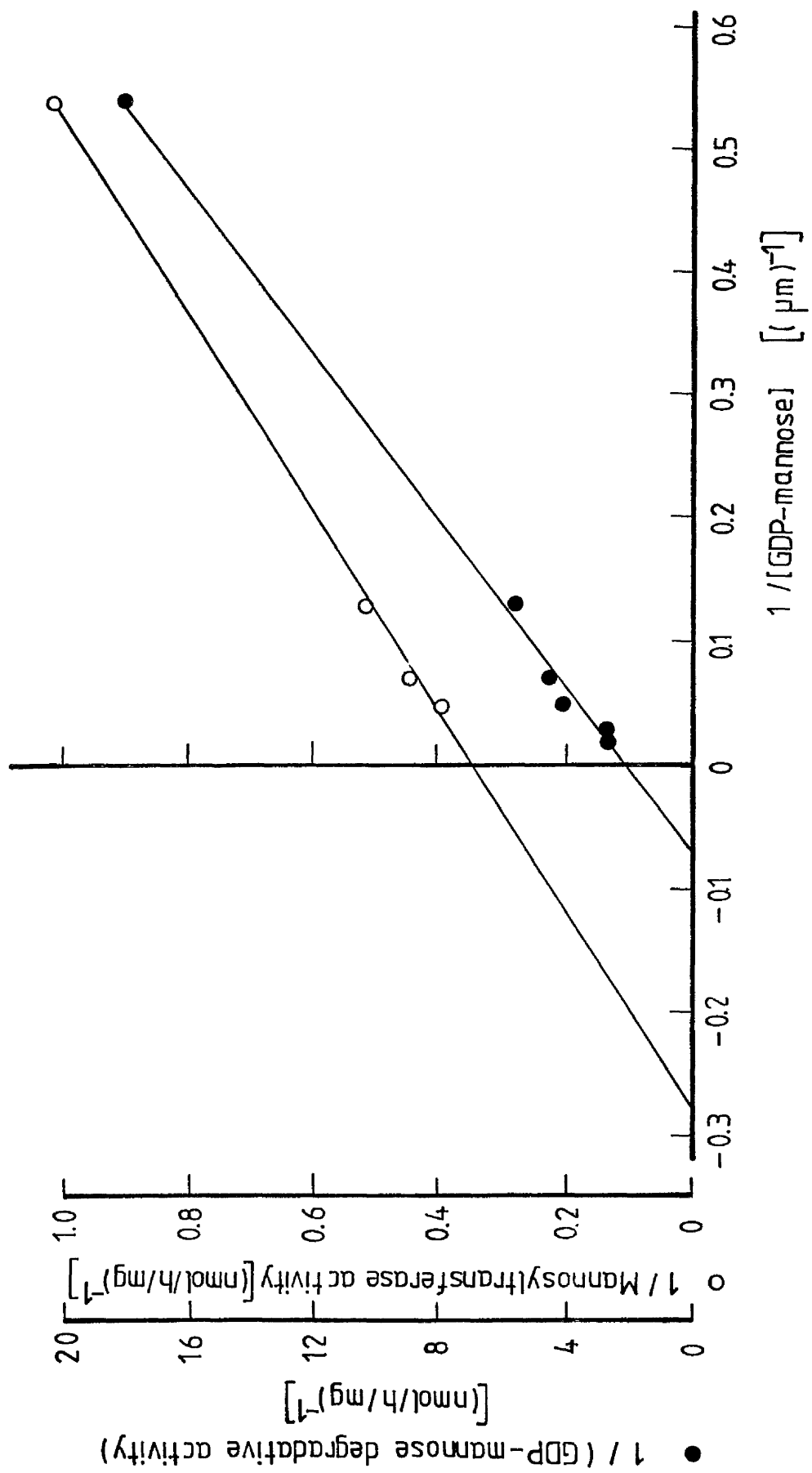
involved ammonium sulphate fractionation of supernatant fraction obtained from a large amount of sphaeroplast lysate. Although the supernatant fraction possessed the same inhibitory activity as previously, no noticeable inhibition of mannosyltransferase activity was caused by protein precipitated, from the supernatant fraction, by 40%, 60% or 80% (w/v) saturated solutions of ammonium sulphate. The loss of activity can be accounted for by the incubation time, at 4°C, that was required to prepare these individual protein fractions (about 36 h).

Since both pepsin and trypsin were capable of destroying the mannosyltransferase inhibitor it seemed likely that the loss of activity on storage at 4°C might be due to a gradual proteolytic destruction. However, when supernatant fraction was incubated at 4°C for 24 h with bovine plasma albumin (13.3 mg/ml) the loss of inhibitory activity was not prevented. This could be taken as evidence against a general, non-specific destruction by yeast proteases. Three vacuolar proteinases (A, B and C) exist in S. cerevisiae (Holzer et al, 1975), each one having its own specific cytoplasmic inhibitor. Trypsin inhibitor and PMSF, which can both act as inhibitors of proteinase B and C, caused only a slight reduction (never more than 20%) in the loss of inhibitory activity of the supernatant fraction, during storage at 4°C for 24 h, when tested over a wide range of concentrations (trypsin inhibitor, 0.2-4.0 mg/ml; PMSF, 1-20 mM). Nevertheless, proteolytic destruction of the inhibitor may be due to an, as yet, unidentified specific protease. Pepstatin A (an inhibitor of yeast proteinase A), TPCK and TLCK (all at 3.3 mM in 33%, v/v, DMSO) and TAME (3.3 mM, in water) were added to supernatant fraction which was incubated at 4°C for 24 h. Unfortunately, the particular batch of supernatant fraction used in these experiments gave poor inhibition of mannan synthesis initially (relative mannosyl-

transferase activity with non-preincubated supernatant fraction was 79.7%) and inhibitory activity was totally lost during 24 h at $4^{\circ}C$. Relative mannosyltransferase activities with supernatant fraction, preincubated with pepstatin A, TPCK, TLCK and TAME, were 92.4%, 81.2%, 93.2% and 99.9%, respectively. These results suggest that the three DMSO-soluble inhibitors did prevent loss of supernatant fraction activity at $4^{\circ}C$. However their usefulness in purification of the inhibitor remains to be established. The percentages quoted above are subject to large errors, due to the low initial inhibitory activity of the supernatant fraction. Furthermore, DMSO (11%, v/v) is slightly inhibitory for mannan synthesis.

F.   Effect of supernatant fraction on synthesis of different carbo-
      hydrate moieties of mannan

The specificity of action of the supernatant fraction on mannosyltransferase activity was investigated by two approaches. Firstly, inhibition and activation of mannan synthesis was compared for sphaeroplast lysates of two strains of S. cerevisiae. The wild-type strain (S. cerevisiae X2180-1A) was used together with S. cerevisiae LB1-3B, an mnn 2 mutant producing mannoproteins that lack side chains in the 'outer chain' portion of mannan due to the absence of α-(1→2)mannosyltransferase activity (Raschke et al, 1973; figure 2, p. 32). Although the enzyme preparation from S. cerevisiae LB1-3B had a specific activity lower than that of the wild-type, supernatant fractions from both strains strongly inhibited mannosyltransferase activity (table 15). If the protein concentration of the two supernatant fractions is normalised to 3 mg/ml, mannosyltransferase activity was inhibited by 85.6% and 85.5% for the wild-type and mnn 2 mutant, respectively.

Table 15. Mannosyltransferase activity in sphaeroplast lysates of S. cerevisiae X2180-1A and LB1-3B

Yeast strain	Specific activity[a] (pmol/h/mg of protein) at protein concentrations of:								Relative[b] activity with 30 µl supernatant fraction[c] (%)
	1 mg/ml				10 mg/ml				
	before preincubation		after preincubation		before preincubation		after preincubation		
	+	-	+	-	+	-	+	-	
S. cerevisiae X2180-1A a	1012.9	310.5	664.7	198.8	34.1	192.6	219.2	159.6	20.1
S. cerevisiae LB1-3B mnn2	663.0	211.4	340.9	164.0	22.4	155.7	149.0	127.4	11.7

[a]Activity was assayed before or after preincubation of the enzyme preparation at 4°C for 24 h using standard assay mixtures containing sphaeroplast lysate protein as indicated. MnCl$_2$ (7.5 mM) was either included (+) or omitted (-) from assay mixtures.

[b]Activity was assayed in standard assay mixtures containing sphaeroplast lysate protein (10 mg/ml), that had been preincubated at 4°C for 24 h, supernatant fraction as indicated, and 7.5 mM MnCl$_2$. Control mixtures containing heat-treated supernatant fraction were used and enzyme activity is expressed as a percentage of activity without supernatant fraction.

[c]Protein contents were 2.8 mg/ml and 3.1 mg/ml for supernatant fractions from S. cerevisiae X2180-1A and LB1-3B, respectively.

Both enzyme preparations shared the same inhibition of mannosyltrans-
ferase activity at high concentrations of sphaeroplast lysate protein
(10 mg/ml).   Likewise, activation of both preparations was achieved
by preincubation at 4°C or by omission of Mn^{2+} ions.

Since only small amounts of $[^{14}C]$-labelled lipid could be
detected in reaction mixtures (section III D, ii; pp   105 - 109   ),
action of the supernatant fraction on the formation of lipid inter-
mediates has not been investigated.   However, its effect on the
synthesis of short, β-eliminable oligosaccharides and of the poly-
saccharide portion of mannan was examined for both the wild-type and
mnn 2 strains.   Most of the mannan synthesised by either strain was
β-eliminable (70-80%).   Values for β-eliminable synthesis corresponded
well with those obtained in Tanner's laboratory for S. cerevisiae
Fleischmann Laboratories, strain 66.24 (70%; Lehle and Tanner, 1974)
and S. cerevisiae X2180 (M.S. Marriott, personal communication).
However, Farkaš and Bauer (1976) reported that the amount of β-eliminable
synthesis by enzyme preparations from S. cerevisiae X2180-1A and an
mnn 2 strain was only 27.6% and 33.2%, respectively.   These low values
probably reflect the high concentration of GDP-mannose used in their
standard assay;   this increases the proportion of polysaccharide chain
synthesised (Farkaš et al, 1976a).   Inhibition of total polymer
synthesis and synthesis of β-eliminable material, for both yeast strains,
was almost identical (table 16).

Synthesis of the polysaccharide chain of mannan was examined
using a method developed by Parodi (1979a) for use with solubilised
enzyme preparations.   Whereas short, β-eliminable oligosaccharides,
when released from the protein by 2M LiOH, are soluble in 66% methanol,
the large polysaccharide chains remain insoluble in 66% methanol.   In

Table 16.   Effect of supernatant fraction on incorporation of radioactivity into different carbohydrate moieties of mannan

Yeast strain	Relative incorporation (%)[a] into:						Incorporation of radioactivity (cpm)[b] in:			
	total polymer			β-eliminable material			methanol-insoluble material		methanol-insoluble, alkali-treated material	
	with			with			with 30 μl		with 30 μl	
	10 μl	20 μl	30 μl of supernatant fraction[c]	10 μl	20 μl	30 μl of supernatant fraction	supernatant fraction	heat-treated supernatant fraction	supernatant fraction	heat-treated supernatant fraction
S. cerevisiae X2180-1A	66.8	22.0	12.0	63.2	17.0	10.7	3,811	5,964	266	454
S. cerevisiae LB1-3B	58.3	30.1	20.8	57.6	30.1	21.4	1,097	2,949	92	675

[a] Standard assay mixtures, containing sphaeroplast lysate protein (10 mg/ml) which had been preincubated at 4°C for 24 h and supernatant fraction as indicated, were treated as described in Materials and Methods for β-elimination procedure. Incorporation of radioactivity is expressed as a percentage of that in control mixtures, which contained 30 μl of heat-treated supernatant fraction.

[b] Standard assay mixtures, containing sphaeroplast lysate protein (10 mg/ml) and supernatant fraction as indicated, were treated by the method of Parodi (1979a), as described in Materials and Methods.

[c] Protein contents were 2.8 mg/ml and 3.1 mg/ml for supernatant fractions from S. cerevisiae X2180-1A and LB1-3B, respectively.

the present study, incorporation of radioactivity into 66% methanol-insoluble, alkali-treated material was very small and not increased by the provision of additional acceptor, in the form of mannan poly-saccharide (5 mg/ml; prepared as described by Parodi, 1979a). Synthesis of the polysaccharide moiety was inhibited by the supernatant fraction with both strains (table 16). The results in tables 15 and 16, therefore, do not indicate that inhibition of mannan synthesis by the supernatant fraction is specific for a particular carbohydrate moiety. Further information on selectivity of inhibitor action must await an investigation of its effect on individual mannosyltransferases.

DISCUSSION

Yeast morphogenesis has been proposed as a model system for studying the development of form and structure in higher eucaryotes (Cabib, 1975). Furthermore, Saccharomyces cerevisiae is increasingly considered to be an ideal eucaryotic microorganism (Rose, 1977b). An understanding of the morphogenetic process of cell-wall growth in yeast requires detailed knowledge of the synthesis of the major wall components. Although the actual architecture of the wall is poorly understood, the structures of chitin, glucan and mannan have been reasonably well elucidated, thus enabling several groups of workers to study the synthesis of these polymers at the molecular level. During the past decade, much information has been acquired on the biosynthesis of both chitin and mannan in S. cerevisiae although mechanisms involved in glucan biosynthesis are only gradually becoming clear. Chitin is synthesised by a single enzyme, chitin synthase, and a scheme for the regulation of chitin synthesis in vivo has been proposed by Cabib and his colleagues (Cabib, 1975, 1976; Cabib et al, 1979). These workers have also recently proposed an entirely different mechanism by which $\beta-(1\rightarrow3)$glucan synthesis may be regulated (Shematek and Cabib, 1980). However, despite extensive research since the initial report of mannan synthesis, in vitro, in 1963 (Algranati et al, 1963), little is known of how formation of the amorphous mannan matrix is regulated. Obviously, a clear picture of the overall process of cell-wall biogenesis will emerge only when control mechanisms for the synthesis of individual wall components are fully understood. It was with this ultimate goal in mind that the present investigation, on the regulation of mannan synthesis, was undertaken.

Any mechanism of regulation postulated for wall formation must explain both spatial and temporal control of synthesis. For example, the localised synthesis of chitin in the primary septa of budding yeasts

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(Cabib and Bowers, 1971) must be subject to some kind of directional control. Similarly, all three polysaccharide components are synthesised discontinuously throughout the cell cycle (Cabib and Farkaš, 1971; Biely, 1978) implying that cell-wall synthesis is subject to some sort of temporal regulation. The aim of this project was to examine what mechanisms might operate, in vivo, to control mannan biosynthesis. The sphaeroplast lysate preparations used in this study, while devoid of most cell-wall and periplasmic components, should nevertheless contain all membrane-bound and intracellular constituents involved in regulation of mannan synthesis.

I. OPTIMAL CONDITIONS FOR MANNOSYLTRANSFERASE ACTIVITY

Due to the complexity of mannan synthesis, slight differences in enzyme preparation and assay conditions can easily lead to changes in the properties of mannosyltransferase activity observed in vitro. Therefore, optimal conditions were established for sphaeroplast lysate and washed membrane preparations. Activity of sphaeroplast lysates was optimal at 30°C and at neutral pH values. These conditions correspond well with those used to measure mannosyltransferase activities in sphaeroplast lysates of S. carlsbergensis (Behrens and Cabib, 1968) and in particulate preparations from several strains of S. cerevisiae : CCY 21-4-13 (Farkaš et al, 1976a and b), X2180 (Farkaš and Bauer, 1976) and LK2 G12 (Elorza et al, 1977a). Nakajima and Ballou (1975b) who developed an assay for a solubilised multimannosyltransferase system using exogenous acceptors, incubated standard reaction mixtures at 25°C for 30 min at pH 7.2. However, they also found that α-(1$\rightarrow$6)mannosyl-transferase activity was stable at 30°C for at least 2 h. In the present study mannosyltransferase activity decreased above 30°C (figure 6, p. 84). It is obviously preferable to avoid unnecessarily

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high temperatures since individual mannosyltransferases will be inactivated at different rates. This is illustrated by results of Farkaš et al (1976a,b) who examined the thermal inactivation of different transferase activities at $45^{\circ}C$. They found that enzymes involved in synthesis of the polysaccharide portion of mannan are much more heat-labile than those responsible for synthesis of the short β-eliminable saccharides attached to serine and threonine. Similarly those enzymes involved in the lipid pathway of mannan synthesis are heat-labile (Farkaš et al, 1976b) and rapidly lose activity, even on storage at low temperature (Sharma et al, 1974; Lehle and Tanner, 1974; Parodi, 1979a).

In most studies on yeast mannosyltransferases, enzyme activity has been measured at neutral pH values. However, Lehle and Tanner (1974) found that the pH optimum for the synthesis of α-(1$\rightarrow$2)-linked mannobiose by a particulate enzyme preparation, with Tris-HCl buffer, was pH 8. In this study, overall mannosyltransferase activity had a broad pH optimum of 6.5-8.5 when Tris-maleate buffer was used, although the activity was less than that with Tris-HCl buffer.

Different assay systems reported have also varied in exogenous acceptor and substrate concentrations. Mannosyltransferase activity, as reported here, represents the incorporation of radioactivity into endogenous glycoprotein and lipid acceptors, and was shown to be linear with incubation time at low concentrations of sphaeroplast lysate protein (1 mg/ml). In studies where high substrate concentrations have been used (0.2 mM GDP-[^{14}C]mannose compared with 1-2 μM in this investigation) endogenous acceptor has been rate determining. Lehle and Tanner (1974, 1978b), for example, reported that exogenous cell-wall mannan (fraction A, prepared according to the procedure of Sentandreu and Northcote, 1968)

slightly stimulated the incorporation of mannose into polymer. This

was markedly increased if the mannan was partially hydrolysed to

shorter mannooligosaccharides. In the present assay system, with low

concentrations of GDP-mannose, neither cell-wall mannan (prepared

according to Kocourek and Ballou, 1969) nor mannan polysaccharide

(prepared by the method of Parodi, 1979a) stimulated mannose incorpor-

ation. Under the same assay conditions used in this study, mannan

fractions A and A-HCl (as described by Lehle and Tanner, 1974) also

had no effect on mannosyltransferase activity of a sphaeroplast lysate

preparation from S. cerevisiae NCYC 366 (L.J. Douglas, unpublished

results).

It is known that mannan is synthesised in intracellular

vesicles (Cortat et al, 1973; Lehle et al, 1977; Marriott and Tanner,

1979) and on the plasma membrane (Santos et al, 1978; Welten-verstegen

et al, 1980); these membranes should all be present in sphaeroplast

lysate preparations. It has been further suggested by Santos et al

(1978) that certain mannosyltransferases are located on the outer

surface of the plasma membrane (ectomannosyltransferases). Since most

of the dolichol-dependent steps, and those involved in the synthesis of

short β-eliminable saccharides, appear to take place on intracellular

membranes (Lehle et al, 1977; Marriott and Tanner, 1979), it is

tempting to suggest that synthesis of the bulky, outer-chain portion of

mannan is catalysed by ectomannosyltransferases. Since 70-80% of the

mannan synthesised by sphaeroplast lysate preparations was β-eliminable

(table 15, p. 142) it would appear that a large amount of the mannan

was probably synthesised on intracellular membranes. When the washed

membrane fraction is prepared, many of the low-density, lipid-rich

vesicles will be removed during centrifugation of the sphaeroplast lysate.

This may explain the lower specific activity of the washed membrane fraction compared with that of the sphaeroplast lysate. Many of the larger vesicles, however, will sediment with the washed membrane fraction so that its activity will not solely be due to plasma membrane-associated mannosyltransferases.

Low activity of enzymes involved in synthesis of the mannan polysaccharide chains (table 16, p. 144) may be explained in several ways. Firstly, the enzymes involved may only be present in small amounts in the strain used, since this haploid strain of S. cerevisiae X2180 produces mannan with an outer chain that has far fewer side chains than that of the diploid (Thieme and Ballou, 1970). Secondly, sphaeroplast lysates may lack sufficient endogenous acceptor. This seems unlikely since addition of exogenous mannan polysaccharide did not affect synthesis of the polysaccharide. Furthermore, sphaeroplast lysates are likely to contain considerable amounts of cell-wall material, as much of it will remain attached to the plasma membrane during preparation of osmotically-sensitive sphaeroplasts with Zymolyase 5000 (Pringle et al, 1979). Thirdly activity may be lost, during preparation, due to the action of periplasmic proteases perhaps activated following the loss in plasma membrane-cell wall contact (Farkaš, 1979). Finally the apparent low activity of ectomannosyl-transferases could be the result of these enzymes having lower affinities for GDP-mannose than those transferases involved in synthesis of the β-eliminable saccharides. Mannosyltransferases with different substrate affinities have been reported by Farkaš et al (1976b) using a particulate preparation of S. cerevisiae incubated with different concentrations of GDP-mannose. Using GDP-mannose concentrations of 15 μM and 1 mM, about 72% and 31%, respectively, of mannose incorporated

into polymer was β-eliminable. This indicates that synthesis of the non-β-eliminable polysaccharide requires high concentrations of GDP-mannose. This result also explains the observations of Farkaš and Bauer (1976). Using high concentrations of GDP-$\left[^{14}C\right]$mannose and yeast particulate preparations, they found that only about 30% of the radio-activity was incorporated into β-eliminable material. When lower concentrations of GDP-mannose are used, as in the present study, the amount of β-eliminable synthesis is between 70-80% (Results section IV F; Lehle and Tanner, 1974).

Yeast mannosyltransferases are known to require Mn^{2+} for activity although in some cases, this may be replaced by Mg^{2+}. For example, transferases involved in the lipid pathways of mannan bio-synthesis are active with either Mn^{2+} or Mg^{2+} (Sharma et al, 1974; Lehle and Tanner, 1975) while those involved in synthesis of the outer chain or transfer of the second, and subsequent, mannose residues attached to serine and threonine have an obligatory requirement for Mn^{2+} (Nakajima and Ballou, 1975b; Parodi, 1979a; Sharma et al, 1974). In most studies, mannosyltransferase activity has been measured with 1-10 mM divalent cation. In the present study, 7.5 mM $MnCl_2$ was more stimulatory than $MgCl_2$ (figure 10, p. 89 ; table 13, p. 130). One intriguing result was the observation (table 13) that both ferric and ferrous ions, at 7.5 mM, were more effective than Mn^{2+}, at low concentrations of sphaeroplast lysate protein. Ferric ions have been shown recently to give optimal activity of a mannosyltransferase in Mucor rouxii (Guttierrez and Ruiz-Herrera, 1979). However, mannosyl-transferases in Saccharomyces species have always shown optimal activity with either Mn^{2+} or Mg^{2+}, regardless of any stimulation by ferric or ferrous ions. Mannosyltransferase activity, in sphaeroplast lysates

of $\underline{S}$. $\underline{carlsbergensis}$, was enhanced by Fe^{2+} although unaffected by Fe^{3+} ions, both at 4 mM (Behrens and Cabib, 1968). A particulate preparation from $\underline{S}$. $\underline{cerevisiae}$ with 1 mM Fe^{3+} showed only 4% of the mannosyltrans-ferase activity observed with 1 mM Mn^{2+} (Lehle and Tanner, 1974).

The kinetics of mannosyltransferase activity of the sphaero-plast lysate preparation differ somewhat from those described by other workers for mannosyltransferases. While the Km values for GDP-mannose (0.13 mM and 0.53 mM) in this study are in the same range of values (0.1 - 0.5 mM) obtained by other workers for crude enzyme preparations (Lehle and Tanner, 1974) and solubilised mannosyltransferases (Nakajima and Ballou, 1975b), enzyme activity in this study shows biphasic kinetics (figure 14, p. 102). Similar biphasic kinetics have been reported for mannosyltransferase activity in membrane fractions of Mucor rouxii (Guttierrez and Ruiz-Herrera, 1979) and were suggested to be the result of a homotropic co-operative effect. Under the assay conditions used here, the combined activies of as many as 10 different mannosyltransferases (Nakajima and Ballou, 1975b) were measured. Thus the most likely explanation for the observed kinetics is the presence of mannosyltransferases with different substrate affinities. Never-theless, the results do not exclude the possibility of substrate activation at high concentrations of GDP-mannose.

II. CONTROL OF MANNOSYLTRANSFERASE ACTIVITY BY LOW MOLECULAR-WEIGHT EFFECTORS

Once the optimal conditions for mannosyltransferase activity in the enzyme preparations under study were established, the aim of this project was to investigate possible regulatory mechanisms that

function to control mannosyltransferase activity, in vitro at least.

Regulation of carbohydrate synthesis for polysaccharides and glyco-

proteins in eucaryotes is poorly understood at the present time,

although one or more of the following basic factors is presumed to be

involved:

(i) the specificity of glycosyltransferases catalysing the individual

    transfer steps

(ii) the availability of polyisoprenoid carriers

(iii) subcellular organisation and compartmentalisation.

      In most cases, glycosyltransferases behave in accordance

with the 'one enzyme-one linkage' concept which postulates that an

enzyme is specific with regard to (a) the sugar transferred (b) the

acceptor and (c) the position and anomeric configuration of the linkage

formed. The specificity for the glycosyl group of the donor in yeast

appears to be nearly absolute (Lehle and Tanner, 1974) whereas the

requirement for a specific nucleotide component may be less precise.

In yeast mannan biosynthesis, GDP-mannose is the usual donor although

there is some incorporation of mannose into polymer when ADP-mannose

is substituted as the substrate (Behrens and Cabib, 1968). Glycogen

synthesis provides another example. UDP-glucose is the physiological

donor in glycogen synthesis by yeasts but may be substituted by ADP-

glucose, which is approximately 50% as effective as the uridine

derivative (Recondo and Leloir, 1961). Nevertheless, Ginsburg (1978)

has suggested that the use of different nucleotides, as carriers of

monosaccharides, separates pathways of synthesis and offers a means

for their independent control.

      The involvement of polyisoprenoid intermediates in hetero-

saccharide and glycoprotein biosynthesis in both procaryotic and

eucaryotic systems is now well established and these compounds may fulfil

an important role in regulatory mechanisms. For example, there is

evidence that the nucleotide precursors for peptidoglycan and teichoic

acid compete for the undecaprenol phosphate carrier common to both

syntheses (Anderson et al, 1972). Thus the availability of the carrier

may control the rate of wall synthesis in Gram-positive bacteria.

Since there is no evidence for the involvement of polyisoprenoid

carriers in the synthesis of chitin, a similar mechanism for controlling

its synthesis seems unlikely. However, the availability of dolichol

phosphate may be of importance in regulating synthesis of the two

different carbohydrate portions of mannan. The possible existence of

a lipid intermediate involved in glucan synthesis cannot, as yet, be

excluded (see, for example, Baguley, 1977).

The importance of compartmentalisation in the synthesis of

macromolecules is well illustrated by the separate localisation of

zymogen, activating factor and inhibitor in the yeast chitin synthase

system (Cabib, 1975, 1976) already discussed in the Introduction

(pp. 35-39). Its importance is also inferred from the distribution of

mannosyltransferases in both intracellular membrane vesicles (Lehle et al,

1977; Marriott and Tanner, 1979) and plasma-membrane fractions (Santos

et al, 1978; Welten-verstegen et al, 1980). Evidence will be

discussed, in section III, for an inhibition-activation system in the

control of mannan synthesis which provides a further role for compart-

mentalisation in yeast cell-wall formation. Meanwhile, this section

describes how the production of cell-wall mannan may be controlled by

low molecular-weight effectors of mannosyltransferase activity.

One obvious mechanism for preventing over-production of

nucleotide precursors of wall components would be by feedback inhibition

of the initial step in the enzymatic sequence.   Thus GDP-mannose might

be expected to inhibit phosphomannose isomerase, the first enzyme unique

to mannan synthesis (figure 26).   The efficient operation of such

feedback controls, would preclude an appreciable intracellular accumu-

lation of wall precursors in yeast.   However, the intracellular concen-

tration of UDP-glucose has been found to be 0.2-0.3 mM in growing yeast

(Rothman and Cabib, 1969; Gancedo and Gancedo, 1973) and may be as high

as 2.4 mM in the early stationary phase of a carbon-limited culture

(Sagardia et al, 1971).   Similarly, Sentandreu and Lampen (1970) found

an accumulation of UDP-N-acetylglucosamine and GDP-mannose when cyclo-

heximide was added to cells.   Under these conditions, mannan synthesis

is inhibited (Sentandreu and Northcote, 1969b) while the level of GDP-

mannose rises 20-fold (Sentandreu and Lampen, 1970).

If feedback inhibition does not occur, then it is possible

that wall polysaccharide synthesis may be regulated at the synthase level

as in the case of glycogen synthesis.   Glycogen synthesis in bacteria is

regulated by control of ADP-glucose pyrophosphorylase, while in yeasts

the locus of regulation is glycogen synthase (Dawes and Senior, 1973).

The difference may arise because UDP-glucose, the glycosyl donor in

yeasts, is also the precursor for β-(1$\rightarrow$3)glucan synthesis (figure 26),

whereas in procaryotes ADP-glucose functions uniquely in glycogen

synthesis.   If the dual role of UDP-glucose requires that glycogen

synthesis be regulated at the synthase level then regulation of glucan

synthesis at the synthase level would seem a logical extrapolation.

Transferases involved in glycoprotein synthesis can be subject

to allosteric control.   For example, the formation of dolichol mono-

phosphate glucose from UDP-glucose, in rat liver endoplasmic reticula,

is powerfully and specifically inhibited by physiological concentrations

Figure 26.   Pathways of yeast polysaccharide biosynthesis

Scheme illustrating the interconversion of hexoses and formation of activated sugars involved in the biosynthesis of yeast polysaccharides.   Enzymes involved are:

              A   :   hexokinases

              B   :   phosphohexomutases

              C   :   sugar nucleotide pyrophosphorylases

              D   :   glycosyltransferases

              E   :   phosphohexose isomerases

Pi, inorganic phosphate;   PPi, inorganic pyrophosphate; GlcNAc, N-acetylglucosamine.

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MANNAN

lipid
intermediates   protein

D

UDP–GLcNAc   GDP–MANNOSE

C   PP$_i$
           GTP

MANNOSE–1–P

ATP   B

MANNOSE ——→ MANNOSE–6–P
         A

         E   glutamine

ATP

GLUCOSE —→ GLUCOSE–6–P ⇌ FRUCTOSE–6–P ————→ GLUCOSAMINE–6–P
         A   E
                                                    Acetyl–CoA

         B   CoA

GLUCOSE–1–P   GLcNAc–6–P

GTP   UTP   B

PP$_i$ C   C   PP$_i$ GLcNAc–1–P

GDP–GLUCOSE   UDP–GLUCOSE   C   UTP
                                                          PP$_i$

? D   D   D   UDP–GLcNAc

                                                D   primer

β(1→6) | β(1→3)   GLYCOGEN   CHITIN

GLUCAN
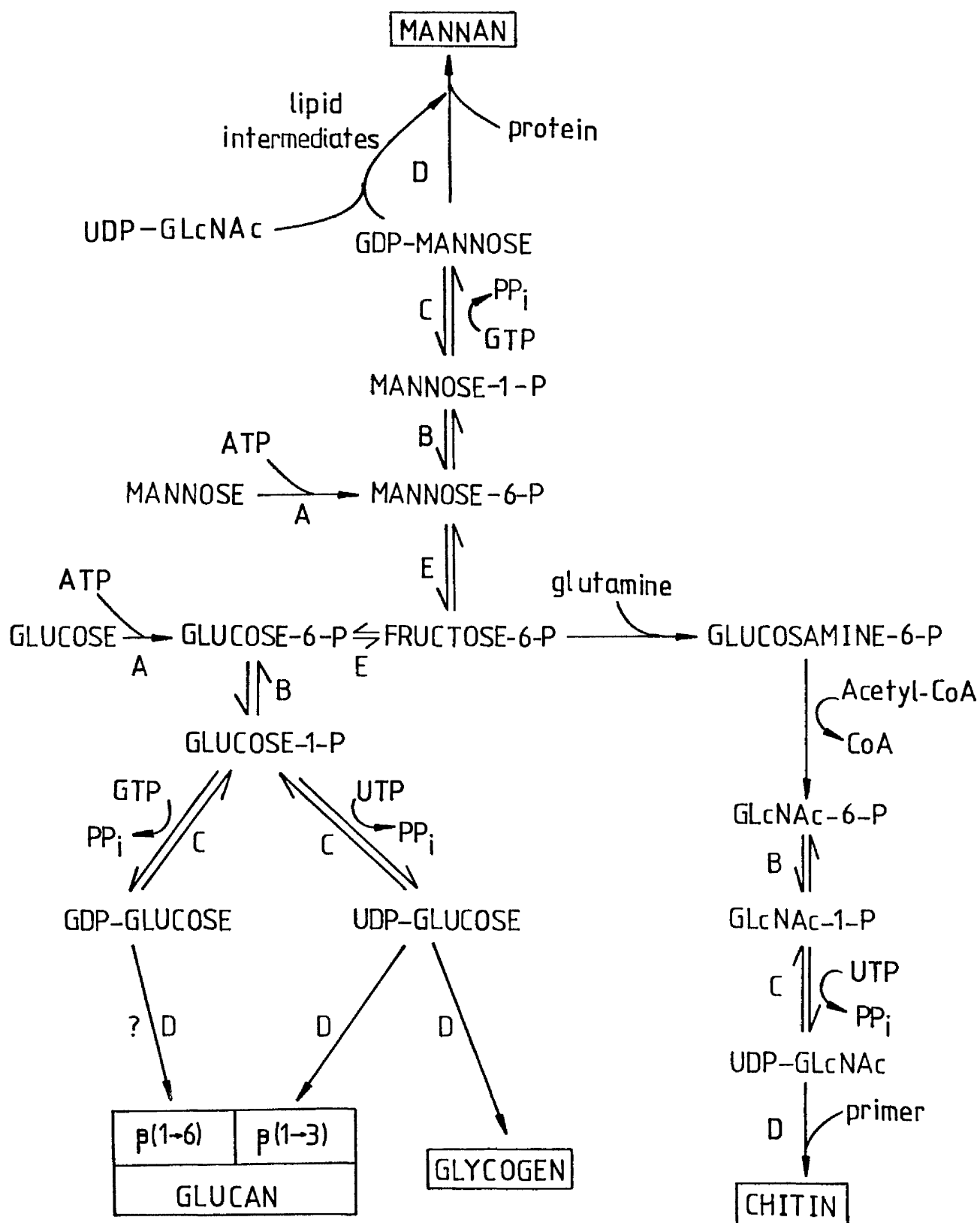
of GDP-mannose (Kerr and Hemming, 1978). This study also revealed that the pool of dolichol phosphate available for the mannosyltransferase is not available for the glycosyltransferase, since UDP-glucose does not affect the formation of dolichol monophosphate mannose. Thus glycoprotein synthesis is controlled at two different levels in this case.

A. Control by sugar nucleotides

Of various sugar nucleotides tested in this study (table 2, p. 95 and figure 13, p. 97), mannosyltransferase activity was inhibited significantly by only 3: ADP-mannose, UDP-mannose and GDP-glucose. The first two probably affect activity competitively by acting as poorly efficient mannosyl donors (Behrens and Cabib, 1968; Karson and Ballou, 1978) while GDP-glucose was clearly demonstrated, by Lineweaver-Burk plots, to act competitively (figure 14, p. 102). The inhibition of mannan synthesis by ADP-mannose, UDP-mannose and GDP-glucose at high concentrations of sugar nucleotide (figure 13, p. 97) never attains a figure of 100%. This may suggest that certain mannosyltransferases are sensitive while others are insensitive to the action of the appropriate sugar nucleotide.

Evidence from other systems suggests that because of the high specificity of glycosyltransferases, competition by other sugar nucleotides is not common in eucaryotes. ADP-glucose is the glucosyl donor for starch synthase from plants and its activity is not modified in the presence of other sugar nucleotides (Recondo and Leloir, 1961). Similarly, cellulose synthesis from GDP-glucose by mung bean seedlings was reported to be unaffected by related sugar nucleotides including GDP-galactose, UDP-glucose, ADP-glucose, TDP-glucose (Barber et al, 1964). However, this finding was contradicted by the same group but

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without comment (Elbein, 1969). Mannosyltransferases in the retina of embryonic chicken also show a high degree of specificity for donor sugar nucleotides and the incorporation of mannose from GDP-mannose into lipid intermediates and glycoproteins is unaffected by GDP-glucose, UDP-glucose or UDP-N-acetylglucosamine (Kean, 1977a).

However, in certain other systems, inhibition of glycosyl-transferases by sugar nucleotides has been observed. Keller and Cabib (1971) reported that UDP-glucose, GDP-mannose and GDP-glucose all inhibited to some extent the incorporation of N-acetylglucosamine into chitin. Although they concluded that these sugar nucleotides were acting competitively, because of their structural analogy to UDP-N-acetylglucosamine, it is interesting that all three inhibitory sugar nucleotides are now known to be precursors of other cell-wall polymers. A further example is the observation that UDP-glucose and ADP-glucose inhibit the mannosylation, from GDP-mannose, of dolichol phosphate that is involved in the glycosylation of rat liver microsomal proteins (Richards and Hemming, 1972). It was suggested that UDP-glucose, which is the precursor for glucosyl residues, acted by competing with GDP-mannose for the available dolichol phosphate. On the other hand, competition by ADP-glucose was probably of no physiological significance. A similar result was obtained recently for the synthesis of dolichol monophosphate ₍mannose₎ by an enzyme solubilised from encysting cultures of Acanthamoeba castellanii (Carlo and Villemez, 1979). Although GDP-glucose does not act as a substrate for this enzyme it still reduces the rate of synthesis of dolichol monophosphate mannose.

Another explanation for the observed inhibition of mannan synthesis by GDP-glucose would be the presence, in the enzyme preparation of GDP-glucose glucohydrolase (Sonnino et al, 1966) which catalyses the following reaction:

$$\text{GDP-glucose} + H_2O \longrightarrow \text{GDP} + \text{glucose}$$
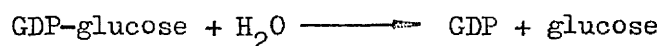
GDP, as an end-product of mannan synthesis, might be expected to
inhibit mannosyltransferase activity. This was shown by Quesada Allue
and Belocopitow (1978) to be the mechanism by which GDP-glucose inhibits
the incorporation of mannose from GDP-mannose into lipid intermediates
by insect microsomal fractions. Glucohydrolase activity, which is
highly specific for GDP-glucose (Sonnino et al, 1966), was detected
when enzyme preparations were incubated with GDP-$[^{14}C]$glucose (figure
17, p. 110). However, it seems unlikely that the inhibitory effect
of GDP-glucose on mannosyltransferase activity is due to the formation
of GDP, by the glucohydrolase, for two reasons. Firstly, although
small amounts of GDP-glucose were hydrolysed rapidly, only a negligible
proportion of GDP-glucose was hydrolysed when present at concentrations
of 1 mM in reaction mixtures. Secondly, GDP caused about 40% inhibition
and glucose none while GDP-glucose caused about 80% inhibition (tables
1 and 2, pp. 94 and 95). It is possible, however, that the extra
inhibition, by GDP-glucose, observed with the sphaeroplast lysate
preparation as compared with the washed membrane fraction (about 4%,
table 2), may be due to the formation of small amounts of GDP which
would then act as an additional inhibitor.

A further possibility, that exogenously-added GDP-glucose
competes for an endogenous polyisoprenoid carrier, presumably dolichol
phosphate, seems unlikely as no radioactively-labelled glucolipids were
detected when the enzyme was incubated with GDP-$[^{14}C]$glucose (figure
16, p. 108). However, under the conditions used (low concentrations
of GDP-glucose), most of the substrate was hydrolysed and hence
unsuitable as a glycosyl donor. There is, as yet, no clear evidence

that such a carrier is required for glucan synthesis. Palamarczyk and

Chojnacki (1973) and Parodi (1976, 1977) have reported incorporation of

glucose from UDP-glucose into a lipid fraction by particulate enzyme

preparations from S. cerevisiae; this incorporation is stimulated by

the addition of yeast dolichol phosphate. It now seems likely that

glucose is incorporated, from UDP-glucose, into lipid-bound oligo-

saccharides involved in mannan synthesis (Parodi, 1979b). Whether GDP-

glucose is also involved in this step is not known. Indeed the role

of GDP-glucose as a possible glucan precursor remains controversial

(Sentandreu et al, 1975; López-Romero and Ruiz-Herrera, 1977;

Shematek et al, 1980). This question might be resolved if glucan

synthesis could be assayed using glucohydrolase-free enzyme preparations.

In the present study, attempts were made to eradicate this activity by

extensive washing of membrane preparations (figure 17, p. 110), but

glucohydrolase activity was never totally removed.

Since GDP-glucose is a strong competitive inhibitor of

mannan synthesis, and probably also a precursor for glucan synthesis,

the two synthetic systems are presumably strictly segregated in vivo.

This further illustrates the importance of compartmentalisation in yeast

cell-wall biogenesis.

B.   Control by nucleotides

Enzyme systems involved in glucan and mannan synthesis show

a slow turnover rate (Elorza et al, 1976). This would suggest that

changes in cell-wall composition, under different growth conditions,

are controlled by some mechanism that allows rapid modulation of the

rate of mannoprotein formation independently of gene transcription and

translation. Evidence for this was recently provided by Elorza et al

(1977a). They found that ADP and, to a lesser extent, GDP and UDP were inhibitors of mannosyltransferases in S. cerevisiae. ADP acted competitively but did not affect reactions involving a lipid carrier. However, GDP and UDP inhibited both lipid-dependent and lipid-independent mannosyltransferases. Elorza and her co-workers, therefore, suggested that changes in the nucleotide pools may act as a control mechanism for mannan synthesis in vivo. When growth conditions deteriorated, the nucleoside triphosphate level would decrease and nucleoside diphosphates would accumulate, leading to decreased mannosyltransferase activity. The same group of workers reported that ATP also inhibits mannosyltrans- ferase activity (Sentandreu et al, 1977) and proposed that this was due to the effect of the hydrolysed product, ADP. At about the same time, the present study had shown that UTP, ADP and, especially, ATP stimulated mannosyltransferase activity while GDP and GTP inhibited activity (table 1, p. 94). It should be noted that ATP and UTP did not always enhance mannosyltransferase activity (table 4, p. 100). It appears from these results and those described above (Elorza et al, 1977a; Sentandreu et al, 1977) that the effect of certain nucleotides is dependent on the method of enzyme preparation.

The possibility that nucleotides may have a role in the control of glycosyltransferase activity in fungal wall-polymer synthesis is also evident from other reports. GDP inhibits the formation of dolichol monophosphate mannose by a particulate preparation from S. cerevisiae, due to a reversal of the reaction involved (Tanner et al, 1971). Formation of the same compound by microsomal preparations from Aspergillus niger is inhibited if either GMP, GDP or GTP is added during incubation of reaction mixtures (Létoublon et al, 1973). This suggests the importance of the guanosine moiety for inhibition. Nucleotides,

especially nucleoside triphosphates, strongly inhibit a xylosyltrans-
ferase involved in synthesis of the acidic extracellular polysaccharide
of Cryptococcus laurentii (Cohen and Feingold, 1967). A range of
nucleotides also inhibit chitin synthase in preparations from the stipe
of Coprinus cinereus (de Rousset-Hall and Gooday, 1975) and the yeast
form of Mucor rouxii (Ruiz-Herrera et al, 1977). In the latter case,
ATP was found to inhibit chitin synthase at high concentrations (6.25 mM)
while slightly enhancing activity at low concentrations (0.2 mM).
This result is similar to the stimulation, by nucleoside triphosphates,
of chitin synthase from the mycelial form of M. rouxii (McMurrough and
Bartnicki-Garcia, 1971).

Although the effects of nucleotides on glycosyltransferase
activities have been reported, their role in controlling cell-wall
synthesis is still not really understood. There are several possible
mechanisms by which nucleotides might affect mannosyltransferase
activity, some of which are listed below. These will be discussed in
relation to the stimulatory effect of UTP, ATP and ADP and the inhibitory
effect of GTP and GDP on mannosyltransferase activity observed in this
study. (i) Exogenously-added nucleotides may affect activity by
complexing Mn^{2+} ions required by the enzyme. (ii) Some nucleotides
may protect the substrate, GDP-mannose, from degradation by hydrolytic
enzymes. (iii) The nucleotides may act indirectly by stimulating the
formation of either substrate or cofactors. (iv) Nucleotides may
alter the equilibrium balance of reversible transferase reactions
involved in mannan biosynthesis. (v) Nucleotides may act directly on
the enzymes themselves, perhaps allosterically, thus conferring different
kinetic properties on the transferases.

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It is unlikely that nucleotides will stimulate mannosyltrans-
ferase activity by complexing divalent cations, since such a decrease
in the available Mn^{2+}, if it did occur, would be expected to decrease
activity.   Vessey and Zakim (1975) reported that mannosyltransferase
activity in liver microsomal fractions was stimulated by low levels of
adenosine nucleotides.   This was due to protection of the GDP-mannose
substrate.   At high concentrations of AMP, however, insoluble complexes
with Mn^{2+} were formed in the reaction mixture.   However these
complexes only formed at concentrations of AMP in excess of 10.mM,
where the mixtures contained 5 mM $MnCl_2$.   In the present study the
maximum concentration of nucleotide used was 5 mM.

There is considerable evidence that nucleotides are effective
in several systems in protecting radioactive sugar nucleotides from
hydrolysis.   For example, the formation of dolichol monophosphate
mannose from GDP-mannose, by liver microsomes is inhibited by rapid
hydrolysis of the substrate by nucleotide pyrophosphatases (Vessey and
Zakim, 1975).   This degradation of the substrate was prevented by AMP
or ATP.   Although ATP was the most effective inhibitor of pyrophospha-
tase activity, ATP also affects the mannosyltransferase reaction: ATP
can be rapidly converted to ADP which in turn is able to discharge the
$\left[^{14}C\right]$-labelled sugar from dolichol monophosphate $\left[^{14}C\right]$mannose.   Similar
activation by AMP and ATP has been observed in the synthesis of dolichol
monophosphate mannose and lipid-linked oligosaccharides by liver mito-
chondrial outer membranes (Gateau et al, 1978).   The incorporation of
mannose, via dolichol monophosphate mannose, into mannobiose by an
α-(1→2)mannosyltransferase in liver microsomes was also enhanced by ATP
(Verma et al, 1977).   AMP also acts as an inhibitor for the soluble
nucleotide pyrophosphatase purified from a hybrid strain of Saccharomyces

(Twu et al, 1977). In the present investigation, AMP had no effect on mannosyltransferase activity while the hydrolysis of GDP-mannose was negligible in incubation mixtures containing low concentrations of enzyme protein (1 mg/ml). These results suggest that ATP does not enhance mannosyltransferase activity by a protective effect on GDP-mannose. There was, in fact, a slight increase in the amount of radio-activity in material which had the same chromatographic mobility as hexose nucleotides when ATP was included in reaction mixtures. Since small amounts of $[^{14}C]$mannose and $[^{14}C]$mannose-1-phosphate are present in reaction mixtures, this result would indicate that ATP activates these radioactive compounds to produce ADP-$[^{14}C]$mannose. ATP may, therefore, act as a substrate itself in the synthesis of ADP-mannose. Although ADP-mannose does act as a poor substrate for mannosyl- and mannosylphosphate-transferases (Behrens and Cabib, 1968; Karson and Ballou, 1978), the amount of $[^{14}C]$-labelled mannose or mannose-1-phosphate available in reaction mixtures would not be sufficient to account for the observed stimulation of mannosyltransferase activity by ATP.

ATP may activate essential pools of dolichol. For example, biosynthesis of retinol monophosphate mannose by liver membrane preparations from vitamin A-deficient rats required ATP (de Luca et al, 1973). This suggests that ATP activates the endogenous retinol, whose level is low in preparations from these rats.

The explanation for inhibition of mannosyltransferase activity by GDP and GTP is most likely the reversal of transferase activity. GDP, and GTP in the presence of phosphatases, are known to reverse the formation of dolichol monophosphate mannose by enzymes in liver preparations (Richards and Hemming, 1972; Vessey and Zakim, 1975; Gateau et al, 1978), retinal preparations (Kean, 1977b) as well as membrane

preparations from <u>Aspergillus</u> <u>niger</u> (Létoublon <u>et</u> <u>al</u>, 1973) and
<u>S. cerevisiae</u> (Tanner <u>et</u> <u>al</u>, 1971).

Finally, nucleotides may interact directly with the mannosyl-
transferases.   In relation to this, Shematek and Cabib (1980) have
proposed that ATP and GTP play a role in the complex regulation of
β-(1→3)glucan synthase activity.   Both nucleotides stimulate β-(1→3)glucan
synthase activity.   GTP does so by actually binding to the enzyme,
during which time it is converted to GDP, which then remains enzyme-
bound.   The action of ATP is not as clearly understood although it does
act differently from GTP. The authors have proposed a model in which
the action of ATP probably first requires chemical modification by some,
as yet, unidentified supernatant factor.   A contrary, but unexplained
result, was obtained by Sentandreu <u>et</u> <u>al</u> (1975) who briefly noted that
glucan synthesis from UDP-glucose, by toluene-ethanol permeabilised
cells, was actually inhibited 50% by 1 mM ATP.   Stimulation of mannan
synthesis by UTP and ATP may occur by phosphorylation or adenylation of
enzymes or the nucleotide may act as an allosteric modifier.   Although
there is 'no evidence that ATP directly modifies mannosyltransferases,
an outline scheme can be suggested to explain how it might subsequently
enhance mannan synthesis.   In the enzyme assay system used here, most
mannan synthesised was β-eliminable.   It has been mentioned previously
that, with higher concentrations of GDP-mannose, the major fraction of
mannan synthesised becomes non-β-eliminable (Farkaš <u>et</u> <u>al</u>, 1976a),
probably because the enzymes involved in the synthesis of the poly-
saccharide moiety have a higher Km value for GDP-mannose.   ATP, by
binding to these enzymes, could modify their conformation in such a way
that would lower their Km values, thus effecting an increase in mannan
synthesis.   At the same time, this would lead to a decrease in the

proportion of β-eliminable saccharides synthesised, an effect which could be tested experimentally.

Similar situations, in which different glycosyltransferases appear to be activated by nucleoside triphosphates, have been described in three reports on mammalian glycoprotein synthesis. Kean (1977b), using enzyme preparations from retinal tissue, found that ATP inhibited formation of dolichol monophosphate mannose yet stimulated the incorporation of mannose into both lipid-bound oligosaccharides and glycoproteins. Richard et al (1978), using an enzyme preparation from liver nuclei, found that formation of dolichol monophosphate mannose was inhibited by nucleoside di- and tri-phosphates, although incorporation of mannose into lipid-bound oligosaccharides and glycoprotein was stimulated. Finally, Godelaine et al (1979a,b) showed that liver microsome preparations normally catalyse the formation of dolichol monophosphate mannose readily, while the formation of lipid-bound oligosaccharides and glycoprotein is slow unless GTP is included in reaction mixtures. The action of GTP is to diminish the synthesis of dolichol monophosphate mannose while at the same time stimulating formation of the other products. The initial effect of GTP, which is not observed with ATP, is to stimulate the formation of dolichol diphosphate di-N-acetylchitobiose from dolichol diphosphate N-acetylglucosamine.

If nucleotides can control mannosyltransferase activity as indicated, do they play a role in the control of mannan synthesis, in vivo? The level of ATP in yeast cells varies from 0.9-4.4 mM, in resting cells, and 1.1-1.4 mM, in growing cells (Gancedo and Gancedo, 1973). Levels of ATP in Saccharomyces pastorianus after addition of cycloheximide remain fairly constant, whereas these of the guanosine nucleotides rise markedly (Siegel and Sisler, 1964). Sentandreu and

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Lampen (1970) found that cycloheximide inhibits mannoprotein synthesis in S. cerevisiae. Although they concluded that cycloheximide inhibited mannan synthesis by preventing the formation of the polypeptide acceptor, it is also possible that inhibition may have been due, in part, to the accumulation of inhibitory guanosine nucleotides. Rapid modulation of the levels of ATP, GTP and UTP in the vicinity of the mannosyltransferases may occur by the action of ATPases, GTPases and UTPases. All of these enzymes have been found enriched in vesicles prepared by a gentle "polybase-induced" lysis of yeast protoplasts (Wiemken et al, 1979).

Conflicting results on the effect of nucleotides on mannosyl-transferase activity complicate the issue but these may be explained, eventually, by differences in nucleotidases and pyrophosphatases present in the enzyme preparations. The role of nucleotides in the control of mannosyltransferases may be better understood when studies are done with purified transferases and well-characterised acceptors.

III. INHIBITION AND ACTIVATION OF MANNOSYLTRANSFERASES

Inhibition of mannosyltransferase activity was observed when high concentrations of sphaeroplast lysate protein were included in reaction mixtures. This inhibition could be relieved by preincubation of the sphaeroplast lysate at low temperatures, by freezing and thawing of the sphaeroplast lysate or by altering the ionic environment. It seemed unlikely that the inhibition was due to low molecular-weight effectors. Although mannosyltransferase activity was enhanced by dialysis of sphaeroplast lysates, the same effect was produced by storing the enzymes at $4^{o}C$ without dialysis. Cohen and Feingold (1967), who

found that nucleoside di- and tri-phosphates inhibited a xylosyltrans-
ferase from Cryptococcus laurentii, also showed that dialysis of crude
cell-extracts resulted in greatly enhanced transferase activity. The
authors suggested that this was due to low molecular-weight, intra-
cellular inhibitors. However, no attempt was made to identify the
nature of the inhibitory substance or substances.

Stimulation of mannosyltransferase activity on storage at
$4^{o}C$ in this study was very similar to many reports describing proteolytic
chitin synthase activation. Chitin synthase was first reported to
exist in an inactive state in S. cerevisiae by Cabib and Farkaš (1971).
The inactivated chitin synthase zymogen is activated by a protease which
was identified as proteinase B (Ulane and Cabib, 1976). Since 1971,
proteolytic activation of chitin synthase has been reported in five of
the twelve different genera of fungi in which the enzyme has been fully
studied (listed by Cabib et al, 1979). Only the enzyme from Coprinus
cinereus has definitely been shown not to exist in the zymogenic state
(Gooday, 1979). The specificity of the protease involved in activation
varies for different fungi. For example chitin synthases from both
S. cerevisiae (Ulane and Cabib, 1976) and Candida albicans (Braun and
Calderone, 1978) are activated by neutral proteases such as trypsin
wheras chitin synthases from Aspergillus flavus (López-Romero and Ruiz-
Herrera, 1976) and Mucor rouxii (Ruiz-Herrera et al, 1977) are
activated to a greater extent by fungal acid proteases. On the other
hand, Campbell and Peberdy (1979) isolated and purified three proteases
from Aspergillus nidulans, with acid, neutral and alkali pH optima;
their results indicate that the neutral protease is involved in activation
of the chitin synthase zymogen in vitro. Specificity was further
suggested by the observation that the neutral protease gave consistently

higher specific activation of the zymogen than trypsin. In the present

study there was no evidence that mannosyltransferases existed in the

zymogenic state. Several proteases (trypsin, pronase, subtilisin BPN',

pepsin, bromelain and an Aspergillus acid protease) were ineffective in

activating mannosyltransferase activity. Furthermore, there was no

activation at low temperatures when mannosyltransferase activity was measu-

red in reaction mixtures containing either low levels of sphaeroplast

lysate protein (1 mg/ml) or washed membrane fraction (1 mg/ml or

10 mg/ml).

Experiments with a supernatant fraction obtained from lysed

sphaeroplasts established that the inhibition of enzyme activity observed

at high protein concentrations was due to a heat-labile, trypsin- and

pepsin-sensitive inhibitor. This finding also discounted the possib-

ility that enzyme activation by freezing and thawing, preincubation at

low temperatures or alterations in the divalent cation concentration

might simply result from the disruption of vesicles and unlysed sphaero-

plasts thus allowing better access of the substrate to enzyme sites.

The inhibitor is not an enzyme responsible for hydrolysis of either

GDP-mannose or newly-synthesised mannan. Although mannan was degraded

by supernatant fraction, the amount was insufficient to explain the

extent of inhibition of mannosyltransferase activity. GDP-mannose, on

the other hand, was hydrolysed by enzyme preparations and supernatant

fraction (figures 23 and 24, pp.133 and 134). However, inhibition

of mannan synthesis and degradation of GDP-mannose by supernatant

fraction differed in their EDTA- and temperature-sensitivies. Hydrolysis

of GDP-mannose was totally lost if the supernatant fraction was

pretreated with EDTA or preincubated at 4°C for 24 h (table 14, p. 137)

but EDTA-treatment did not affect the ability of the supernatant fraction

to inhibit mannosyltransferase activity and only about 50% of the inhibitory activity was lost at $4^{o}C$ for 24 h.

In light of these findings, a model for the inhibition of mannan synthesis is proposed which is diagrammatically illustrated in figure 27. In this model it is envisaged that lysed sphaeroplasts contain an inhibitory protein which can bind to one or more mannosyl-transferases and form an inactive complex. This complex formation would require the presence of Mn^{2+}, perhaps to form a divalent cation bridge between the two proteins or to produce conformational changes in one or both molecules allowing complex formation. At low protein levels (1 mg/ml in the assay) the concentrations of the enzyme and inhibitor are too low to permit complex formation, even with Mn^{2+}. At high protein levels (10 mg/ml in the assay), however, complex formation will take place although it can be prevented by the addition of EDTA or monovalent cations. Incubation of the sphaeroplast lysate at low temperature, would result in a gradual proteolytic destruction of the inhibitor. During the subsequent assay, no inactive complexes could be formed with or without Mn^{2+}.

The activity of the inhibitor may be modulated either by storage at low temperature or by varying the available Mn^{2+} concentration. $MnCl_2$, at a concentration of 7.5 mM, gave optimal mannosyltransferase activity when low levels of sphaeroplast lysate protein were used in the standard assay. At high protein levels, however, this Mn^{2+} concentration proved to be inhibitory, although the inhibition could be relieved by preincubation at $4^{o}C$ (figure 22, p. 121). The ionic environment, therefore, controls mannosyltransferase activity in two ways. Firstly, individual mannosyltransferases have specific requirements for particular divalent cations (section I, p. 151); secondly, Mn^{2+} concentration

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Figure 27.   Hypothetical scheme for inhibition of mannan synthesis

in S. cerevisiae sphaeroplast lysates

⊤⊙⊤   Membrane-bound mannosyltransferase

◆   Inhibitor

∿   Degraded inhibitor

SPHAEROPLAST LYSATE
(20 mg/ml)

Assay
+MnCl$_2$

Assay
-MnCl$_2$

Preincubation
at 4°C

INHIBITION

NO INHIBITION

Assay +/-
MnCl$_2$

NO INHIBITION
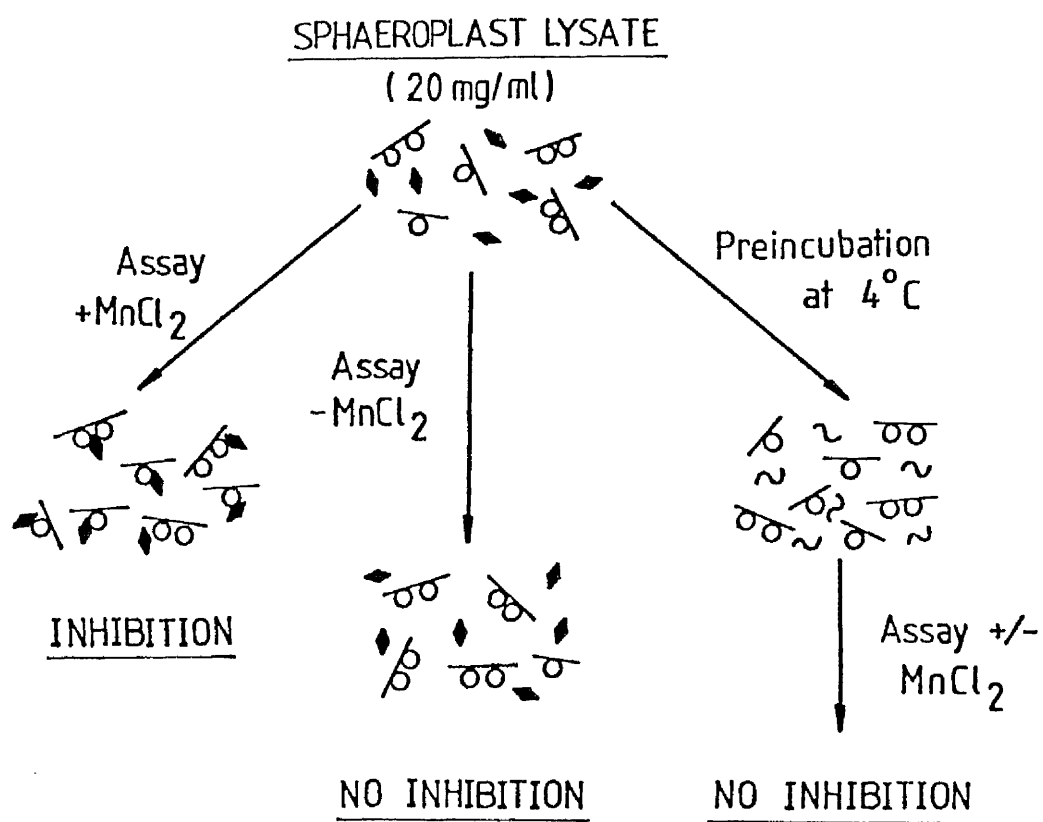
regulates the formation of enzyme-inhibitor complexes. Okorokov
et al (1977) found that the concentrations of Mn^{2+} in yeast cytoplasm
and vesicles were 2 mM and 14 mM, respectively, while Roomans (1980)
found that most divalent cations were bound to polyphosphate in vesicles,
although some binding to other compounds did occur. Thus the amount
of cellular polyphosphate, first located in vesicles by Indge (1968),
will probably determine the location and levels of divalent cation
within the cell. This might then affect glycosyltransferase activities
in cell-wall synthesis; it is known, for example, that S. cerevisiae
grown under conditions of phosphate-limitation has a reduced content of
wall glucan (Ramsey and Douglas, 1979).

Although the inhibitor in the supernatant fraction was
destroyed by both trypsin and pepsin treatment (tables 10 and 11, pp.
125 and 127), proteases did not activate mannosyltransferase activity
in incubation mixtures containing 10 mg/ml of sphaeroplast lysate protein.
One possible explanation of this result would be that while free
inhibitor (in the supernatant fraction) is sensitive to proteases, bound
inhibitor (as an enzyme-inhibitor complex in the sphaeroplast lysate),
is resistant. Alternatively, sphaeroplast lysates may contain a
protease inhibitor which is inactivated during preparation of the super-
natant fraction. Inactivation of yeast proteinases is a complicated
process in which inhibitors for one proteinase are destroyed by the
action of another proteinase (Holzer et al, 1975). In addition to
vacuolar proteinases in yeast, there are other peptidases located at
the cell surface (Frey and Röhm, 1978; Ciejek and Thorner, 1979),
probably in the periplasmic space. Hence there is a complex system of
proteases and peptidases, all of which may be active in sphaeroplast
lysates, which may explain apparently contradictory results in this study.

Similar, unexplained results, were reported by Sentandreu and Ruiz-
Herrera (1978). They found that the activating factor of chitin
synthase, present in cell-free extracts of M. rouxii was an acid protease
sensitive to pepstatin A. However, pepstatin A had no inhibitory
effect on the activation of the enzyme in toluene-treated cells.

Destruction of the mannosyltransferase inhibitor at low
temperature may be accomplished by a highly specific protease. Of the
protease inhibitors tested, none affected enzyme activation at 4°C for
24 h or completely stopped the loss of inhibitory activity of the super-
natant fraction when stored at 4°C. Loss of the latter activity was
partially stopped by trypsin inhibitor, PMSF, pepstatin A, TPCK and
TLCK. Several new intracellular proteases have been detected in
S. cerevisiae lately by Wolf (1980) and it is possible that one of those
might fulfil the role envisaged for a protease in figure 27 (p. 171).
An apparent lack of specificity in the proteolytic activation of chitin
synthase zymogen in S. cerevisiae has been found by Ulane and Cabib (1976).
The zymogen is not only activated by trypsin and proteinase B but also
by chymotrypsin, subtilisin and papain. Mutants have been isolated
recently which lack, or possess altered, proteinase B yet show normal
vegetative growth (Wolf and Ehmann, 1979; Zubenko et al, 1979).
These results indicate that the protease involved in chitin synthase
activation may not be proteinase B as proposed by Cabib and his coworkers
(Ulane and Cabib, 1976) but rather a minor unidentified protease.

There are no previous reports of a protein inhibitor of yeast
mannosyltransferases. Recently, however, Parodi (1979a) has described.
the activation, upon storage at 2°C, of those mannosyltransferases
involved in outer chain synthesis. After three weeks at this tempera-
ture, enzyme activity had increased more than twofold. This finding

is similar to some of the results obtained in this study and may be explained by the inhibition-activation mechanism now proposed. In this instance an inhibitor of one or more mannosyltransferases required for synthesis of the mannan outer chain would undergo a slow proteolytic destruction at low temperature. In this study different mannosyltransferase activities were measured in the presence of supernatant fraction by three methods. Firstly β-elimination was used to estimate the amount of mannose incorporated into short oligosaccharides attached to serine and threonine. Secondly, synthesis of the mannan polysaccharide chain was measured (Parodi, 1979a). Finally, mannosyltransferase activity of S. cerevisiae LB1-3B, a mutant that produces a mannan outer chain without any side chains, was measured. Unfortunately, little difference could be detected between enzymes from the two strains and the results showed that synthesis of both the β-eliminable and outer polysaccharide portions of mannan were susceptible to inhibition by the supernatant fraction. It is still possible, however, that the inhibitor may have a selective effect. For example, mannosyltransferases involved in the synthesis of lipid intermediates, which were not assayed specifically here, may be resistant to its action.

In a recent review, Farkaš (1979) proposed a scheme for the regulation of cell-wall biosynthesis in fungi which involves inactivation of plasma membrane-bound polysaccharide synthases by specific proteinaceous inhibitors located in the cell-wall or periplasm. Such an inhibitor has been convincingly identified only in Mucor rouxii, where it was isolated from the cytoplasm (López-Romero et al, 1978). This inhibitor specifically inhibits activated chitin synthase by competing with UDP-N-acetylglucosamine and was relatively thermostable after purification. The existence of chitin synthase inhibitors in fungi was first postulated

by Porter and Jaworski (1966) who suggested that the instability of chitin synthase in the mitochondrial fraction from Allomyces macrogynus might be attributable to the liberation of an inhibitor on freezing and thawing these particles. Since then, heat-stable, non-dialysable, cytoplasmic inhibitors of chitin synthase activity have been detected in both yeast and mycelial forms of M. rouxii (McMurrough and Bartnicki-Garcia, 1973; Ruiz-Herrera and Bartnicki-Garcia, 1976) and Mortierella vinacea (Peberdy and Moore, 1975). A thermolabile compound, which can inactivate particulate glucan synthase has been observed in soluble fractions from Saprolegnia monoica (Fèvre and Dumas, 1977).

Although the inhibition of mannosyltransferase activity, described here, was detected in a 100,000 x g supernatant fraction prepared from sphaeroplast lysates, there is no information as yet on its precise location in vivo. It is conceivable that the supernatant fraction contains components derived from the cell wall or periplasm. Moreover, a surface location for the inhibitor might be expected if its activity is directed towards those mannosyltransferases responsible for the formation of mannan outer chain. Such an inhibitor might still be non-specific when assayed in vitro. However, mannosyltransferases found in vesicles and especially those requiring lipid involvement (Lehle et al, 1977; Marriott and Tanner, 1979) would not be affected by a periplasmic inhibitor in vivo. Precise information on the exact location and mode of action of the inhibitor must await purification of individual components involved in mannan synthesis. Attempts already made to purify the inhibitor were unsuccessful due to its lability at low temperature; further studies will require some means of preventing its proteolytic destruction.

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IV. CONCLUSIONS ON YEAST CELL-WALL BIOGENESIS

The results of this study suggest that mannosyltransferase activity is regulated by two mechanisms which may, eventually, prove to be inter-related. Firstly, the soluble nucleotide pool appears to function in the regulation of mannosyltransferase activity (section II) as well as participating in the control of glucan synthase activity (Shematek and Cabib, 1980). Secondly, mannan synthesis is subject to inhibition by a soluble proteinaceous inhibitor, perhaps located in the periplasm (section III). This final section represents an attempt to integrate the results in the study into a generalised scheme of cell-wall formation which involves synthesis of all three polysaccharide components, glucan, chitin and mannan. A diagrammatic representation of cell-wall formation is illustrated in figure 28. In this model, existing cell-wall polymers at the region of growth are hydrolysed to allow the insertion of newly-synthesised material. Wall-lytic enzymes, glucan synthase and chitin synthase zymogen are delivered to the plasma membrane by vesicles. Mannosyltransferases are already active in the rough and smooth endoplasmic reticula; the mannan products are conveyed to growing regions of the wall and synthesis is completed at the cell surface by ectomannosyltransferases. For simplicity, only one protease has been included in the figure. This is the vacuolar, chitin synthase activating factor (Cabib et al, 1973).

Evidence from studies of cell-wall synthesis in several fungi indicates that activation-inactivation processes represent a principal mechanism by which fungal morphogenesis is regulated. While Farkaš (1979) has suggested how these processes might control biosynthesis of the skeletal polysaccharides, glucan and chitin, it now seems that the amorphous mannan matrix is also subject to an inhibition-activation

Figure 28.   Illustrative representation of the formation of a

primary growth region in a pre-existing cell wall.

Adapted from diagrams by Rose (1976) and Farkaš (1979).

 mannan matrix   wall-lytic enzymes

 glucan, chitin   mannosyltransferase

microfibrils   inhibitors


 chitin synthase activating factor

 activating factor inhibitor

 active

polysaccharide synthases

 inactive

 chitin synthase activating factor -

inhibitor complex

CELL WALL

PERIPLASMIC SPACE

PLASMA MEMBRANE

CYTOPLASM

WITH VESICLES
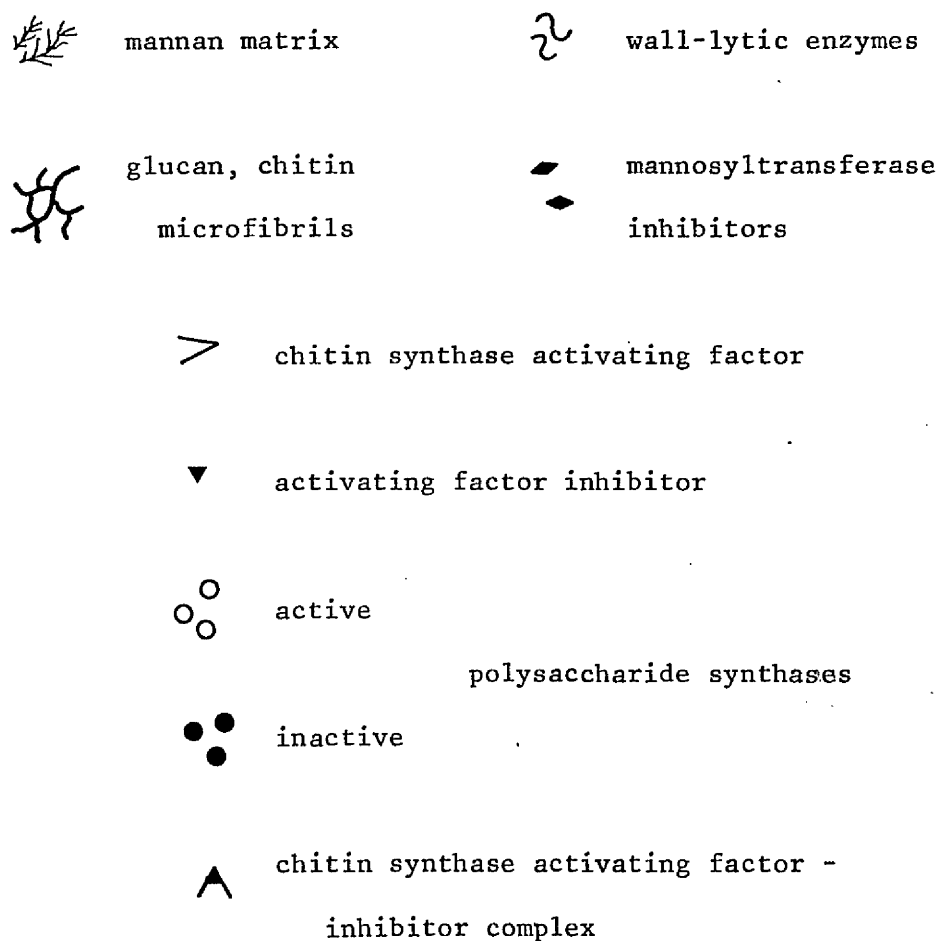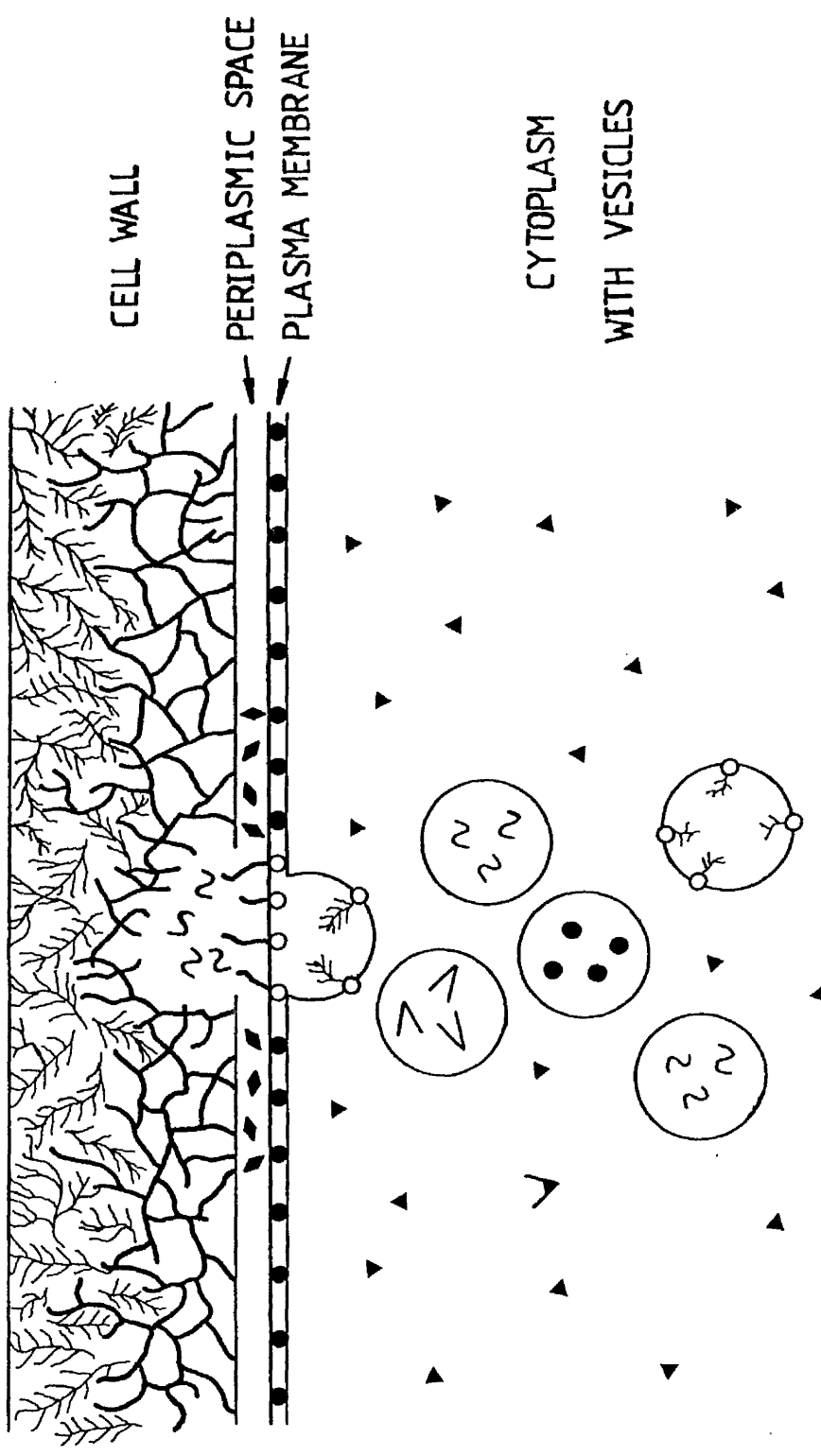
mechanism of control. Although proteolytic activation of the chitin synthase zymogen in S. cerevisiae and other fungi is now well established, zymogenicity of glucan synthase or mannosyltransferases in S. cerevisiae has not yet been shown.

The mechanism by which activation of polymer synthesis is regulated is still poorly understood. Mannosyltransferases appear to be already active on intracellular membrane-bound vesicles and glycosylation of mannan commences, on the rough endoplasmic reticulum, before the protein moiety is fully synthesised. Maturation of the mannoprotein is completed by ectomannosyltransferases at the cell surface. In mannan synthesis, therefore, there is no proteolytic activation of transferases although it is now proposed that synthesis is terminated by the periplasmic, mannosyntransferase inhibitor. Glucan synthase, on the other hand, is incorporated into the plasma membrane in an active form, presumably transported there by vesicles. Since no protein inhibitors or activators of glucan synthase have been reported and the enzyme shows a low turnover rate, rapid modulation may be effected by ATP and GTP levels. Location of the chitin synthase zymogen is still disputed. However it seems likely that it is found both in the plasma membrane (Cabib et al, 1979) and in chitosomes (Bartnicki-Garcia et al, 1979).

Activation of synthases may occur by proteolysis, as for chitin synthesis or by loss of cell wall – plasma membrane contact (Farkaš, 1979). The localised dissolution of wall polymers at the growth site has been considered by Farkaš (1979) to cause a loosening of the cell wall – plasma membrane contact thus activating 'cryptic' polysaccharide synthases located in the plasma membrane. The dissolution of polymers might be caused by β-glucanases, mannanases and chitinases delivered by vesicles guided to a particular growth region. Such a

mechanism would imply that the lipid and protein composition of tonoplast and plasma membrane should be compatible to allow reverse pinocytosis of vesicles at the growth point.

All three polysaccharide synthases are membrane-bound and glucan and chitin synthases have been found on the cytoplasmic face of the plasma membrane (Durán et al, 1975; Shematek et al, 1980). Enzyme activity is therefore subject to control by cytoplasmic factors such as soluble nucleotides. In the present study, ATP and UTP stimulated mannosyltransferase activity while ATP and GTP are known to enhance glucan synthase activity (Shematek and Cabib, 1980). By binding to the transferases, perhaps allosterically, the nucleotides could control the activity of these enzymes. In turn, the nucleotide levels could be controlled by release of appropriate nucleoside triphosphatases from a vesicular location (Wiemken et al, 1979). In addition, the cytoplasm contains an inhibitor of the chitin synthase activating factor. This inhibitor has been suggested by Cabib (1975) to function as a safety device to trap any activating factor that might be released from vesicles into the cytoplasm. This would prevent non-specific activation of the chitin synthase zymogen in the plasma membrane. The exact location of the mannosyltransferase inhibitor in vivo is not yet known, although it is speculated that it may be found in the periplasm to prevent synthesis of the outermost parts of mannan molecules.

Inactivation of polysaccharide synthases, at regions where growth is not required, could be achieved by several mechanisms. Firstly, synthases could be inactivated by prolonged exposure of the zymogen to an activating protease as suggested by Hasilik (1974) for chitin synthesis in S. cerevisiae. Secondly, non-zymogenic synthases could simply be destroyed by gradual proteolysis. Thirdly, periplasmic

inhibitors may act on certain mannosyltransferases. Fourthly, in the absence of cell-wall polymer hydrolases, the cell wall - plasma membrane contact could prevent activation of cryptic polysaccharide synthases.

Much information is still required before a precise picture of cell-wall biogenesis is obtained. However, the results described here provide evidence for two mechanisms involved in the regulation of mannan synthesis. These further illustrate the important role played by cellular compartmentalisation in the ordered deposition of cell-wall components.

APPENDICES

I. BUFFER SOLUTIONS

	Buffer	Composition	pH range	Reference
1)	Tris	Tris(hydroxymethyl)amino-methane and HCl	7.1–8.9	Bates and Bower (1956)
2)	Citrate	Citric acid and trisodium citrate	3.0–6.2	Dawson et al (1969)
3)	Maleate	Sodium hydrogen maleate and NaOH	5.2–6.8	Mertz and Owen (1940)
4)	Imidazole	Imidazole and HCl	6.2–7.8	Temple (1929)
5)	Hepes	Sodium N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonate and NaOH	6.6–8.6	BDH Chemical Co. Ltd. (Biochemical tables)
6)	Borate	Sodium tetraborate and HCl	8.1–9.0	Bates and Bower (1956)

II. COMPOSITION OF REAGENTS

A. FEHLING'S SOLUTION REAGENTS (Dawson et al, 1969)

Solution A: 34.65 g $CuSO_4.5H_2O$ dissolved in water and diluted to 500 ml.

Solution B: 173 g sodium potassium tartrate and 125 g KOH dissolved in water and diluted to 500 ml.

Equal volumes of solutions A and B were mixed immediately before use.

B. REAGENTS FOR PROTEIN ESTIMATION (Lowry et al, 1951)

Solution A: 2% (w/v) Na_2CO_3 in 0.1 M NaOH

Solution B: 0.5% (w/v) $CuSO_4 5H_2O$ in 1% (w/v) sodium potassium tartrate

Solution C: 50 ml of reagent A mixed with 1 ml of reagent B. Renewed daily.

Solution D: Folin-Ciocalteau reagent diluted 1:1 (v/v) daily with water.

To samples (0.6 ml) containing 25-100 μg of protein, 3 ml of solution C was added, mixed well and allowed to stand 10 min at room temperature. 0.3 ml of solution D was then added rapidly with immediate mixing. After 30 min, the absorbance at 750 nm was read using 1 cm cuvettes.

C. ALKALINE SILVER NITRATE REAGENT DIP (Trevelyan et al, 1950)

Solution A: 0.1 ml of saturated aqueous solution of $AgNO_3$, diluted to 20 ml with acetone and water added dropwise until $AgNO_3$ redissolves.

Solution B: 0.5 M NaOH in aqueous ethanol, made by diluting 40% (w/v) NaOH with ethanol before use.

Solution C: 5% (w/v) sodium thiosulphate.

Chromatograms were passed through solution A, allowed to dry and the procedure repeated. They were then passed through solution B until black spots of silver, due to reducing substances, appeared on a brown silver oxide background. Papers were finally passed through solution C to remove excess silver oxide, and washed with water.

D. PERCHLORIC ACID - MOLYBDATE SPRAY (Hanes and Isherwood, 1949)

Spray: 60% (v/v) perchloric acid (5 ml), 1 M HCl (10 ml) and 4% (w/v) ammonium molybdate (25 ml), made up to 100 ml with water.

Chromatograms were sprayed and then heated at 100°C, until just dry. Glucose-1-phosphate gives a yellow spot while mannose-1-phosphate develops a blue spot after exposure to ultraviolet light for 10-15 min.

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