# BIOSYNTHESIS OF MANNAN IN *SACCHAROMYCES CEREVISIAE*. ISOLATION OF A LIPID INTERMEDIATE AND ITS IDENTIFICATION AS A MANNOSYL-1-PHOSPHORYL POLYPRENOL*

R. SENTANDREU** and J.O. LAMPEN

*Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903, USA*

Received 31 August 1972

## 1. Introduction

Glycosylated lipids have been shown to be immediate biosynthetic precursors of a variety of bacterial polysaccharides, e.g., *Salmonella* O-antigen [1], cell wall peptidoglycan [2], and the mannan of *Micrococcus lysodeikticus* [3]. These lipid intermediates have been characterized as phosphoryl [3] or pyrophosphoryl derivatives [1, 2] of $C_{50}$ to $C_{60}$ polyprenols.

Eukaryotic cells contain dolichols which are $C_{70}$ to $C_{100}$ *cis–trans* polyprenols with a saturated unit at the hydroxyl end of the molecule. Behrents et al. [4] have shown that dolichols can also participate in transfer reactions leading to synthesis of lipid-linked monosaccharides.

Tanner [5] presented evidence that a lipophilic mannosyl derivative might be the immediate precursor of yeast mannan, and later [6] showed that dolichols from yeast or liver can serve as mannosyl acceptors in a particulate system from yeast. We reported previously [7] that mannan is synthesized from GDP-mannose through a lipid intermediate by a particulate preparation from *S. cerevisiae*. We now describe some kinetic aspects of the reaction leading to the synthesis of mannan, the purification of the lipid intermediate and its identification as a mannosyl-1-phosphoryl polyprenol.

* Supported in part by USPHS Grant AI-04572.

** R.S. was a Visiting Fellow under the Mutual Education and Cultural Exchange Act (The Fulbright Hays Act). Present address: Department of Microbiology, University of Salamanca, Salamanca, Spain.

## 2. Materials and methods

Reagents, growth conditions and preparation of the particulate membrane fraction have been described [7]. Mannosyl transfer was assayed as stated with each experiment. The reaction was usually stopped by adding 1 vol of cold 10% TCA. After 30 min at 0°, the precipitates were collected on glass fiber discs, washed with cold 5% TCA, dried and the radioactivity counted (total incorporation).

Lipids were separated by thin-layer chromatography on Silica Gel G plates using the following solvent systems: 1) chloroform–methanol–water (65:25:4); 2) isopropanol–50% aqueous acetic acid (100:7); 3) 1-butanol–acetic acid–water (3:1:1) and 4) chloroform–methanol–28% ammonium hydroxide (65:35:5). Lipid spots were visualized by charring [8] or spraying with a reagent consisting of 0.5 g *p*-anisaldehyde and 0.5 ml conc. sulfuric acid in 9 ml of 90% ethanol [9]. Radioactive lipids were localized by scraping 0.5 cm strips of the adsorbent layer into scintillation vials.

## 3. Results and discussion

The kinetics of mannose incorporation from GDP-[$^{14}$C]mannose into endogenous acceptor by the particulate preparation have already been described [7]. Some of the incorporated radioactivity could be extracted by chloroform–methanol (2:1) (GDP-mannose is not extracted). Formation of this material was linear

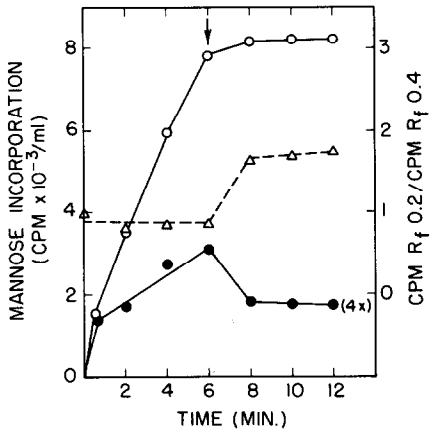


Fig. 1. Transfer of mannose from GDP-[$^{14}$C]mannose into lipid fractions. Particulate preparation (48 mg protein), 0.5 mmoles Tris-maleate buffer pH 6.8, 50 $\mu$moles mercaptoethanol in a final volume of 8 ml were incubated for 10 min at 30°. GDP-[$^{14}$C]mannose (200 nCi, 1.32 nmoles) was added and samples taken as indicated. At (↓) unlabelled GDP-mannose (200 $\mu$moles) was added and incubation continued. At the indicated times samples were treated with 20 vol. 5% TCA for measurement of total incorporation. Other samples were extracted with 50 vol. $CHCl_3$–MeOH (2:1) at 28° for 2 hr; the organic extracts were washed and evaporated. The residues were dissolved in $CHCl_3$ and the radioactivity determined (total lipid fraction). The radioactive lipids were separated by thin-layer chromatography using Silica Gel G and solvent 1. (○—○—○), Total incorporation; (●—●—●), total lipid fraction (4 × cpm); (△—△—△), ratio of radioactivity at $R_f$ 0.2/radioactivity at $R_f$ 0.4.

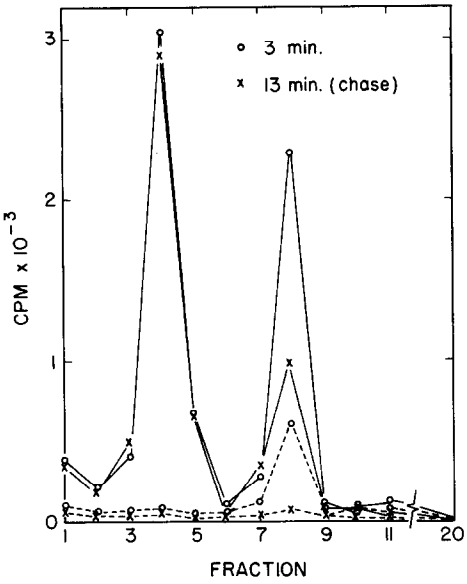


Fig. 2. Alkaline ethanolysis of lipids labelled by transfer from GDP-[$^{14}$C]mannose. Particulate preparation (240 mg protein) and 3.3 nmoles (0.5 $\mu$Ci) of GDP-[$^{14}$C]mannose in 60 ml of Tris-maleate buffer–$MnCl_2$–mercaptoethanol were incubated 3 min at 30°. A sample (30 ml) was withdrawn for analysis, and unlabelled GDP-mannose (400 nmoles) added to the remaining mixture which was incubated an additional 10 min. The samples were heated at 100° for 2 to 3 min to stop the reaction, and the lipids extracted with 600 ml $CHCl_3$–MeOH (2:1) at 28° for 45 min. The organic extracts were washed with saline and Folch upper phase, and fractionated on silicic acid as in the preparation of the lipid intermediate (see text). The $CHCl_3$–MeOH eluates were analyzed by thin-layer chromatography before (solid lines) and after (broken lines) alkaline ethanolysis [10].

with time in a prolonged experiment. After short incubation (fig. 1), part of the radioactivity could be chased out of the fraction by addition of cold GDP-mannose to the reaction mixture. The kinetics of mannose incorporation into this crude lipid fraction are not those expected if a single compound were being formed or for a lipid(s) acting as a true intermediate in mannan synthesis.

Crude lipid fractions obtained by extraction at intervals before and after addition of cold GDP-mannose were spotted on Silica Gel G plates which were developed with solvent 1. Two radioactive spots of $R_f$ 0.2 and $R_f$ 0.4 were found in all samples. The ratio of the radioactivity of the slow and fast moving spots ($R_f$ 0.2/$R_f$ 0.4) increased after the chase (fig. 1), implying that the material with $R_f$ 0.4 was heterogeneous and that the mannosyl moiety of one or more of its components could be chased into the mannan fraction.

Attempts to improve the fractionation by using other solvent systems were unsuccessful. Solvent 2 gave only a single radioactive spot; solvents 3 and 4 gave two spots with different $R_f$ values than with solvent 1, but the same ratio of radioactivity. The lipid mixtures were, therefore, adsorbed on a silicic acid column and eluted with chloroform, acetone, and finally chloroform–methanol (1:1). The radioactivity was recovered in the last solvent which elutes phospholipids [7].

Since some of the radioactive lipids formed were probably mannosyl phosphatides, we tried to remove

these by mild alkaline ethanolysis [10]. Under these conditions pyrophosphoryl linkages are cleaved but phosphodiester linkages are not affected. Samples taken during incubation with GDP-[$^{14}$C]mannose, then deacylated [10] and chromatographed gave only one radioactive spot of $R_f$ 0.4. Samples obtained after the chase and treated similarly had no detectable radioactivity (fig. 2). Thus the particulate preparation incorporated mannose into at least three lipids; one of them runs at $R_f$ 0.2, is cleaved by alkaline ethanolysis, and is not chased by GDP-mannose. The other two are in the $R_f$ 0.4 spot; one is readily cleaved, the other is alkali stable and its label can be chased into the mannan fraction. The lipid intermediate which participates in mannan synthesis by *M. lysodeikticus* is also alkali-stable [3].

Our interest was thus focused on the lipid ($R_f$ 0.4) stable to alkaline ethanolysis. We found that the yeast preparation has a small pool of lipid which saturates with [$^{14}$C]mannose almost instantaneously, before the incorporation of radioactive mannose into mannopeptides. This manno-lipid turns over rapidly since addition of cold GDP-mannose produced a rapid disappearance of label from the fraction and a concomitant increase in radioactivity associated with mannopeptides [7].

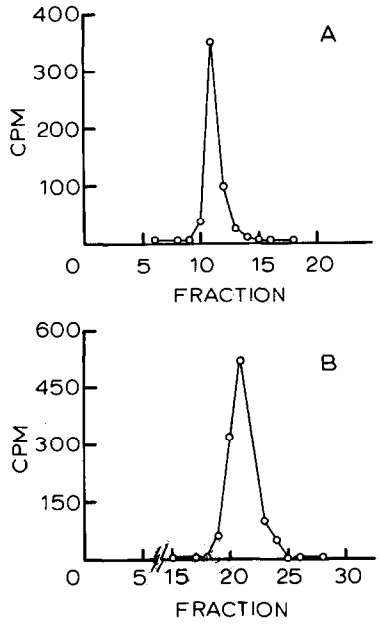


Fig. 3. Purification of the lipid intermediate from *S. cerevisiae* LK2G12. A) Elution pattern from DEAE-cellulose of the radioactive lipid purified by alkaline ethanolysis and silicic acid chromatography (details in text). B) Elution pattern from Sephadex LH-20 of the radioactive lipid from DEAE-cellulose chromatography (fractions A 11 and 12).

### 3.1. *Preparative purification of the lipid intermediate*

The particulate preparation from 70 g wet weight of yeast was made up to 120 ml with buffer and incubated 10 min at 30°. GDP-[$^{14}$C]mannose (500 nCi) was added with thorough mixing and the reaction halted after 30 sec by immersing the mixture in a boiling bath for 2 min. Lipids were extracted at 30° with 2.5 l chloroform–methanol (2:1) for 45 min on a gyratory shaker. The suspension was filtered and washed with saline (0.2 vol) and with 0.2 vol of Folch upper phase [11]. Two parallel incubations were carried out using non-radioactive GDP-mannose.

The crude lipid preparation (1.75 × $10^5$ cpm) was applied to a silicic acid column (2 cm × 10 cm) and eluted with chloroform (5 vol), acetone (5 vol), and (1:1) chloroform–methanol (10 vol). The chloroform–methanol eluates from the radioactive and the two non-radioactive incubations were combined and taken to dryness. The residue was redissolved in carbon tetrachloride and the lipids deacylated according to Dawson [10]. The organic phase was applied to a silicic acid column (1:1) as before. Radioactivity in the chloroform and acetone eluants was neglible. The

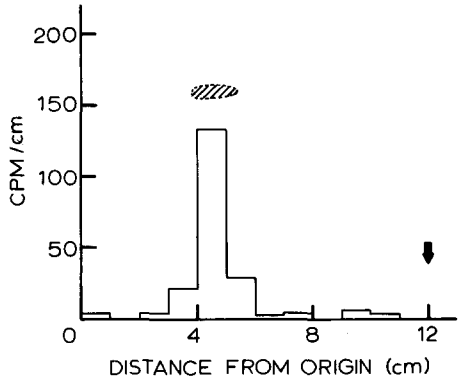


Fig. 4. Thin-layer chromatography of the purified lipid intermediate. Fractions B21 and 22 (fig. 3) were pooled and streaked on duplicate Silica Gel G plates which were developed with solvent 1 (front shown by ↓). One plate was used to determine distribution of radioactivity; the second was developed with the *p*-anisaldehyde-sulphuric acid reagent [9].

chloroform–methanol eluant contained $8.6 \times 10^4$ cpm.

The chloroform–methanol fraction was applied to a DEAE-cellulose column (2 cm × 30 cm) prepared according to Rouser et al. [11], and eluted with 500 ml of methanol, then chloroform–methanol (1:1) containing 10 μmoles ammonium acetate and 1 ml of conc. $NH_4OH$ per liter; 20 ml fractions were collected (fig. 3A). Fractions 11 and 12 were combined, desalted by washing with water ($4.4 \times 10^4$ cpm), and the solvent removed. The residue was dissolved in 2 ml chloroform–methanol (1:1) containing 50 mM ammonium acetate and applied to a Sephadex LH-20 column (2.5 cm × 26 cm) which was eluted with the same mixture (flow rate: 0.5 ml per minute; fraction size: 2 ml). The single radioactive peak (fig. 3B) contained $2.9 \times 10^4$ cpm.

The peaks obtained from DEAE-cellulose and Sephadex LH-20 were almost symmetrical; nevertheless, only the two central fractions from each were pooled to avoid any contaminants present in the leading or trailing edges.

The purified lipid intermediate was homogeneous by chromatography on Silica Gel G with solvents 1 (fig. 4) or 4. The single radioactive spot was coincident with an elongated spot visualized by charring or by *p*-anisaldehyde-sulphuric acid (olive-green color). The latter reagent is semi-specific for prenoid alcohols [9].

Although the intermediate resists alkaline ethanolysis, it is acid-labile. Total hydrolysis (2 N HCl, 100°, 2 hr) released a single radioactive compound that chromatographed on paper in solvent 1 as authentic mannose. Mild acid hydrolysis (0.1 N HCl, 100°, 20 min) rendered all the label water-soluble. The ratio of total mannose [12] to total phosphorus [13] in the aqueous phase after mild hydrolysis was 1.27:1.0. Thus, the lipid intermediate contains single phosphate and mannose groups.

The acid lability of the intermediate shows that the hydrophilic portion is a mannosyl-1-phosphate unit and the relative stability to alkali points to a phosphoryl rather than a pyrophosphoryl linkage to the lipid. These results together with the information obtained by the reaction with *p*-anisaldehyde indicate that the lipid intermediate is a phosphodiester of mannose and an isoprenoid alcohol.

The precise structure of the lipophilic moiety of the intermediate and the nature of the other manno-lipids formed by the membrane preparation are being investigated.

## Acknowledgement

We thank Mrs. J.O. Vensel for skilled technical assistance.

## References

[1] A. Wright, M. Dankert, P. Fennessey and P.W. Robbins, Proc. Natl. Acad. Sci. U.S. 57 (1967) 1798.
[2] Y. Higashi, J.L. Strominger and C.C. Sweeley, Proc. Natl. Acad. Sci. U.S. 57 (1967) 1878.
[3] M. Scher, W.J. Lennarz and C.C. Sweeley, Proc. Natl. Acad. Sci. U.S. 59 (1968) 1313.
[4] N.H. Behrens, A.J. Parodi, L.F. Leloir and C.R. Krisman, Arch. Biochem. Biophys. 143 (1971) 375.
[5] W. Tanner, Biochem. Biophys. Res. Commun. 35 (1969) 144.
[6] W. Tanner, P. Jung and N.H. Behrens, FEBS Letters 16 (1971) 245.
[7] R. Sentandreu and J.O. Lampen, FEBS Letters 14 (1971) 109.
[8] T. Ziminski and E. Borowski, J. Chromatog. 23 (1966) 480.
[9] G.P. McSweeney, J. Chromatog. 17 (1963) 183.
[10] R.M.C. Dawson, in: Lipid Chromatographic Analysis, Vol. 1, ed. G.V. Marinetti (M. Dekker, New York, 1967) p. 163.
[11] G. Rouser, G. Kritchevsky, A. Yamamoto, G. Simon, G. Galli and A.J. Bauman, in: Methods in Enzymology, Vol. XIV, ed. J.M. Lowenstein (Academic Press, New York, 1969) p. 272.
[12] R.J. Winzler, Meth. Biochem. Anal. 1 (1954) 279.
[13] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466.