PARTICIPATION OF A LIPID INTERMEDIATE IN THE BIOSYNTHESIS OF *SACCHAROMYCES CEREVISIAE* LK2G12 MANNAN*

R. SENTANDREU** and J.O. LAMPEN
Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903, USA

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1. Introduction

One of the most interesting developments in the study of the synthesis of complex polysaccharides has been the discovery of the role of polyprenoid derivatives. These compounds function as intermediate acceptors of carbohydrate residues transferred from their activated form (sugar nucleotides) and subsequently passed on to the final acceptor.

The existence of such lipid intermediates was postulated in the synthesis of Salmonella O-antigen [1, 2] and of bacterial peptido-glycan [3]. The intermediates were later isolated from both enzyme systems in sufficiently pure form that their lipid moieties could be identified as C_{55} polyprenoid alcohols [4, 5]. Two of the internal double bonds are trans and the remaining eight are cis [6, 7].

The existence of lipid intermediates has also been shown or postulated in several eukaryotic cell types [8–10]. Behrens and Cabib [11] studied the synthesis of yeast mannan, one of the major polymers of the cell wall, and were unable to detect a lipid-like intermediate. Ankel et al. [12] described a particulate preparation from *Cryptococcus laurentii* which transferred radioactive mannose from GDP-mannose-^{14}C into a lipid fraction. They studied the kinetics of the transfer and found no evidence for a true lipid intermediate. Tanner [13] has presented evidence that in yeast, as in other systems [1–10], a lipophilic mannosyl intermediate might be the immediate precursor of mannan.

We report that mannan is indeed synthesized from GDP-mannose through a lipid intermediate by particulate membrane preparations from yeast (*Saccharomyces cerevisiae*). These preparations also incorporated mannose into a phospholipid fraction by a process apparently independent of the lipid intermediate.

2. Materials and methods

GDP-mannose-^{14}C was bought from New England Nuclear. Whenever possible, other reagents were of analytical grade.

Saccharomyces cerevisiae LK2G12 was grown in Winge's medium (yeast extract, 3 g; glucose, 20 g; water, 1 litre) on a gyratory shaker to early exponential phase. The washed cells were suspended in 50 mM tris-maleate buffer pH 6.8–5 mM $MnCl_2$–1 mM mercaptoethanol and broken in a Braun Model MSK mechanical cell homogenizer (1 g wet weight cells/ml). The suspension was centrifuged at 10,000 *g* for 10 min and the resulting supernatant spun at 39,000 *g* for 45 min. This pellet (particulate membrane preparation) was homogenized and used as such for all experiments.

The enzyme preparation was incubated in the presence of GDP-mannose-^{14}C as indicated for the individual experiments. The radioactive lipids formed were usually extracted by shaking the mixture with 20 volumes of chloroform–methanol (2:1) at 28° for 30 to 60 min. The extraction was conducted in a gyratory shaker to avoid sedimentation of the particles.

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** R.S. was a Visiting Fellow under the Mutual Education and Cultural Exchange Act (The Fulbright-Hays Act). Present address: Instituto de Biologia Celular, C.S.I.C., Madrid, Spain.

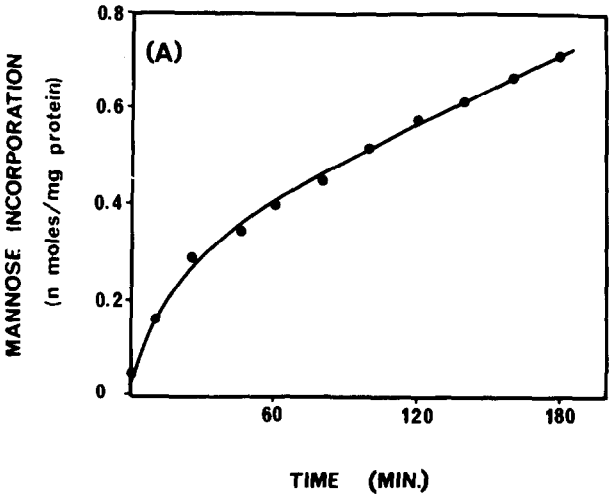

Fig. 1. Incorporation of mannose from GDP-mannose-^{14}C into endogenous acceptors. Time course of the reaction. Reaction mixtures containing, per ml, 50 μmoles tris-maleate buffer pH 6.8, 5 μmoles $MnCl_2$, 1 μmole mercaptoethanol and particulate preparation (132 μg protein) were preincubated 10 min at 30°. GDP-mannose-^{14}C (0.53 nmoles; 80 nCi) was then added. Samples (200 μl) were withdrawn at the indicated times and mixed with 600 μl 10% TCA. The resulting precipitates were collected and the radioactivity determined with a Packard Tri-Carb scintillation spectrometer.

Hydrolyses were carried out in 2 N HCl at 100° for 2 hr in sealed ampules and the residual acid removed by freeze-drying.

3. Results and discussion

The particulate membrane preparation isolated from broken cells was able to incorporate mannose from GDP-mannose-^{14}C into endogenous acceptor(s). The incorporated radioactivity can be determined by collecting the particulate preparation on a glass fiber disc or by precipitation with alcohol or 5% trichloroacetic acid (TCA).

Under the conditions of assay, activity was still detectable up to 180 min (fig. 1). Almost all the radioactivity incorporated by the particulate preparation (TCA precipitate) remained at the origin when aliquots of the reaction suspension were streaked on a paper chromatogram and run in either solvent 1 (ethyl acetate–pyridine–water; 8:2:1) or solvent 2 (ethyl acetate–butanol–water–acetic acid; 3:4:4:2.5).

More than 95% of the radioactivity released from the particulate preparation by hydrolysis ran in solvent 1 in the position of authentic mannose (fig. 2).

A small amount of the radioactivity incorporated by the particulate preparation and precipitable by TCA is soluble in chloroform–methanol (2:1). It is known that polar lipids are coprecipitated with proteins by TCA, and their solubilization can only be achieved in solvents like methanol or ethanol which break the association between lipids and polypeptides [14]. For this reason lipids were routinely extracted from the membrane preparation with chloroform–methanol (Materials and methods). When the radioactivity extracted into the organic solvents was released by hydrolysis, it also travelled in solvent 2 like mannose.

The lipid materials obtained before and after chase of the incorporated radioactivity with cold GDP-mannose were fractionated in a silicic acid column. The column was eluted with chloroform, then acetone and finally chloroform–methanol (1:1). The bulk of the radioactivity was recovered in the last solvent which elutes polar lipids (table 1). This rules out the possibility that some of the mannose-^{14}C had been incorporated as mannosyl-diglycerides or dimannosyl-diglycerides as these would have been eluted by acetone.

It was possible that some of the lipids formed by the particulate preparation and eluted from the silicic acid column by chloroform–methanol were phosphatides, because a large percentage of the radioactivity incorporated during incubation from GDP-mannose-^{14}C was not chased by excess of the cold sugar nucleotides (table 1). We tried therefore to remove any such phosphatides by mild alkaline alcoholysis. Samples obtained before and after the chase with cold GDP-mannose were treated as described by Dawson [16]. Alkaline ethanolysis of the samples obtained during incubation with GDP-mannose-^{14}C gave some alkali-stable radioactive material. Similar treatment of radioactive lipids extracted after the chase gave no detectable radioactivity. This result indicates that two or more lipids are formed when the particulate preparation is incubated with GDP-mannose. One at least seems to be resistant to the deacylation, and its radioactivity can be chased with cold GDP-mannose. The lipid inter-

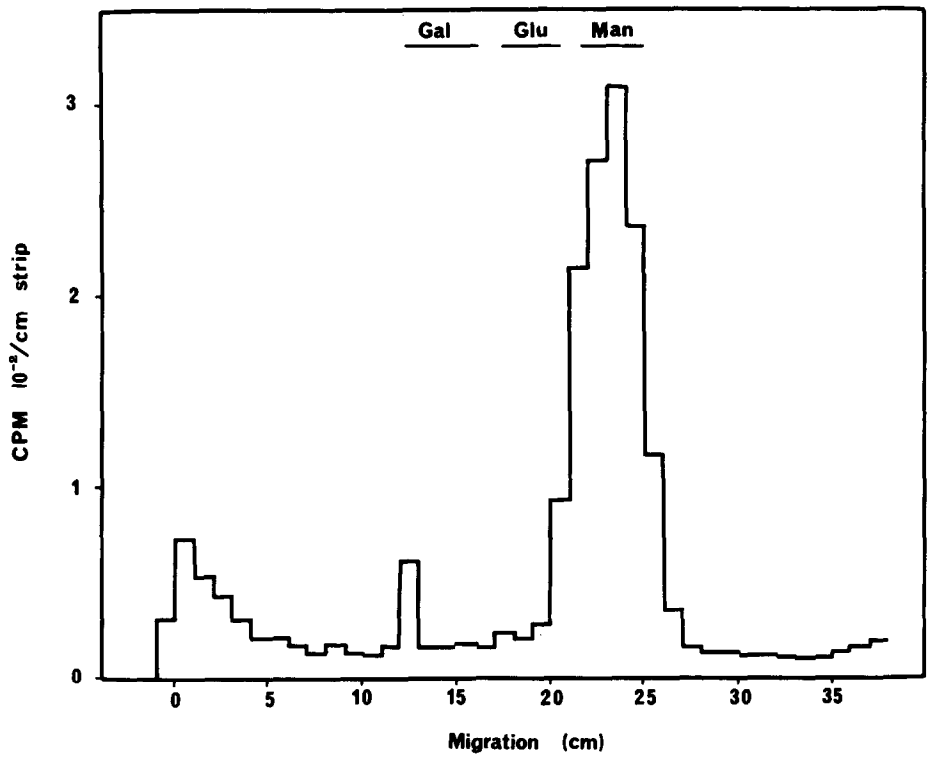

Fig. 2. Histogram of the radioactivity released by hydrolysis from the membrane preparation after incubation with GDP-mannose-^{14}C. Experimental conditions were similar to those described in fig. 1, and hydrolysis of the product was carried out as described in Materials and methods. The radioactive residue was dissolved in water and passed through short columns of Dowex AG 50 W X 8 H^+ form and Dowex AG 1 X 8 formate form. The resulting solution was freeze-dried and samples chromatographed with added galactose, glucose and mannose (as standards) on Whatman no. 1 paper in solvent 1.

Table 1
Silicic acid column chromatography of the lipids formed by the particulate membrane preparation.

Substrate	Total radioactivity in crude lipid (cpm)	Eluate (cpm)			Recovery (%)
		$CHCl_3$	CH_3COCH_3	CM* (1:1)	
GDP-mannose-^{14}C (3.3 nmoles)	22,800	24	95	17,258	75.9
GDP-mannose (chase) (400 nmoles)	15,800	32	76	12,358	78.4

Particulate preparation (240 mg protein) and 3.3 nmoles (0.5 μCi) of GDP-mannose-^{14}C in 60 ml of tris-maleate buffer–$MnCl_2$–mercaptoethanol (as fig. 1) were incubated at 30° for 3 min. A sample (30 ml) was withdrawn and the incorporated mannose-^{14}C chased by adding 400 nmoles of unlabelled GDP-mannose to the remaining mixture and incubating an additional 10 min. The reaction was stopped by heating the samples at 100° for 2 to 3 min, and the lipids extracted with 600 ml chloroform–methanol (2:1) at 28° for 45 min. The organic extracts were washed with 0.9% NaCl (0.2 vol) and Folch upper phase (0.2 vol), taken to dryness in a rotary evaporator at room temperature, and redissolved in chloroform. This crude lipid preparation was fractionated on a silicic acid column (1 X 5 cm) which was eluted with chloroform (5 vol), acetone (5 vol) and finally (1:1) chloroform–methanol (10 vol). The elutant solutions were taken to dryness and redissolved in the same solvents for determination of radioactivity.

* Chloroform:methanol

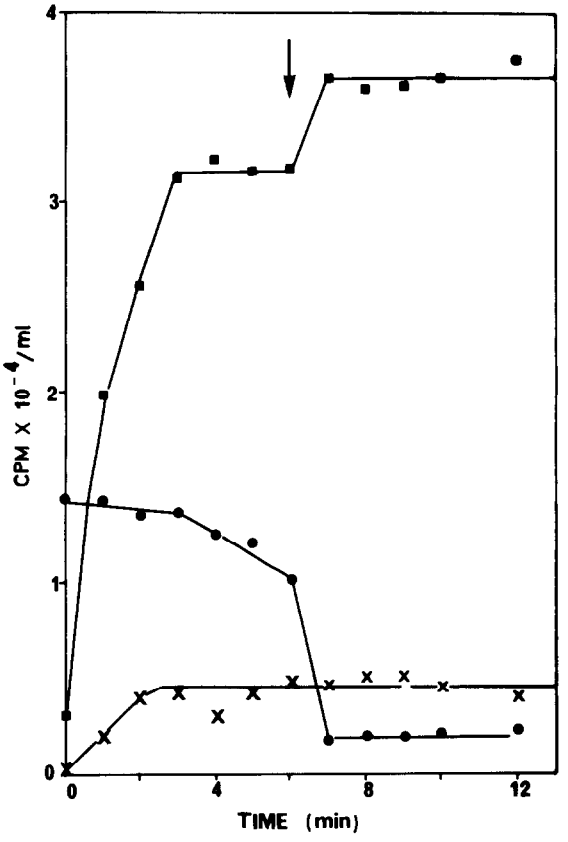

Fig. 3. Formation of lipid intermediate from GDP-mannose-^{14}C and transfer of mannose-^{14}C moiety to mannan. Particulate preparation (240 mg protein) and 3.3 nmoles (0.5 μCi) of GDP-mannose-^{14}C in 60 ml of 50 mM tris-maleate buffer pH 6.8, 5 mM $MnCl_2$, 1 mM mercaptoethanol were incubated at 30°. Samples (4 ml) were withdrawn at one minute intervals. At 6 min (arrow) 400 nmoles of GDP-mannose were added to the remaining suspension and samples again withdrawn. The reaction was terminated and crude lipid preparations obtained by the procedure described in table 1. Aliquots were subjected to alkaline ethanolysis (Dawson [15]) and the residual (alkali-stable) radioactivity left in the organic phase was determined. ●——● Alkali-stable lipid fraction (lipid intermediate); X —— X alkali-labile lipid fraction; ■——■ mannan formed by the particulate preparation. Determined in aliquots of the original suspension (TCA precipitable radioactivity – total lipid fraction).

mediate that participates in mannan synthesis by *Micrococcus lysodiekticus* is also alkali-stable [6].

To determine if the alkali-stable mannose containing lipid(s) was acting as an intermediate in the synthesis of mannan, the kinetics of incorporation of mannose from GDP-mannose-^{14}C into the 'lipid intermediate', alkali-labile fraction, and mannan was followed (fig. 3). Labeling of the 'lipid intermediate' preceded incorporation of radioactivity into mannan; its concentration is small and it showed an almost instantaneous saturation. When GDP-mannose-^{14}C was no longer present, the 'lipid intermediate' decayed slowly. After addition of excess non-radioactive GDP-mannose, the mannose-^{14}C associated with this fraction decreased; at the same time a comparable increase of radioactivity was detected in the mannan fraction, but not in the alkali-labile phospholipid fraction.

The chase of mannose-^{14}C from the 'lipid intermediate' to mannan by addition of cold GDP-mannose rules out the possibility that GDP-mannose is the immediate precursor of mannan and points to the 'lipid intermediate' as an essential stage in mannan synthesis.

Behrens and Leloir [10] have shown that a microsomal fraction from liver can utilize dolichols (polyprenoids with a chain length of C_{85} to C_{110}) to form the lipid intermediate. The fact that yeast has a mixture of dolichols with chain length of C_{70} to C_{90} [16] suggests that these substances also form part of the lipid intermediate in yeast mannan biosynthesis. The nature of the 'lipid intermediate' and of the other lipids formed by the particulate membrane preparation (apparently without involvement of the intermediate) is being investigated.

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