THE ENZYMIC SYNTHESIS OF YEAST MANNAN

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When guanosine diphosphate mannose (GDP-mannose) was first isolated from yeast, it was proposed that this compound might be the precursor of mannan (Cabib and Leloir, 1954). Evidence supporting this hypothesis will be outlined in the present communication.

Preparation of the extract.-Since mannan is a constituent of the cell wall, a probable site for its synthesis is the cell membrane. Therefore, in an effort to preserve the membrane structure as much as possible, yeast protoplasts were prepared, and then burst by freezing and thawing.

Saccharomyces carlsbergensis (National Collection of Yeast Cultures S 74) was grown in a malt extract medium (Wickerham, 1951) and harvested during the logarithmic phase. Protoplasts were prepared by incubation with snail gut juice, as described by Eddy and Williamson (1959), but using 0.6 M KCl as osmotic stabilizer. A washed protoplast pellet obtained from 6 g. of yeast (wet weight) was resuspended in 0.05 M Tris buffer at pH 7.5 up to a total volume of 6 ml. Complete disruption of the protoplasts was obtained by freezing and thawing the suspension three times.

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This preparation will be called "total extract". Three milliliters of "total extract" were centrifuged at 25,000 x g for 10 minutes, and the pellet was washed twice with 0.05 M Tris buffer at pH 7.5 and resuspended in 3 ml. of the same buffer. This suspension will be referred to as "particulate fraction".

Radioactivity incorporation into polysaccharide fraction.- When GDP-mannose-C^{14} (labeled in the mannose moiety) was incubated with the "total extract" and the reaction mixture was heated to 100° C, the resulting voluminous precipitate was found to be radioactive. After treating the precipitate with hot alkali, most of the radioactivity was solubilized, and could be precipitated with ethanol (fraction A), as shown in Table I. The supernatant liquid from the heated reaction mixture was also treated with ethanol, and a small amount of radioactivity was found in the resulting precipitate (fraction B). The addition of p-hydroxymercuribenzoate (pHMB) stimulated the incorporation, probably by inhibiting enzymes that destroy GDP-mannose. A small enhancement was sometimes noted by adding mannodextrins, but these results were not very reproducible. When the "particulate fraction" was used instead of the "total extract", the distribution of radioactivity was somewhat different, fraction B being more labeled than in the previous case. This result might be due to a less extensive entrainement of the polysaccharide by the smaller precipitate obtained with the "particulate fraction". The total amount of radioactivity was, however, almost the same as with the "total extract", a result to be expected if the enzyme responsible for the incorporation were located in the cell membrane.

Identification of radioactive mannan.-A sample of fraction A was dissolved in water and treated with an alkaline copper reagent, as described by Trevelyan (1952). This procedure, when applied to yeast alkaline digests is specific for mannan and has been used for its quantitative determination (Trevelyan,

TABLE I

Radioactivity Incorporation into Polysaccharide Material

The incubation mixture contained: 5.5×10^{-4} M GDP-mannose-C^{14} (0.33 μmole, specific activity 78,000 c.p.m./μmole), 2×10^{-4} M ethylene diaminetetraacetic acid, 2×10^{-3} M $MgSO_4$, 0.05 M Tris at pH 7.5, and enzyme from 500 mg. of yeast (wet weight) in a total volume of 0.6 ml. Incubation was carried out at 30° C for two hours with gentle shaking. After incubation, 1.5 ml. of water was added, the tubes were heated at 100° C for 2 minutes and then centrifuged at 30,000 x g for 15 minutes. The precipitate was resuspended in 1.5 ml. of 1.5 N KOH and heated at 100° C for 30 minutes. Insoluble material was removed by centrifugation and two volumes of ethanol were added to the supernatant liquid. The precipitate thus formed was washed with 66% ethanol, plated and counted (fraction A). The supernate from the heating step was treated with three volumes of ethanol and the precipitate was washed and counted (fraction B). The values were not corrected for self-absorption.

Additions	Enzyme	Radioactivity incorporated into ethanol-insoluble fraction (c.p.m.)		
		Fraction A	Fraction B	Total
None	"Total extract"	419	54	473
pHMB, 2.3×10^{-4} M	"	613	82	695
pHMB, 2.3×10^{-4} M +mannodextrins[a], 2.4 mg.	"	687	102	789
GDP-mannose-C^{14} added after incubation	"	7	15	22
None[b]	"Particulate fraction"	442	182	624
Mannodextrins[a], 2.4 mg.	"	459	180	639
Yeast mannan, 4 mg.	"	357	133	490
GDP-mannose-C^{14} added after incubation	"	0	0	0

[a] Prepared by hydrolysis of yeast mannan with fuming HCl and subsequent paper chromatography; degree of polymerization *ca.* 10.

[b] No effect of pHMB was observed with the "particulate fraction".

1952). Repetition of this purification step did not change the specific activity of the polysaccharide (see Table II).

TABLE II

Identification of radioactive mannan

A sample of fraction A (see Table I) was purified by precipitation with Fehling solution, followed by redissolution and precipitation with ethanol as described by Trevelyan (1952). The procedure was repeated once. The purified polysaccharide was hydrolyzed with 2 N sulfuric acid for 5 hours at 100° C and the solution was passed through an Amberlite IR-4B resin column, in the OH^- form. The solution was evaporated and submitted to paper chromatography with butanol-pyridine-water (6:4:3). The paper was cut in segments and eluted. The eluates were counted and that from the radioactive segment was then submitted to paper electrophoresis with 0.05 M sodium borate, and the zone corresponding to mannose was again eluted and counted, after elimination of the borate (Zill et al., 1953). For the specific activity determinations, mannose was measured with the phenol-sulfuric acid reagent (Dubois et al., 1956).

Treatment	Radioactivity c.p.m.	Mannose µmoles	Specific activity c.p.m./µmole
First copper purification	576	18.6	31
Second copper purification[a]	340	11.3	30
Hydrolysis and passage through resin	218	7.6	28.7
Paper chromatography (mannose zone)	155	4.9	31.6
Paper electrophoresis (" ")	108	-	-

[a] A mechanical loss occurred during this step.

After acid hydrolysis and paper chromatography of the product, the only radioactive zone found on paper was that corresponding to mannose. The eluted mannose still had the same specific activity and could be further characterized by electrophoresis with sodium borate (Table II).

Specificity of precursor.-When either mannose 1-phosphate-C^{14} or mannose-C^{14} was substituted for GDP-mannose, the incorporation of radioactivity was small, as shown in Table III. Moreover, addition of cold mannose 1-phosphate to C^{14}-labeled GDP-mannose, did not decrease the amount of incorporation.

This is the first reported case of mannosyl transfer from GDP-mannose.

TABLE III

Specificity of Mannan Precursor

The basic reaction mixture was as in Table I, but with the addition of 2.3×10^{-4} M pHMB and omitting GDP-mannose. The enzyme used was the "total extract" and the polysaccharide was isolated as for fraction A of Table I.

Substrate added	Total c.p.m. added	Incorporation into fraction A	
		c.p.m.	μmoles
GDP-mannose-C^{14}, 0.31 μmole	24,000	890	11.4
GDP-mannose-C^{14}, 0.31 μmole +mannose 1-phosphate, 0.84 μmole	24,000	1,001	12.8
Mannose 1-phosphate-C^{14}, 0.42 μmole	16,000	76	2.0
Mannose-C^{14}, 0.5 μmole	17,500	53	1.5
GDP-mannose-C^{14}, after incubation	24,000	11	
Mannose 1-phosphate-C^{14}, after incubation	16,000	0	

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