Polyprenol Phosphate as an Acceptor of Mannose from Guanosine Diphosphate Mannose in *Aspergillus niger*

By R. M. BARR* and F. W. HEMMING

Department of Biochemistry, University of Liverpool, Liverpool L69 3BX, U.K.

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Growth of *Aspergillus niger* in the presence of [2-¹⁴C]mevalonate and ³²P_i led to the formation of a lipid, containing ¹⁴C (0.14% of dose) and ³²P (0.009% of dose), that had chromatographic properties identical with those of *exo*-methylene-hexahydropolyprenol phosphate. When a particulate enzyme preparation from the thallus of *A. niger* was incubated with GDP-[¹⁴C]mannose, the main radioactive products were mannose 1-phosphate (57% of products) and mannose (18%). In addition radioactive mannan (8%) and a mannosylipid (2%) were formed. The latter was identified as *exo*-methylene-hexahydropolyprenol phosphate mannose on the basis of its chromatographic properties, acid lability and on the increase in formation of the mannosylipid when the phosphate of *exo*-methylene-hexahydropolyprenols was added to the incubation mixture. The phosphates of ficaprenols and cetyl alcohol caused no corresponding increase. These observations are interpreted as evidence that the thallus of *A. niger* contains a mannose transferase that uses the phosphate of *exo*-methylene-hexahydropolyprenols as an acceptor. This situation is discussed in the light of the analogous involvement of a prenol phosphate mannose as an intermediate in the biosynthesis of bacterial wall mannan.

The function of prenol phosphates as lipid acceptors of sugar molecules in the formation of bacterial wall polymers is now well established and has been extensively reviewed (Osborne, 1969). Strong evidence has also been presented for a similar role in glycoprotein biosynthesis (Behrens & Leloir, 1970) and there are reports indicating a similar function in mannan biosynthesis in *Saccharomyces cerevisiae* (Tanner, 1969; Sentandreu & Lampen, 1971) and in *Phaseolus aureus* (Kauss, 1969). In those systems in which the lipid intermediate has been fully characterized, the prenol phosphate involved is a poly-*cis*-polyprenol containing very few *trans*-isoprene residues. The presence of poly-*cis*-polyprenols in the aspergilli (Stone *et al.*, 1967b; Barr & Hemming, 1972) raised the question of whether the phosphates of these prenols have a similar role in polysaccharide biosynthesis in these fungi.

It has been reported that the cell walls of aspergilli contain mannose in polymer form (Crook & Johnston, 1962). By direct analogy with the formation of mannan in *Micrococcus lysodeikticus* (Lahav *et al.*, 1969) the system shown in Scheme 1 is possible. The present paper is concerned with evidence relating to reaction 1 in the scheme.

* Present address: Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Caerns., U.K.

Experimental

Methods

Incorporation of radioisotopes. *A. niger* was grown statically in liquid medium (200 ml) in Roux bottles as described by Barr & Hemming (1972).

In experiment A [U-¹⁴C]mannose (10 μ Ci, 3.7 μ mol) was added to the medium with the spore suspension. The thallus (62 g damp weight) was harvested after 7 days of growth.

In experiment B [2-¹⁴C]mevalonate (10 μ Ci, 0.8 μ mol) (see Barr & Hemming, 1972) was added to the growth medium at the same time as inoculation with the spore suspension. After 6 days of growth the thallus (45 g damp weight) was harvested.

In experiment C ³²P_i (1 mCi, 0.3 mmol), obtained from The Radiochemical Centre, Amersham, Bucks., U.K., was added to the growth medium at the same time as the spore suspension. In this experiment the concentration of KH₂PO₄ in the medium was lowered from 2.5 g/l to 1 g/l. The thallus was harvested after 6 days of growth.

Isolation of materials from the thallus. (a) Mannan. The thallus obtained in experiment A was homogenized in phenol–water (22:3, w/v) with a Polytron homogenizer (Kinematica G.m.b.H., Luzern, Switzerland). The homogenate was stirred for 2 h at 70°C and was then filtered. The filtrate was washed

Scheme 1. Possible scheme for the biosynthesis of mannan in *A. niger*

The scheme is analogous with the system operating in *Micrococcus lysodeikticus* (after Scher *et al.*, 1968).

three times with phenol-saturated water. The aqueous washings, after dialysis against water overnight, were then incubated with ribonuclease (5mg), deoxyribonuclease (2mg) and Pronase (10mg) at 20°C for 30min. The solution was then dialysed against water for 3 days before being freeze-dried, to produce a brown powder (104mg). A portion was taken for measurement of radioactivity and a second portion for acid hydrolysis.

(b) Lipid. The chopped thallus from experiments B and C was homogenized in butan-1-ol (150ml) with a Polytron homogenizer. The suspension was filtered and the residue was extracted twice further with butan-1-ol. The butanol extracts were combined and washed with water (300ml). The butanol solution was then evaporated to dryness with a rotary evaporator.

Lipid fractionation. The lipid (approx. 1g) from experiments B and C was chromatographed on a column (2.5cm×12cm) of silicic acid (50g; 100 mesh; A. R. Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.). After elution of lipids from the column with chloroform (800ml) and then acetone (500ml) a fraction was eluted with chloroform-methanol (1:1, v/v) (see, e.g., Scher & Lennarz, 1969). The solvent flow rate was 200ml/h. This last fraction contained approx. 0.5g of lipid, which was chromatographed on a column (2cm×10cm) of DEAE-cellulose (acetate form) as described by Lahav *et al.* (1969). The column was eluted successively with chloroform-methanol (2:1, v/v; 200ml), methanol (200ml), 7.5mM-ammonium acetate in methanol-water (99:1, v/v; 500ml) and 0.05M-ammonium acetate in methanol-water (99:1, v/v; 500ml). The last two eluents were prepared from a stock solution of 5M-ammonium acetate in methanol containing 3% (v/v) acetic acid. Solvent flow rate was 100ml/h. The volume of the last two fractions was decreased on a rotary evaporator before they were washed with water to remove the ammonium acetate and acetic acid. The chloroform solution was then evaporated to dryness under N₂.

The lipids (approx. 20mg from each fraction) were treated with alkali to deacylate the phospholipids (Dawson, 1967). The lipid was dissolved in chloro-

form-methanol (1:4, v/v; 5ml), and was maintained at 40°C after addition of NaOH (1M; 0.5ml) for 15min. Acetic acid (1M; 0.5ml) was added to the solution followed by chloroform-methanol (9:1, v/v; 10ml), 2-methylpropan-1-ol (5ml) and water (10ml). The mixture was shaken well and was then clarified by centrifugation. The chloroform layer was washed with methanol-water (1:2, v/v; 5ml) before evaporation to dryness under N₂. The lipid (10mg) was then subjected to t.l.c.

Synthesis of polyprenol phosphates. The method of Cramer & Böhm (1959) as modified by Popjak *et al.* (1962) and Behrens & Leloir (1970) was used to phosphorylate a sample of *exo*-methylene-hexahydropolyprenols isolated from *A. niger*, as described by Barr & Hemming (1972). Samples of ficaprenyl phosphate and cetyl phosphate were prepared in the same way. The prenol (5μmol) was dissolved in benzene (1.5ml) and to this was added trichloroacetonitrile (100μl). The solution was left for 15min and bistriethylamine phosphate (0.75mmol, 0.4mCi of ³²P) dissolved in acetonitrile (7.5ml) was added slowly over a period of 30min. The mixture was left for 2h and the addition of benzene, trichloroacetonitrile and bistriethylamine phosphate was repeated. The mixture was then left overnight and the solvent was removed with a rotary evaporator. The residue was dissolved in chloroform-methanol (2:1, v/v; 20ml) and the solution was washed twice with water (10ml). The chloroform solution was evaporated to dryness and was then subjected to chromatography on a column of DEAE-cellulose (acetate form) as described under 'Lipid fractionation'. The prenol phosphate was eluted in the 7.5mM-ammonium acetate fraction and was recovered as described in the same section. The purity of the prenol phosphate was checked by t.l.c. Compounds were located by radioautography and by radio-scanning.

Thin-layer chromatography. Chromatography was done on layers (0.25mm thick) of either Kieselgel G (E. Merck A.G., Darmstadt, Germany) or of cellulose CC41 (Whatman; W. and R. Balston Ltd., Maidstone, Kent, U.K.). With the former adsorbent the solvent systems were A, di-isobutyl ketone-

acetic acid–water (60:45:6, by vol.), or B, chloroform–methanol–water (65:25:4, by vol.). With the latter adsorbent, solvents C, ethyl acetate–butanol–water–acetic acid (6:8:8:5, by vol.), or D, iso-butyric acid–water–aq. NH_3 (sp.gr. 0.88)–0.1 M-EDTA (15:14:1:1, by vol.), were used.

Particulate enzyme preparation. The 3–4-day-old thallus from one Roux bottle was separated from the medium by decantation and was then washed twice with ice-cold water. The thallus (30–40 g) was then cut up finely with scissors and was ground in an ice-cold mortar with quartz sand (150 g; 40–100 mesh; pre-washed with 0.1 M-EDTA and exhaustively with ice-cold water) and buffer [0.1 M-tris–maleate (pH 7.4)–0.2 M-sucrose; 50 ml]. The resulting mixture was filtered through fine muslin and the sand and thallus retained by the muslin was ground further with additional buffer solution (25 ml) before being filtered again. The process of grinding and filtering was repeated once more and the filtrates were combined. A sediment of fine sand and unbroken thallus was removed by centrifugation at 1500g for 20 min. The supernatant was then centrifuged at 60000g for 100 min. This centrifugate was suspended in a small volume (approx. 0.5 ml) of tris–maleate buffer (0.1 M, pH 7.4) and used as the enzyme preparation.

Enzyme incubations. Incubations were done in a total volume of 100 μl that contained enzyme preparation (50 μl), tris–maleate buffer (pH 7.4; 60 mM), MgCl_2 (5 mM), MnCl_2 (2 mM), Triton X-100 (0.1%) and GDP-[U- ^{14}C]mannose (usually 0.1 μCi , 0.7 nmol). Incubations were done aerobically for 20 min at 30°C and were terminated by heating at 100°C for 2 min with addition of water (1 ml) and chloroform–methanol (2:1, v/v; 2 ml) or by transferring the incubation mixture directly to a thin layer of cellulose and chromatographing directly in systems C or D.

Polyprenol phosphates were added to the incubation tube before addition of the other components. To aid solubility, they were complexed as a chelate with MgCl_2 and with EDTA as described by Behrens & Leloir (1970). The prenyl phosphate (40 nmol), dissolved in chloroform–methanol (2:1, v/v; 10 μl), was mixed with MgCl_2 (2 mM; 10 μl) and EDTA (1 mM; 10 μl) and the mixture was evaporated to dryness under vacuum. The usual incubation components (as detailed above) were then added and the incubation was performed normally.

Extraction of mannoside from the incubation mixture. The incubation mixture was diluted by addition of water (1 ml) and this was followed by chloroform–methanol (2:1, v/v; 2 ml). After vigorous shaking, the chloroform layer was recovered and the aqueous layer was extracted with a further 1 ml of chloroform. The combined chloroform extracts were washed with water (1 ml) before the chloroform was removed by evaporation under N_2 . The residue was

dissolved in a small volume of chloroform–methanol (2:1, v/v) before chromatography in systems A or B.

Acid hydrolysis of mannan. When chromatographed in systems C or D, polysaccharides formed in the incubations remain at the origin. The band of cellulose adsorbent at the origin was removed and was dissolved in H_2SO_4 (50%, v/v; 0.5 ml) at room temperature. Water (3 ml) was added and the solution was refluxed at 100°C for 1 h. The solution was cooled and carefully neutralized with $\text{Ba}(\text{OH})_2$ (saturated solution). The precipitate of BaSO_4 was removed by centrifugation and the supernatant was freeze-dried. The residue was dissolved in the minimum volume of water and was chromatographed in system C alongside a marker sample of authentic mannoside.

A sample of mannan isolated in experiment A was hydrolysed in the same way but in the absence of any cellulose.

Acid hydrolysis of mannoside. Dilute acid hydrolysis of the mannoside formed from GDP-[^{14}C]mannose in enzyme incubations was accomplished by heating at 100°C for 15 min with a mixture of methanol (0.1 ml) and HCl (0.02 M; 0.1 ml). The solution was then cooled, neutralized with NaOH (0.01 M in methanol; 0.2 ml) and was extracted with chloroform, methanol and water as described for the extraction of the lipid from the enzyme incubation. The chloroform and aqueous layers were then assayed for ^{14}C .

Determination of radioactivity. Measurement of ^{14}C and radioautography were as described by Barr & Hemming (1972). Some chromatograms were scanned for radioactivity with a Nuclear–Chicago Actigraph III Radioscanner. Radioactivity in areas of thin-layer chromatograms was measured after the area of adsorbent containing radioactive compound had been transferred directly to the counting vial.

Materials

All other materials used were as described by Barr & Hemming (1972).

Results and Discussion

Presence of a prenyl phosphate

Recoveries of ^{14}C and ^{32}P during the purification of a lipid fraction from experiments B and C are recorded in Table 1. The lipid concerned had chromatographic properties identical with those of the phosphate of pig liver dolichol when chromatographed on columns of silicic acid and DEAE-cellulose acetate and on t.l.c. in system A.

In experiment B, radioautography of the thin-layer chromatograms (system A) of fraction (viii) (Table 1) showed clearly the presence of three bands of material, one of which corresponded precisely with

Table 1. Incorporation of ^{14}C and $^{32}\text{P}_i$ into the lipid fractions at various stages of purification of the lipid extract of *A. niger* grown in the presence of 3RS-[2- ^{14}C]mevalonate and $^{32}\text{P}_i$

The results of experiment C are corrected to day 0 of the growth period. N.D., not determined.

	Experiment B ^{14}C (d.p.m.)	Experiment C ^{32}P (d.p.m.)
Label added to medium	2.2×10^7	2.2×10^9
Butanol extract	7.9×10^5	4.3×10^7
Silicic acid chromatography		
(i) Chloroform fraction	1.3×10^5	0.4×10^6
(ii) Acetone fraction	4.2×10^5	3.4×10^6
(iii) Chloroform-methanol fraction	1.7×10^5	1.9×10^7
DEAE-cellulose chromatography of fraction (iii) above		
(iv) Chloroform-methanol fraction	1.5×10^5	6.1×10^6
(v) Methanol fraction	3.5×10^3	0.2×10^6
(vi) Ammonium acetate (7.5 mM) fraction	6.7×10^3	1.3×10^6
(vii) Ammonium acetate (0.05 M) fraction	N.D.	4.8×10^6
Alkali treatment of fraction (vi) above		
(viii) Chloroform layer	5.0×10^3	7.8×10^4
(ix) Aqueous layer	N.D.	1.2×10^6
Thin-layer chromatography (system A) of fraction (viii) above corresponding to dolichol phosphate	1.1×10^3	3.5×10^3

that of the [^{32}P]phosphate of pig liver dolichol, which was later shown to be identical chromatographically in this system with the synthetic phosphate of the *exo*-methylene-hexahydropolyterpenols of *A. niger*. In experiment C, radioautography of the corresponding chromatogram showed the presence of several bands of material containing ^{32}P but again one of these corresponded precisely with that of dolichol [^{32}P]phosphate.

The incorporation of ^{14}C from [2- ^{14}C]mevalonate and of ^{32}P from $^{32}\text{P}_i$ into this lipid material, together with its chromatographic properties, provide strong evidence for identifying it as the phosphate of the *exo*-methylene-hexahydropolyterpenols.

The proportion of the radioactivity that behaved like prenol phosphate on chromatography in system A was low (0.14 and 0.009% of that extracted in experiments B and C respectively). This reflects both the small amount of the prenol phosphate present and its slow rate of biosynthesis. It is noteworthy that the recovery of ^{32}P in undecaprenol phosphate recovered from *Micrococcus lysodeikticus* after growth on $^{32}\text{P}_i$ was also very low (Lahav *et al.*, 1969).

The fact that alkali treatment of fraction (vi) (experiment C, Table 1) rendered most of the ^{32}P present water-soluble supports the idea that much of the impurity present in this fraction before the treatment was phospholipid. Of the ^{32}P remaining in the chloroform layer most remained at the origin when subjected to chromatography in system A. This may have been water-soluble phosphates originating from contamination by the aqueous layer. Some of

the other ^{32}P -labelled material in the chloroform layer was probably phospholipid that had not been deacylated by the alkali treatment.

Of the two unidentified ^{14}C -labelled compounds in fraction (viii), experiment B, one had chromatographic properties consistent with its being a prenol phosphate sugar, but its identification was not taken further. We were unable to separate and purify each radioactive compound in fraction (viii) by preparative t.l.c. because the recoveries of radioactivity in extracts of bands of adsorbent from the chromatograms were very low.

In experiment B, fractions (i), (ii), (iv) and (v) were combined. They contained the neutral lipid of the extract and from them polyterpenol was recovered (Barr & Hemming, 1972). This contained 34200 d.p.m. of ^{14}C . Assuming the specific radioactivities of this polyterpenol and of the polyterpenol as phosphate to be the same it can be calculated that approx. 3% of the total polyterpenol is present as phosphate. This corresponds to a maximum of 0.8 μg of polyterpenol phosphate/g damp-dry weight.

Mannan biosynthesis

A polysaccharide portion (104 mg) isolated from the thallus of *A. niger* that had been grown in the presence of [U- ^{14}C]mannose (experiment A) yielded mannose after acid hydrolysis. The polysaccharide contained ^{14}C (32800 d.p.m.) and the radioactivity liberated by acid hydrolysis was chromatographically identical with mannose in system C. The incorpora-

Table 2. Thin-layer-chromatographic results for radioactive components of the incubation mixture after incubation of guanosine diphosphate [$U-^{14}C$]mannose with a particulate enzyme preparation

Samples of the whole incubation mixture were subjected to chromatography immediately after the incubation was completed.

Band	R_F value		^{14}C recovered (%)	Identification
	System C	System D		
1	0	0	4.5	Mannan
2	0.10	0.44	46	GDP-mannose
3	0.22	0.56	31	Mannose 1-phosphate
4	0.42	0.65	10	Mannose
5	0.58	0.85	2.0	Unknown
6	0.95	0.96	6.5	Unknown

tion of ^{14}C into the wall polysaccharide was disappointingly low, but this low value may have resulted from the relatively high concentration of glucose in the growth medium. Nevertheless the result confirmed the report (Crook & Johnston, 1962) that *A. niger* forms a polymer that contains mannose.

Metabolism of GDP-mannose

When a portion of the incubated mixture was chromatographed directly in systems C or D six radioactive compounds were detected. The bands of radioactive material were removed from the chromatogram and counted for radioactivity. The results are summarized in Table 2. Only band 2 was present in a similar sample from a control incubation with boiled enzyme. By using authentic compounds as marker substances, bands 2, 3 and 4 were identified as GDP-mannose, mannose 1-phosphate and mannose respectively. Bands 5 and 6 were not identified, whereas band 1 had the characteristics of a mannan and yielded mannose on strong acid hydrolysis.

It was considered that the low incorporation of ^{14}C into bands 1, 5 and 6 might be in part the result of hydrolase activity on GDP-mannose, producing mannose 1-phosphate and mannose. NaF (final concentration 10mM) decreased the production of mannose 1-phosphate and mannose by 58% but had no significant effect on the incorporation of radioactivity into bands 1, 5 and 6.

T.l.c. in system C of the total lipid extracted from incubation mixtures showed that only band 6 was present in the extract. The amount of ^{14}C recovered in this way indicated the transfer to lipid of approx. 1% of the mannose of the GDP-mannose added to the incubation medium.

Behrens & Leloir (1970) in their studies on dolichol phosphate glucose as an intermediate in the transfer of glucose from UDP-glucose to glycoprotein

demonstrated an increase in the amount of glucose incorporated when dolichol phosphate was added to the incubation mixture. It was argued that dolichol phosphate was acting as an acceptor for glucose from UDP-glucose. A similar experiment was done with the system from *A. niger*. The phosphate of the mixture of *exo*-methylene-hexahydropolyprenols caused a marked increase in the transfer of [^{14}C]-mannose from GDP-[^{14}C]mannose to the lipid (6080 d.p.m. compared with 3680 d.p.m. in a control incubation). The effect seemed relatively specific to this compound because the phosphate of ficaprenol (Stone *et al.*, 1967a) had little effect (4030 d.p.m.) and cetyl phosphate caused almost no difference (3570 d.p.m.). Had the effect of the prenol phosphate been the result, for example, of the detergent properties of the compound, one would expect cetyl phosphate to be effective.

The radioactive lipid that was newly formed on the addition of the phosphate of the *A. niger* prenol had the same chromatographic properties in systems A and B as the lipid formed in the other incubations. The R_F values of the lipid in these systems were 0.52 and 0.15 respectively, and differ from those reported by Lahav *et al.* (1969) for undecaprenol phosphate mannose. This may be partly due to the differences in the prenol region of the molecule, but principally to differences in chromatographic technique and materials. When undecaprenol phosphate [^{14}C]-mannose was formed by using a preparation from *M. lysodeikticus* as described by Lahav *et al.* (1969) it was found to have an almost identical R_F value (0.16) with that of the mannanlipid of *A. niger* in system B.

A characteristic of prenol phosphate sugars is the ease with which they are hydrolysed in acid (Lahav *et al.*, 1969). When the β -double bond of the prenol is saturated (as in the *A. niger* prenol) hydrolytic cleavage occurs preferentially at the bond between the phosphate and the sugar. With a typically allylic

prenol (as in the bacterial prenil) hydrolytic cleavage occurs at both the prenil-phosphate and the phosphate-sugar links. Whether or not the prenil has a β double bond, dilute acid hydrolysis of the [^{14}C]-mannolipid should render the ^{14}C water-soluble. After treatment with dilute acid (see the Experimental section) of the *A. niger* [^{14}C]-mannolipid formed in a standard incubation mixture in the absence of added prenil phosphate, 65% of the radioactivity was extracted into the aqueous layer when it was shaken with chloroform-methanol (2:1, v/v; 2 ml) and water (1 ml). Before acid treatment all of the radioactivity was recoverable from the chloroform layer when partitioned in this way.

There is thus good evidence for the presence of a polyprenol phosphate (presumably the phosphate of the family of *exo*-methylene-hexahydropolyprenols) in *A. niger*. Evidence is also in favour of the presence in the thallus of a mannose transferase, which catalyses the transfer of mannose from GDP-mannose to this prenil phosphate to form a prenil phosphate mannose. The identification of the product is based on chromatographic properties, acid lability and increase in yield on addition of prenil phosphate to the incubation mixture producing the lipid. These observations are in accord with step 1 in the proposed scheme (Scheme 1). Whether or not the transfer of mannose from GDP-mannose to mannan, which was also observed during the work, involves this manno-

lipid as an intermediate, as shown in Scheme 1, has yet to be established.

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