Phytochemistry, 1973, Vol. 12, pp. 1641 to 1649. Pergamon Press. Printed in England.

POLYPRENOL PHOSPHATES AND MANNOSYL TRANSFERASES IN *PHASEOLUS AUREUS*

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(*Received* 27 *October* 1972. *Accepted* 20 *February* 1973)

Key Word Index—*Phaseolus aureus*; Leguminosae; mannosyl transferases; mannolipid; prenol phosphates; betulaprenol phosphate.

Abstract—The transfer of mannose from GDP[^{14}C]mannose to lipid and to insoluble polymer by a particulate preparation of *Phaseolus aureus* has been investigated. The evidence favours the lipid being a prenol phosphate mannose. Of a range of prenol phosphates tried, betulaprenol phosphate was the most effective exogenous acceptor of mannose. Most of the insoluble [^{14}C]polymer formed was glycoprotein in nature although small quantities of ^{14}C were associated with glucomannan and galactoglucomannan fractions. Time studies failed to reveal a typical precursor-product relationship between the lipid and polymer fractions but on incubation of [^{14}C]mannolipid with the particulate fraction a small transfer (0·5–0·7%) of [^{14}C] to polymer was detected. *p*-Hydroxymercuribenzoate inhibited (by 90%) the transfer of [^{14}C] from GDP[^{14}C]-mannose to polymer and simultaneously increased (3-fold) the [^{14}C] recovered in the lipid fraction. The effect was nullified by mercaptoethanol. Attempts to solubilize the transfer system were only partially successful. The formation of a chromatographically identical mannolipid was demonstrated in particulate fractions of *Codium fragile* and tomato roots.

INTRODUCTION

THE DEMONSTRATION of a role for poly, *cis,trans*-prenol phosphate sugars as lipid intermediates in the biosynthesis of cell wall polymers of bacteria (see e.g. Heath[1]) raised the possibility of a similar phenomenon taking place in higher organisms. Many animals, plants and fungi are known to contain poly, *cis,trans*-prenols[2] and, as part of an investigation into possible functions of these prenols, the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to lipid and to insoluble polymer has been studied in all three types of organism. Particular attention has been paid to the nature of the [^{14}C]mannolipid formed. The evidence makes it clear that mammalian systems catalyse the transfer of mannose from GDP-mannose to endogenous prenol phosphate and also the transfer of mannose from this 'lipid intermediate' to glycoprotein.[3–5] The formation of a prenol phosphate mannose has also been demonstrated in preparations of *Aspergillus niger*, the one fungal system so far studied.[6] This work has not been taken further yet. It was decided to use a particulate preparation of 4-day-old shoots of *Phaseolus aureus* as an example of a system from higher plants for this had been shown to incorporate several sugars into polymers.[7]

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Some results of the studies of mannose transfer in this system have been reported.[8,9] Since the work began, other workers have also reported on the formation of a mannolipid in the same system.[10–12]

RESULTS AND DISCUSSION

(a) *Properties of the Mannolipid Formed*

The [^{14}C]mannolipid formed during the incubation of GDP[^{14}C]mannose was extracted and its chromatographic properties were studied. When chromatographed on DEAE-cellulose acetate none of the radioactivity was eluted by methanol but all was eluted between 400 and 600 ml of the 0·0075 M ammonium acetate eluent. In this respect the mannolipid closely resembled undecaprenol phosphate mannose isolated from *Micrococcus lysodeikticus*.[13]

TLC of the [^{14}C]mannolipid revealed an R_f of 0·22 in system A and 0·37 in system B. The corresponding R_fs reported for undecaprenol phosphate mannose[13] are 0·20 and 0·36.

Two types of mannolipid of natural origin have been described. One is a glyceride derivative, mannosyl diglyceride[14] and the other a prenol phosphate mannose.[13] Treatment of a [^{14}C]mannosyl diglyceride with mild alkali according to the procedure of Dawson modified and described by Dankert *et al.*[15] should deacylate the compound and so render the [^{14}C]mannose water soluble. In contrast undecaprenol phosphate [^{14}C]mannose resists this treatment (possibly due to the α-configuration of the mannosyl linkage) and the [^{14}C]mannose remains lipid-soluble.[13] On the other hand, if treated mildly with dilute acid[5] undecaprenol phosphate [^{14}C]mannose is readily hydrolysed and the [^{14}C]mannose becomes water soluble whereas mannosyl diglycerides would be expected to resist this treatment and remain lipid-soluble. When the [^{14}C]mannolipid under investigation was subjected to mild alkali treatment, 95–99% of the radioactive material remained lipid soluble and its R_f value in system B remained unchanged. Mild treatment with dilute acid rendered all of the ^{14}C of the [^{14}C]mannolipid water soluble. Upon TLC (system C) the major part (90–95%) of the water soluble radioactivity had an identical mobility (R_f 0·35) to that of authentic mannose marker. The remaining part of the ^{14}C (5–10%) had an R_f value (0·24) coincident with that of authentic mannose-1-phosphate marker.

(b) *Variation in Incubation Conditions*

The incubation procedure was essentially that described by Hassid's group.[7] Table 1 shows that omission of bovine serum albumin from the preparation caused a drop in the transfer of mannose to mannolipid. Storage of the enzyme for 4 days at −20° led to complete loss of activity. For this reason incubations were always carried out with fresh preparations suspended in medium containing bovine serum albumin.

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Experiments described later in this paper involved addition of prenol phosphates and of mannolipid to the medium in the presence of detergents (sections e and f). The effect of low concentrations of detergents alone on mannose transfer to lipid was shown to be small (Table 1). None of those used inhibited transfer and Triton X100 caused a slight increase. Because of this Triton X100 was used in experiments described in sections e and f. It was shown in experiment B that Triton X100 did not stimulate a change in transfer of mannose to insoluble polymer.

TABLE 1. THE TRANSFER OF [^{14}C]MANNOSE FROM GDP[^{14}C]MANNOSE TO MANNOLIPID UNDER VARIOUS CONDITIONS OF INCUBATION

Experiment	Conditions		^{14}C recovered in lipid dpm	% incorporation
A	Standard		5720	6·4
A	Minus albumin		3930	4·4
A Enzyme	4 days at −20°		—	—
B	Standard		7250	8·2
B	+ Triton X100	all at 0·1% (w/v)	9200	10·6
B	+ Tween 80		7740	8·7
B	+ Tween 20		8410	9·5
B	+ Na deoxycholate		7140	8·1

Standard incubation conditions were '30 min at 45 000 *g*' pellet from 6·7 *g* tissue in 300 μl K phosphate (0·05 M, pH 7·4) containing bovine serum albumin (1% w/v) $MgCl_2$ (0·01 M), sucrose (0·4 M) and GDP[^{14}C]mannose ($8·8 \times 10^4$ dpm, 3·6 pmol) incubated for 10 min at 27°. Experiments A and B were performed with different preparations of enzyme.

(c) *The Variation of Transfer of* [^{14}C]*mannose from GDP*[^{14}C]*mannose to Lipid and Polymer with Time*

The change with time of the level of incorporation of [^{14}C]mannose into lipid and insoluble polymer is illustrated in Fig. 1. It can be seen that the incorporation into polymer continues to rise in a linear fashion throughout the 5-min period investigated. On the other hand, incorporation into lipid levels off within 2–3 min, possibly due to saturation of the existing lipid acceptor or the attainment of a steady equilibrium position between accepting mannose and transferring it to some other acceptor. The results of sulphydryl inhibitors (section h) favour the latter alternative. However, it is clear that if the terminal acceptor is a polymer, not all of the insoluble polymer being assayed in this experiment is receiving [^{14}C]mannose via the [^{14}C]mannolipid, since the two time-course curves in Fig. 1 do not cross. That the polymer being assayed here is a heterogeneous mixture is supported by experiments reported later in this paper (section i). In similar experiments, Kauss[12] and Villemez[10] reported a crossover point at about 90 sec.

(d) *The Incubation of* [^{14}C]*mannolipid with the Particulate Preparation*

In an attempt to resolve the uncertainty regarding the precursor-product relationship between the mannolipid and the insoluble polymer it was decided to incubate a partially purified preparation of [^{14}C]mannolipid with the particulate enzyme. The polymer was then recovered, washed sequentially with chloroform:methanol (2:1), methanol, hydrochloric acid (0·1 M) and distilled water and assayed for ^{14}C. In one experiment 0·47%

(780 dpm) of the ^{14}C of the [^{14}C]mannolipid added (167 700 dpm) was transferred to the polymer. In a second experiment the transfer was 0·71 %. Although the level of transfer of mannose from lipid to polymer was low it was probably real and not simply the result of contamination. It is possible that the insolubility of the [^{14}C]mannolipid in the aqueous medium was mainly responsible for the poor incorporation.

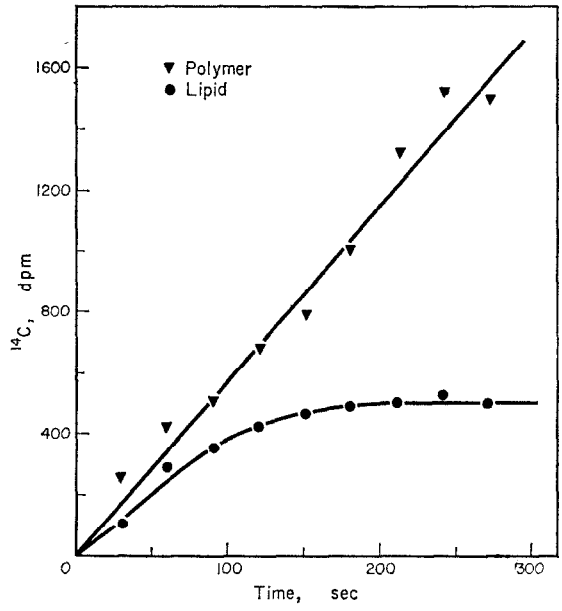

FIG. 1. THE VARIATION WITH TIME OF THE INCORPORATION OF ^{14}C INTO LIPID AND INSOLUBLE POLYMER FROM GDP[^{14}C]MANNOSE WHEN INCUBATED WITH THE *Phaseolus aureus* PARTICULATE PREPARATION AS INDICATED IN TABLE 1.

(e) *The Isolation of Mannolipid from Shoots Germinated in the Presence of* [^{32}P]ortho *phosphate*

The properties of the [^{14}C]mannolipid formed during incubations (section a) are consistent with its being a prenolphosphate mannose. The amount of mannolipid formed in these experiments was extremely small, being insufficient, for example, to give a stain with anisaldehyde reagent or to give a positive test for phosphate. It was decided to investigate the possible presence of phosphate in the lipid molecule by using [^{32}P]phosphate.

Seeds were allowed to absorb Tris[^{32}P]*ortho*phosphate (0·5 mCi) prior to germination. The 4-day-old shoots of the germinated seeds were used as a source of enzyme preparation in the usual way. The preparation was then used for a standard incubation but with non-radioactive GDP mannose as substrate. The lipid was isolated and partially purified as before. TLC of the lipid in systems A and B followed by radioautography of the chromatogram indicated the presence of five [^{32}P]lipids, one of which corresponded precisely in position to that of marker [^{14}C]mannolipid in both systems. This [^{32}P]lipid accounted for $1{\cdot}4 \times 10^3$ dpm ^{32}P, which corresponds to 0·0075 % of that added to the original seeds. The result supports the suggestion that the mannolipid contains phosphate and emphasizes the small quantity of mannolipid present.

(f) *The Effect of Adding Prenol Phosphates to the Incubation*

In studies showing that *cis,trans*-prenol phosphates act as acceptors from nucleotide diphospho sugars in both bacterial and mammalian systems (see Introduction), the addition of prenol phosphate led to an increased formation of the glycosyl prenol phosphate. It was decided to investigate the effect of adding polyprenol phosphates to the *P. aureus* preparation on the transfer of mannose to lipid and polymer. Several polyprenols were available and are listed in Table 2. The preparation and purification of the monophosphates of these prenols has been described.[6] The monophosphates of cetyl alcohol and farnesol were also prepared and tested.

TABLE 2. STRUCTURES OF POLYPRENOLS REFERRED TO IN THE TEXT AND IN TABLE 3

Betulaprenols–6 → 9	ω–*t*–*t*–*c*–[*c*]$_n$–*c*–OH	$n = 1 \rightarrow 4$
Dolichols–17 → 21	ω–*t*–*t*–*c*–[*c*]$_n$–*s*–OH	$n = 12 \rightarrow 16$
Ficaprenols–10 → 13	ω–*t*–*t*–*t*–[*c*]$_n$–*c*–OH	$n = 4 \rightarrow 8$
Solanesol	ω–*t*–*t*–*t*–[*t*]$_n$–*t*–OH	$n = 4$
Bacterial undecaprenol	ω–*t*–*t*–*c*–[*c*]$_n$–*c*–OH	$n = 6$

ω—ω-isoprene residue; *t*—*trans*-isoprene residue; *c*—*cis*-isoprene residue; *s*—saturated isoprene residue.

The transfer of [^{14}C]mannose to lipid and to polymer in the absence and presence of these alkyl phosphates is shown in Table 3. Both betulaprenol phosphate and dolichol phosphate caused a significant increase in the transfer of [^{14}C]mannose to lipid presumably by acting as acceptors for the mannosyl transferase. The phosphates of solanesol and of farnesol had only a slight stimulatory effect whereas ficaprenol phosphate caused a slight inhibition and cetyl phosphate a quite marked inhibition. The results suggest that not only is the presence of *cis*-isoprene residues important to the function of the prenol phosphates but also that the presence of two and no more, internal *trans*-residues may be critical. It is significant, in this respect, that the bacterial undecaprenol which, as the phosphate is known

TABLE 3. THE EFFECT OF ADDING ALKYL PHOSPHATES TO THE INCUBATION MIXTURE ON THE TRANSFER OF [^{14}C]MANNOSE TO LIPID AND POLYMER

Alkyl mono-phosphate added	^{14}C recovered dpm lipid	polymer	Alkyl mono-phosphate added	^{14}C recovered dpm lipid	polymer
None	3990	7670	Ficaprenol phosphate	3360	6330
Betulaprenol phosphate	11680	6400	Farnesol phopshate	4470	7360
Dolichol phosphate	11160	5840	Cetyl phosphate	2640	6660
Solanesol phosphate	4450	7350			

Incubation conditions as in Table 1. Alkyl phosphate, concentrations were 264 μM.

to act as an acceptor for bacterial glycosyl transferases, is also a poly *cis*-prenol with just two internal *trans*-residues. Surprisingly the molecular size of the polyprenol and the form of the α-residue, as evidence by the results with dolichol and betulaprenol phosphates, are not critical with regard to their function in this system.

The lack of stimulation of incorporation of mannose into insoluble polymer by any of the prenol phosphates offers no support for a role for prenol phosphate sugars as intermediates in the biosynthesis of polymers in this system.

The concentration of alkyl phosphates employed in Table 3 was 264 μM. It has been shown since that a concentration of betulaprenol phosphate of 33 μM gives a maximal effect and that at increasingly higher concentrations the effect declines progressively such that at 264 μM the stimulation in transfer of [^{14}C]mannose to lipid is only 60% of that at 33 μM. In the case of dolichol phosphate the effect was maximal at 128 μM and only slightly greater (15%) than that at 256 μM. It seems from these results that betulaprenol phosphate is a better acceptor than dolichol phosphate.

That exogenous betulaprenol phosphate will act as an acceptor was confirmed by demonstrating the incorporation of [^{3}H]betulaprenol phosphate into the mannolipid. The synthesis of the [^{3}H]betulaprenol phosphate and details of the incorporation experiment have been reported elsewhere.[9]

(g) *Attempted Solubilization of the GDP Mannose: Mannolipid Mannosyl transferase Activity*

Attempts to solubilize the transferase were only partially successful. The material solubilized from a standard particulate fraction by stirring with Triton X100 (0·1%) for 16 hr at 4–5° transferred only 0·02% of the ^{14}C of GDP[^{14}C]mannose (8·8 $\times$ 10^4 dpm) to lipid when incubated for 20 min at 27°. Extraction with digitonin (1·6%) for 20 min[16] was more successful, the transfer of ^{14}C from GDP[^{14}C]mannose to lipid reaching 2·3%. Some of this activity could be precipitated by ammonium sulphate (80% saturation) and was redissolved in buffer. The transfer of ^{14}C to lipid by this preparation was 0·2%. This last figure was increased to 0·43% when the incubation mixture contained 10 nmol of betulaprenol phosphate. This aspect of the work was not taken further but it showed that the solubilized enzyme will use betulaprenol phosphate as an acceptor. It also indicated that solubilization with digitonin might be a useful stage in purification of the enzyme for further studies.

TABLE 4. THE EFFECT OF SULPHYDRYL INHIBITION ON THE MANNOSE TRANSFERASE SYSTEM

Additions	Recovery of ^{14}C in mannolipid dpm	(%)	insoluble polymer dpm	(%)
None	6340	5·7	8270	7·5
p-Hydroxymercuribenzoate (7 mmol)	17620	16·0	880	0·8
p-Hydroymercuribenzoate (7 mmol) and mercaptoethanol (10 mmol)	8050	7·3	5950	5·4

Additions were to the standard incubation medium (see Table 1) and were present at the commencement of the 5-min incubation.

A report has been published recently[16] concerning another solubilized mannose transferase from *P. aureus*. The solubilizing agent was Triton X100 (12·5%) and the product was a polymannan. No evidence was presented concerning the production of a mannolipid by this solubilized preparation, although the same laboratory reports the production of mannolipid by the original particulate preparation.[10]

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(h) *The Effect of* p-*Hydroxymercuribenzoate on the Transfer of* [^{14}*C*]*mannose from GDP-*[^{14}*C*]*mannose to Lipid and to Insoluble Polymer*

The importance of the sulphydryl group in the transferase system is illustrated by the results in Table 4. The presence of *p*-hydroxy-mercuribenzoate caused in increased accumulation of [^{14}C]mannose in the lipid and a decrease in ^{14}C associated with polymer. The effect could be counteracted by the presence of mercaptoethanol. The results are consistent with, but do not prove, the idea that the mannolipid is an intermediate between GDP-mannose and insoluble polymer, the step between the intermediate and polymer being sensitive to sulphydryl group inhibitors. That the ^{14}C associated with mannolipid at 5 min is higher in the presence of *p*-hydroxy-mercuribenzoate than in the control shows that the steady level of ^{14}C associated with mannolipid after 2–3 min (section c) is not due to saturation of lipid acceptor with [^{14}C]mannose. It appears rather to be due to the attainment of an equilibrium position between the lipid accepting and passing the [^{14}C]mannose. This equilibrium is disturbed in the presence of *p*-hydroxymercuribenzoate.

The inhibition of the transfer of glucose to polymer by a sulphydryl inhibitor in this plant has been reported.[17] In this case the transfer of [^{14}C]glucose from GDP[^{14}C]glucose to cellulose was reduced by 81% in the presence of *p*-chloromercuribenzoate. The effect could be reversed by dithiothreitol. No mention of a [^{14}C]glucolipid was made.

(i) *The Nature of the Insoluble Polymer Formed*

The insoluble [^{14}C]polymer was accumulated from several standard incubations and a portion was hydrolysed with strong acid. The hydrolysate was neutralized, deionized and chromatographed in system C. The only radioactive product present corresponded in R_f value (0·35) to mannose and was clearly separated from other marker monosaccharides including glucose and galactose.

A second portion of the [^{14}C]polymer was treated with pronase as described before.[18] The water soluble material and insoluble material resulting from this treatment were freeze-dried and assayed for radioactivity. The recovery of ^{14}C was 77% and of this 84% (2070 dpm) was water soluble and 16% (370 dpm) remained associated with insoluble polymer. TLC of a portion of the water soluble product in system C left all of the ^{14}C at the origin of the chromatogram. This shows that the pronase treatment had not liberated free mannose (R_f 0·35 in this system) and that the pronase was free of contamination with mannosidase. The result confirms the observation of Villemez[11] that the major mannopolymer formed in this system is a mannoprotein.

In another experiment a portion of the [^{14}C]mannopolymer was subjected to a fractionation scheme described by Timell.[19] According to this scheme galactoglucomannan, which is dissolved in aq. KOH (25% w/v) is precipitated by a saturated solution of $Ba(OH)_2$. Of the material that is insoluble in the KOH the glucomannan is dissolved in NaOH (8% w/v) containing sodium borate (4% w/v). Some material, including cellulose, remains insoluble in all of these solutions. Of the ^{14}C associated with mannopolymer only 3·7% was recovered in the glucomannan fraction, 3·0% in the galactoglucomannan fraction and 1·7% remained insoluble in all of the treatments. The results confirmed that these polysaccharides are only minor products of the mannosyl transfer reaction under study.

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[19] Timell, T. E. (1965) *Adv. Carbohyd. Chem. Biochem.* **40,** 409.

(j) *The Formation of a Mannolipid in Other Plants*

Studies of the metabolism of GDP[^{14}C]mannose were extended to *Codium fragile* and to cultured tomato root. The former was chosen as a plant which contains predominantly mannans in its cell wall.[20]

The results in Table 5 indicate that both plant preparations transferred [^{14}C]mannose to lipid and that exogenous betulaprenol phosphate doubled the amount transferred by the tomato preparation. Radioautography of the TLC plate (system A) of the lipid formed by the tomato preparation showed that most of the [^{14}C] lipid had an identical R_f to that of the mannolipid of *P. aureus* and a small portion of [^{14}C] remained at the origin. The *Codium* lipid gave three equally intense radioactive spots on the chromatogram, one of which corresponded to the *P. aureus* mannolipid—the others being at R_f 0·00 and 0·45.

TABLE 5. THE TRANSFER OF ^{14}C FROM GDP[^{14}C]MANNOSE TO LIPID BY PARTICULATE PREPARATIONS OF *Codium fragile* AND CULTURED TOMATO ROOTS

Source of preparation	Betulaprenol phosphate added (nmol)	[^{14}C]Lipid formed dpm	% incorporation
Codium fragile	—	880	1·0
(a) Tomato root	—	610	0·7
(b) Tomato root	—	520	0·6
(c) Tomato root	40	1280	1·4
(d) Tomato root	40	1140	1·2

Incubation conditions were as described in Table 1. (a–d) were different samples of the same preparation of tomato root.

The level of incorporation of [^{14}C]mannose into the lipids was lower than in the case of *P. aureus*. This may be because both the *Codium* and tomato tissue were tougher and more difficult to macerate than were the *P. aureus* shoots. However, despite this it is clear that both plant tissues produce a mannolipid chromatographically similar to that of *P. aureus*. The tomato root culture may prove particularly useful for studying changes in mannolipid formation at different stages of development as defined by the distance of the tissue studied from the root meristem.

EXPERIMENTAL

Particulate preparations from tissues. 4-Day-old dark-grown shoots of *Phaseolus aureus* were ground with sand and a '45 000 *g* for 30 min' pellet obtained by the method of Hassid.[7] The final particulate preparation was resuspended in a small vol. (0·5/10 g of shoots) of K phosphate buffer (0·05 M, pH 7·4) containing bovine serum albumin (1%, w/v), $MgCl_2$ (0·01 M) and sucrose 0·4 M. *Codium fragile* was provided by the Marine Biological Station, Plymouth. The mature tissue was kept fresh in sea water prior to preparation of the particulate fraction as described above. Excised tomato roots obtained from Dr. D. A. Thurman, Dept. of Botany, were cultured according to well established methods.[21] 2-Month-old rapidly growing cultured tomato root (20 g) was used for the preparation of the particulate fraction as described above.

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Incubation conditions and isolation of products. Incubations were carried out as described in Table 1. The reaction was terminated by the addition of *n*-BuOH (2 ml) and the mixture was shaken vigorously for 30 sec. The BuOH layer was separated from the insoluble polymer by centrifugation and decantation and the polymer was extracted further with a $CHCl_3$–MeOH mixture (2:1). To the combined extracts was added $CHCl_3$ (5 ml) and the mixture was washed several times with H_2O. The combined extract was then evaporated to dryness and redissolved in $CHCl_3$–MeOH (2:1) for further study. The insoluble polymer was washed thoroughly with $CHCl_3$–MeOH (2:1) MeOH and H_2O respectively. It was then freeze dried prior to futher study. Both mannolipid and polymer isolated in this way were shown, chromatographically, to be free of radioactive mannose, mannose-1-phosphate and GDP-mannose.

Chromatographic methods. Chromatography on DEAE-cellulose acetate was as described previously[6] except that $CHCl_3$–MeOH (2:1) rather than MeOH, was used as solvent for ammonium acetate. This modification increased the solubility of the lipid fractions in the eluting solvent without altering significantly the position at which they were eluted from the column.

TLC systems were as follows: A: silica gel G; diisobutyl ketone HOAc–H_2O (20:15:2); B: silica gel G; $CHCl_3$–MeOH–H_2O (65:25:4); C: cellulose CC41; EtOAc–*n*-BuOH–HOAc–H_2O (6:8:5:8). The positions of radioactivity on chromatograms were detected by radioautography or by radioscanner. Stainable materials were detected using either the anisaldehyde spray reagent[22] or, in the case of sugars, the aniline–diphenylamine reagent.[23] Details of other experimental methods have been published as indicated by the references in the text.

Acknowledgements—The authors acknowledge the support of an overseas scholarship from The Dawood Foundation, Karachi, Pakistan and from the British Council to S.S.A. and a research grant from the Science Research Council to F.W.H.

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