OCCURRENCE OF A β-D-MANNOPYRANOSYL PHOSPHATE RESIDUE IN THE POLYPRENYL MANNOSYL PHOSPHATE FORMED IN CALF PANCREAS MICROSOMES AND IN HUMAN LYMPHOCYTES*

Annette HERSCOVICS, Christopher D. WARREN and Roger W. JEANLOZ**
Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Mass. 02114, USA

and

Josiah F. WEDGWOOD, Ingrid Y. LIU and Jack L. STROMINGER
Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Mass. 02138, USA

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1. Introduction

Polyprenyl mannosyl phosphates, which have been implicated as intermediates in the biosynthesis of glycoproteins [2–5], are formed from GDP-D-[^{14}C]-mannose in a variety of animal tissues [2–13], including calf pancreas microsomes [12] and human lymphocyte homogenates [13]. Although in some cases the polyprenyl mannosyl phosphates were shown to have properties similar to those of synthetic Dol-P-α-Man [3,9,12,13], the configuration of the anomeric carbon of the D-mannose residue was not determined. The present report demonstrates differences between the chromatographic and hydrolytic properties of the α- and β-D anomers of synthetic Dol-P-Man, and shows that the polyprenyl [^{14}C]-mannosyl phosphates from calf pancreas and human lymphocytes contain a β-linked D-mannose residue.

2. Materials and methods

2.1. *Chromatography*

TLC was carried out on precoated plates of silica gel G (E. Merck A. G., Darmstadt, Germany) with an anisaldehyde–sulfuric acid–ethanol (1:1:18) [14] spray reagent at 125°C for detection, unless otherwise stated. The solvent systems were: (A), (B), and (C), $CHCl_3$–CH_3OH–H_2O (60:25:4), (10:10:3), and

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** To whom correspondence should be addressed.

Abbreviations: Dol-P-α-Man, dolichyl α-D-mannopyranosyl phosphate; Dol-P-α-[^{14}C]Man, dolichyl α-D-[^{14}C]-mannopyranosyl phosphate; Dol-P, dolichyl phosphate; Cit-P-α-Man, citronellyl α-D-mannopyranosyl phosphate; Cit-P-β-Man, citronellyl β-D-mannopyranosyl phosphate; α-Man-1-P, α-D-mannopyranosyl phosphate; β-Man-1-P, β-D-mannopyranosyl phosphate; Man-1,2-P, β-D-mannopyranosyl 1,2-phosphate; Man-2-P, D-mannose 2-phosphate; AcOH, acetic acid.

Table 1
Alkaline hydrolysis of dolichyl α- and β-D-mannopyranosyl phosphates[a]

Dol-P-Man anomer	Solvent	Vol (ml)	NaOH (conc. M)	Temp. (°C)	Time (hr)	Method of neutralization	Degree of hydrolysis[b]	Lipid products[b]
α	1-Propanol[c]	0.5	0.1	100	0.03	0.1 M AcOH (0.5 ml)	> 90%	Dol-P
β	1-Propanol[c]	1.0	0.1	100	0.03	AG50W-X8 (py^+)[d]	60%	Dolichol[e]
β	1-Propanol[c]	0.5	0.1	37	4	0.1 M AcOH (0.5 ml)	75%	Dolichol[e]
β	1-Butanol[f]	1.0	0.25	54	3	AG50W-X8 (py^+)	> 90%	Dolichol[e]

[a] 0.5 mg used for each experiment.
[b] After neutralization, solutions were evaporated (N_2 gas) and residues extracted with $CHCl_3$–CH_3OH (5:1, 0.2 ml). The resulting solutions were examined by tlc (solvents A and G) and removed from the tube. The remaining residue was dissolved in H_2O (50 μl) and examined by TLC (solvents C and F). For identity of carbohydrate products, see table 2.
[c] Mixture of 1-propanol and M NaOH (10:1).
[d] Carried out after concentration to 0.2 ml. Addition of the resin AG50W-X8 (pyridinium$^+$) prior to concentration resulted in loss of carbohydrate products.
[e] TLC also revealed the presence of other minor products.
[f] A solution of solid NaOH in H_2O-saturated 1-butanol, with addition of a small volume of methanol to give a single phase.

(10:14:5), respectively; (D) 2,6-dimethyl-heptanone–acetic acid–H_2O (20:15:2); (E) $CHCl_3$–CH_3OH–15 M NH_4OH–H_2O (65:35:4:4); (F) 1-propanol–15 M NH_4OH (1:1); and (G) toluene–CH_3OH (49:1). Paper chromatography was performed on Whatman No. 1 paper in solvents (H) 95% ethanol–M ammonium acetate, pH 7.5 (7:3); and (I) isopropanol–15 M NH_4OH–H_2O (7:1:2), and the carbohydrates were detected with periodate-benzidine [15].

2.2. *Chemical synthesis*

The following compounds were synthesized: Dol-P-α-Man and Cit-P-α-Man [16]; Dol-P-α-[^{14}C]Man [16,17]; Dol-P-β-Man and Cit-P-β-Man [18]; α-Man-1-P [19]; β-Man-1-P and Man-1,2-P [20,18]; Man-2-P by acid treatment of Man 1,2-P (0.1 M HCl, 100°C 5 min); Dol-P [13].

2.3. *Biosynthesis of polyprenyl [^{14}C]mannosyl phosphates*

The biosynthesis of ^{14}C-labeled polyprenyl mannosyl phosphates from GDP-D-[^{14}C]mannose (New England Nuclear, Boston, Mass. 02118) and endogenous lipid is described elsewhere for calf pancreas rough microsomes [12] and human lymphocyte homogenates [13]. Chloroform–methanol extracts of pancreas microsomes containing a single polyprenyl [^{14}C]mannosyl phosphate were used without further purification. The polyprenyl [^{14}C]mannosyl phosphate from human lymphocytes was partially purified by DEAE-cellulose chromatography.

3. Results and discussion

The α and β anomers of Dol-P-Man could be distinguished from each other by the products of their alkaline hydrolysis, by their chromatographic mobility, and by the action of α-mannosidase.

Following alkaline treatment, Dol-P-α-Man gave mainly Dol-P (R_F 0.63, solvent A) (table 1), and traces of carbohydrate breakdown products (table 2). D-Mannose, the expected carbohydrate product, was never recovered because it was unstable under the conditions used. Thin-layer chromatography (TLC) (solvents B and D) indicated that more than 95% of D-mannose (1 mg) was degraded after treatment at 80°C for 5 min in 1-propanol–:M NaOH (1:10, 1.4 ml). In contrast, Dol-P-β-Man gave mainly dolichol (solvent G) (table 1), and a mixture of β-Man-1-P (predominant at 100°C) and Man-2-P (predominant at 37°C) (table 2). TLC separated the α and β anomers of Man-1-P; Man-2-P was differentiated from α-Man-1-P by its resistance to acid treatment and its positive

Table 2
Mobility on silica gel tlc of D-mannosyl phosphates, D-mannose, and products of alkaline hydrolysis of dolichyl α- and β-D-mannopyranosyl phosphates

Solvent	Man-1-P		Man-1,2-P	Man-2-P	Man	Hydrolysis products of Dol-P-Man	
	α-Anomer	β-Anomer				α-Anomer	β-Anomer
B	0.18	0.10	0.50	0.18	0.4–0.5	[0.33][a], [0.52], 0.87	0.18, 0.10
C[b]	0.33[c,d]	0.21[c,d]	0.61[c]	0.33[c,e]	0.5–0.6		0.33[c,e], 0.21[c,d], [0.61]
F	0.31	0.29	0.61	0.35	0.46	[0.35], [0.50]	0.35, 0.29
D					0.10	[0.41], [0.68]	

[a] Only traces of material detected, in brackets.
[b] The following tests were only performed with the compounds obtained with solvent C:
[c] Giving a positive reaction with a spray reagent specific for phosphate groups [21].
[d] Converted to D-mannose by treatment with dilute acid (0.1 M HCl, 100°C, 5 min) and not detected by aniline phthalate reagent (100°C, 5 min).
[e] Resistant to treatment with dilute acid (0.1 M HCl, 100°C, 5 min) and giving a positive reaction with aniline phthalate reagent (100°C, 5 min).

reaction with aniline phthalate (table 2).

It is most probable that Man-2-P and β-Man-1-P obtained from Dol-P-β-Man were formed by ring opening of Man-1,2-P during alkaline treatment, since Man-1,2-P is not stable under the alkaline conditions used for the hydrolysis of Dol-P-Man. The formation of Man-1,2-P during the hydrolysis of Dol-P-β-Man explains the cleavage of the bond between dolichol and phosphate, which is usually stable in hot dilute NaOH. Furthermore, it is not possible for an α-D-mannopyranosyl phosphate residue, which has a trans diaxial arrangement of the phosphate and C-2 hydroxyl groups, to give rise to a cyclic 1,2-phosphate [22].

These results indicate that alkali treatment of the α anomer of Dol-P-Man gives dolichyl phosphate, and of the β anomer, dolichol, the products being diagnostic of the linkage present in the original Dol-P-Man. It is of interest that alkali treatment has been reported to yield dolichyl phosphate and D-mannose from polyprenyl mannosyl phosphate of porcine liver [9], but dolichol and Man-1-P from that of bovine liver [2].

Treatment of the pancreatic polyprenyl [^{14}C]-mannosyl phosphate and of synthetic Dol-P-α-[^{14}C]-Man with 0.1 M NaOH at 65°C resulted in a similar rate of release of radioactivity into a water-soluble form, the degradation being complete at 20 min. However, different radioactive products were formed in each case (fig. 1). The pancreatic polyprenyl mannosyl phosphate yielded a radioactive product that accounted for about 67% of the original radioactivity and migrated to the same position as synthetic Man-2-P (solvents H and I; fig 1A). This radioactive product had properties similar to those of synthetic Man-2-P: it was resistant to acid treatment (0.1 M HCl, 5 min at 100°C), which converts Man-1-P into D-mannose, and it was converted by alkaline phosphatase into a compound with the R_F of D-mannose. When alkali treatment was followed by neutralization with AG-50 (pyridinium), only 20% of the radioactivity was recovered in the aqueous phase. Radioautography of TLC plates showed spots that cochromatographed with β-Man-1-P and Man-2-P (solvent C). After acid treatment (0.1 M HCl, 5 min at 100°C), the first of these two spots disappeared, whereas the intensity of the latter was unaffected, and some radioactivity appeared in the region of D-mannose. Similar results were obtained with the lymphocyte polyprenyl [^{14}C]mannosyl phosphate. In contrast, Dol-P-α-[^{14}C]Man gave completely different products, which probably arose from breakdown of D-mannose (fig. 1B). Since alkali treatment of the biosynthetic polyprenyl [^{14}C]mannosyl phosphate, like that of synthetic Dol-P-β-Man, yielded mainly Man-2-P and β-Man-1-P, it is concluded that the polyprenyl mannosyl phosphates from calf pancreas and human lymphocytes contain a β-D-mannosyl linkage.

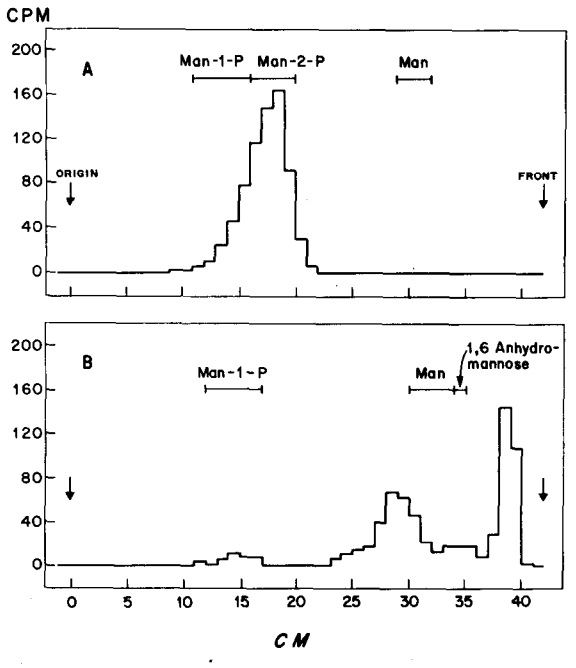
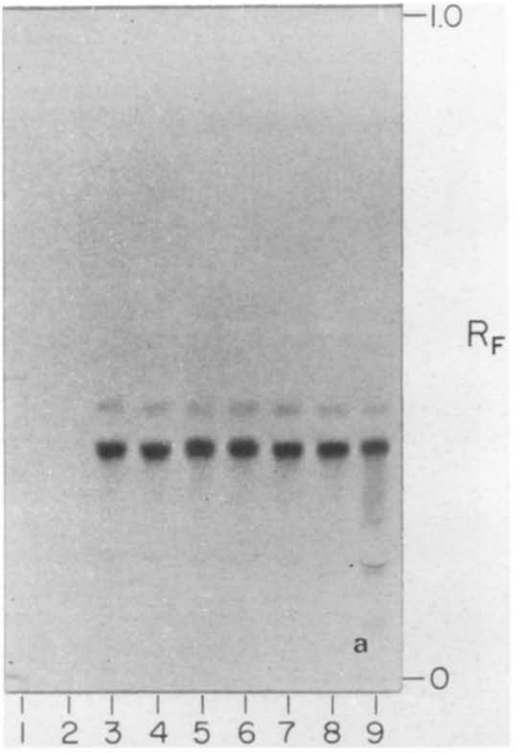
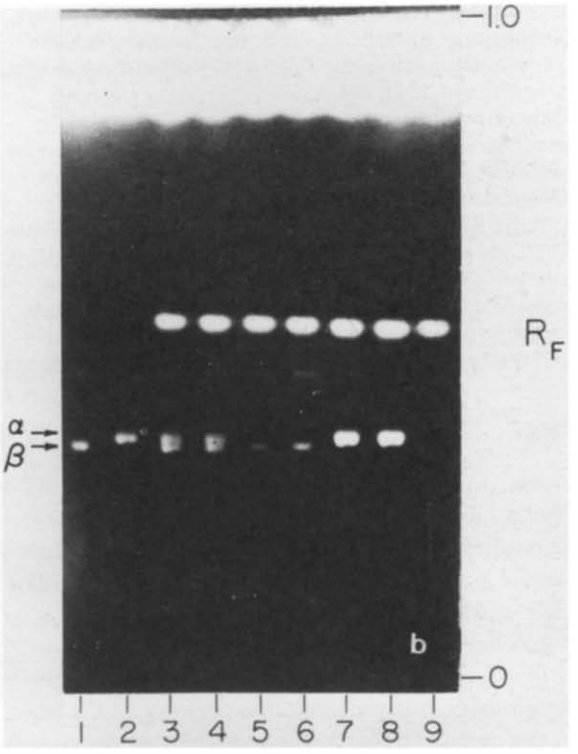


Fig. 1. Effect of alkali on polyprenyl [^{14}C]mannosyl phosphates. Solutions of pancreatic polyprenyl [^{14}C]mannosyl phosphate (A) and Dol-P-α-[^{14}C]Man (B) (about 8500 cpm) were dried under N_2, resuspended in 1-propanol (0.45 ml) and M NaOH (0.05 ml), and placed in a water bath at 65°C. After 20 min, 0.1 M acetic acid (0.5 ml), and $CHCl_3$–CH_3OH–H_2O (10:5:1) were added with mixing. The phases were separated, the lower phase was rinsed with $CHCl_3$–CH_3OH–H_2O (3:48:47, 0.5 ml) and those rinsings were combined with the upper phase which was reduced in volume for paper chromatography in solvent H. Radioactivity on the paper was located by counting 1 cm strips in 10 ml Aquasol (New England Nuclear, Boston, Mass. 02118) using a Packard model 3375 liquid scintillation spectrometer. 1,6-Anhydro-D-mannose was a gift of C. P. J. Glaudemans.





This conclusion was confirmed by the results shown in fig. 2. Small differences in the speed of migration of the anomers of Dol-P-Man were observed on TLC, R_F values for Dol-P-α- and β-Man being respectively 0.54, 0.57 (solvent A); 0.67, 0.60 (solvent D); and 0.60, 0.54 (solvent E). Different batches of silica gel plates showed considerable variations of their ability to separate these compounds. The polyprenyl [^{14}C]mannosyl phosphates from calf pancreas or human lymphocytes cochromatographed with the Dol-P-β-Man in solvents D and E (fig. 2).

α-Mannosidase caused the release of all the radio-

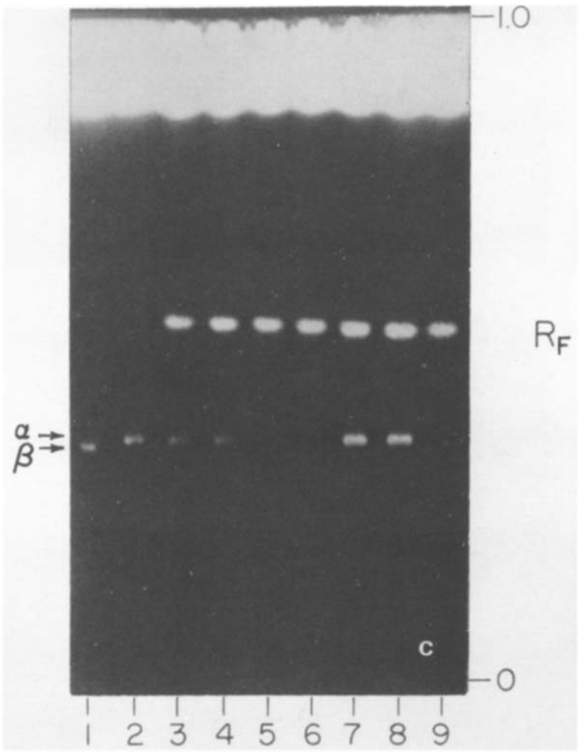


Fig. 2. TLC of polyprenyl mannosyl phosphates. Polyprenyl [^{14}C]mannosyl phosphate from human lymphocytes (1000 cpm) was mixed with either Dol-P-α-Man or Dol-P-β-Man in a total volume of 25 μl, and chromatographed in solvent E. Lipids were visualized with iodine vapor. Radioautography was performed with Kodak No-screen X-ray film. Samples are as follows: Dol-P-β-Man (1), Dol-P-α-Man (2), Dol-P-β-Man, Dol-P-α-Man, and polyprenyl [^{14}C]mannosyl phosphate (3,4), Dol-P-β-Man and polyprenyl [^{14}C]mannosyl phosphate (5,6), Dol-P-α-Man and polyprenyl [^{14}C]mannosyl phosphate (7,8), polyprenyl [^{14}C]mannosyl phosphate (9). a: radioautograph, b: iodine vapor contact print, c: radioautograph overlaid on contact print. Note that the iodine vapor spot representing Dol-P-β-Man is obliterated by the radioactive material.

activity from synthetic Dol-P-α-[^{14}C]Man into a water-soluble form (table 3). In contrast, treatment of the pancreatic polyprenyl [^{14}C]mannosyl phosphate released no significant amount of water-soluble radioactive compounds. This was not due to inactivation of the enzyme by constituents of the chloroform–methanol extract, since release was observed from the synthetic α-D anomer in the presence of the pancreatic extract (table 3). The specificity of the α-mannosidase for the α-D anomer was retained with α-Man-1-P and its derivatives, since it catalyzed the release of D-mannose from GDP-D-[^{14}C]mannose and from synthetic α-Man-1-P, Cit-P-α-Man, and Dol-P-α-Man, but not from the corresponding β-D anomers.

Table 3
Effect of α-mannosidase on polyprenyl [^{14}C]mannosyl phosphates

Polyprenyl [^{14}C]mannosyl phosphate	α-Mannosidase units/ml	Radioactivity in aqueous phase (cpm)
Dol-P-α-[^{14}C]Man	0	676
Dol-P-α-[^{14}C]Man	28.5*	532
Dol-P-α-[^{14}C]Man	10.7	4936
Dol-P-α-[^{14}C]Man	20.5	4140
Pancreatic	28.5	176
Dol-P-α-[^{14}C]Man & Pancreatic	28.5	4156

Polyprenyl [^{14}C]mannosyl phosphate from calf pancreas (2980 cpm) and synthetic Dol-P-α-[^{14}C]Man (4830 cpm) were dried in tubes with N_2 in the presence of sodium taurocholate. The residues were then suspended in a total volume of 105 μl containing sodium acetate, pH 5 (37.5 mM), sodium chloride (50 mM), zinc sulfate (0.05 mM), bovine serum albumin (100 μg/ml), sodium taurocholate (0.5%), cycloheximide (250 μg/ml), chloramphenicol (250 μg/ml), and jack bean α-mannosidase (Boehringer Mannheim Corp., New York), as indicated. After incubation at 35°C for 18 hr, 5 vol of chloroform–methanol (2:1) were added, and the radioactivity released into the aqueous phase was measured.

* Boiled enzyme

The chromatographic and hydrolytic data presented for the polyprenyl [^{14}C]mannosyl phosphates formed in calf pancreas microsomes and human lymphocytes support the conclusion that these contain a β linkage. To our knowledge this is the first report of a β-D-mannopyranosyl phosphate linkage in a naturally occurring product.

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