Biochem. J. (1983) **212**, 123–128
Printed in Great Britain

Novel mannose carrier in the trypanosomatid *Crithidia fasciculata* behaving as a short α-saturated polyprenyl phosphate

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(*Received 7 October 1982/Accepted 23 November 1982*)

Crithidia fasciculata cells incubated with [^{14}C]glucose or membranes derived from the same protozoan incubated with GDP-[^{14}C]mannose were found to synthesize a lipid monophosphate mannose. No glucosylated mild acid-labile compound was formed *in vivo* or *in vitro* when UDP-[^{14}C]glucose was used instead of GDP-[^{14}C]mannose. The lipid moiety of the mannosyl derivative formed behaved as a polyprenol having 11 isoprene residues as judged by t.l.c. and by gel filtration in sodium deoxycholate-containing buffers. The mannolipid was not broken on treatment with hot phenol, suggesting the existence of an α-saturated isoprene unit. This is the first case reported in which a mannosyl phospholipid involved in sugar transfer in a eukaryotic cell behaves as if it was similar to that of bacterial polyprenols, although having its putative α-isoprene unit saturated to the same extent as dolichols from higher organisms.

Polyprenols of different sizes have been found among different taxonomic groups and an evolutionary tendency seems to exist towards the enlargement of chains. Totally unsaturated undecaprenol (C_{55}) is typical of bacteria (Wright *et al.*, 1967), whereas there is a great variety of sizes in α-saturated and α-unsaturated polyprenols in algae and plants. In the latter organisms, compounds with 10–13 isoprene residues (C_{50}–C_{65}) are predominant (Dunphy *et al.*, 1967). In non-photosynthetic organisms, long α-saturated polyprenols (dolichols) have been described: C_{65}–C_{80} in *Tetrahymena pyriformis* (Adrian & Keenan, 1981), C_{65}–C_{90} in *Saccharomyces cerevisiae* (Jung & Tanner, 1973; Tavares *et al.*, 1977; Reuvers *et al.*, 1978), C_{80}–C_{100} in some marine invertebrates (Walton & Pennock, 1972), C_{80}–C_{95} in insects (Beedle *et al.*, 1975) and C_{55}–C_{110} in vertebrates (Dunphy *et al.*, 1967; Mankowski *et al.*, 1976).

The role of polyprenyl derivatives as intermediates in the formation of glycoconjugates is already widely known (Hemming, 1973; Lucas & Waechter, 1976; Schachter, 1978; Parodi & Leloir, 1979). Derivatives of Und-*P* are involved in the synthesis of a variety of bacterial glycans (Hemming, 1973). Eukaryotic dolichol-linked sugars are intermediates in the glycosylation of proteins (Lucas & Waechter, 1976; Schachter, 1978; Parodi & Leloir, 1979). It is not clear if all the polyprenols having different sizes participate in the synthesis of glycoconjugates because the chain length of free polyprenols and not that of the sugar carriers has been reported mainly. The Dol-*P* carriers whose length is known are those from *S. cerevisiae*, *Phaseolus vulgaris* and mammalian liver, which have mainly 17, 18 and 17–20 isoprene units respectively (Behrens *et al.*, 1971; Jung & Tanner, 1973; Delmer *et al.*, 1978; Adair & Keller, 1982). The most dramatic case in which an abundant free polyprenol is apparently not involved in glycan biosynthesis is that of plant ficaprenol, an α-unsaturated compound having 11–12 isoprene residues. Dolichol derivatives with about 18 isoprene units are known to participate in glycoprotein assembly in plants (Brett & Leloir, 1977; Delmer *et al.*, 1978).

We have recently described the formation *in vivo* of a lipid diphosphate-linked oligosaccharide in the flagellate protozoan *Crithidia fasciculata* (Trypanosomatidae) and its involvement in protein glycosylation (Parodi *et al.*, 1981). Here we report the formation *in vitro* and *in vivo* of a mannosylated lipid with properties similar to those of eukaryotic

Abbreviations used: Dol-*P*, dolichyl phosphate; Und-*P*, undecaprenyl phosphate.

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α-saturated polyprenyl monophosphate mannose, but also having the chromatographic behaviour of bacterial derivatives.

Experimental

The materials and methods not explicitly mentioned here were exactly the same as described previously (Belocopitow *et al.*, 1977; Quesada-Allue, 1978; Parodi *et al.*, 1981).

Chemicals

All organic solvents were distilled before use. Dol-*P* was from Sigma. The lipid-linked sugars used as standards were synthesized as described previously (Belocopitow *et al.*, 1977; Quesada-Allue *et al.*, 1975). Und-*P*-[^{14}C]Gal was a gift from Norita Iñón from this Institute. GDP-[^{14}C]Man (sp. radioactivity 230 Ci/mol) and UDP-[^{14}C]Glc (sp. radioactivity 248 Ci/mol) were prepared by the method of Couso *et al.* (1980).

Preparation of C. fasciculata membranes

C. fasciculata (Kinetoplastida, Trypanosomatidae) cells (symbiont-free; A.T.C.C. 11745) were grown in the medium described by Bacchi *et al.* (1974) without agar at 28°C. The culture containing about 6 g of cells (exponential phase) was ice-cooled and centrifuged. The collected cells were washed several times by resuspension in 0.1 M-Tris/HCl buffer, pH 7.4, containing 1 mM-disodium EDTA, 2 mM-$MgCl_2$, 1 mM-2-mercaptoethanol and 0.4 M-sucrose, followed by centrifugation at 1200 *g*. The washed pellet was mixed with glass beads and the cells ground in an ice-cooled mortar. The whole mixture was suspended in the above indicated solution and centrifuged at 3000 *g* for 10 min to eliminate the bulk of glass beads and haemin together with unbroken cells. The supernatant was then centrifuged at 8000 *g* for 15 min, the pellet discarded and the new supernatant fluid thus obtained centrifuged at 100000 *g* for 1 h, to obtain a membrane-rich pellet. The latter was resuspended in the above mentioned solution but without sucrose at protein concentrations of 30–40 mg/ml, and used as enzyme source. The preparation was kept at −70°C.

Transfer of mannose residues from GDP-[^{14}C]Man

The standard incubation mixture contained 60 mM-Tris/HCl buffer, pH 7.6, 4 mM-disodium EDTA, 20 mM-$MgCl_2$, 40 mM-2-mercaptoethanol, 6 μM-GDP-[^{14}C]Man (about 1.95×10^5 c.p.m.) and 1.0 mg of membrane proteins in a final volume of 100 μl. When liver Dol-*P* (4 μg) was also present, it was dried under an N_2 stream and solubilized with a final concentration of 1% (w/w) Triton X-100. The mixture was incubated for 30 min at 25°C in a shaker and the reaction was stopped with 2 ml of chloroform/methanol (3:2, v/v). The tubes were then centrifuged to separate the insoluble material. $MgCl_2$ (4 mM; 300 μl) was added to the supernatant fluid, and the lower phase thus formed was washed by the method of Folch *et al.* (1957).

Labelling in vivo of lipid-linked sugars

The growing conditions, labelling procedures and isolation of labelled substances were as described by Parodi *et al.* (1981). The lower phase of the first chloroform/methanol/water (3:2:1, by vol.) partition obtained in the preparation of lipid-bound oligosaccharides was evaporated under an N_2 stream and the residue was dissolved in 2 ml of chloroform/methanol (1:3, v/v) containing 0.1 M-NaOH. After 10 min at 37°C the saponification was stopped with 20 μl of acetic acid and 4 ml of chloroform/methanol (9:1, v/v). Water (1.1 ml) was then added to obtain an organic phase containing the bulk of unsaponifiable lipids.

The organic phase was washed several times with chloroform/methanol/water (1:16:16, by vol.)

Chromatography

Paper chromatography was performed on Schleicher and Schüll 2043a paper. T.l.c. was carried out on silica gel 60 glass plates and silica gel 60F-254 plastic plates purchased from Merck. The following solvents were used: A, chloroform/methanol/ethanol/water (1:2:2:1, by vol.); B, chloroform/propan-2-ol/ethanol/1 M-acetic acid (2:2:3:1, by vol.); and C, butan-1-ol/pyridine/water (10:3:3, by vol.).

Results

The synthesis of mannolipids in vitro

The synthesis of [^{14}C]mannose-labelled lipophilic substances with enzymes present in *C. fasciculata* membranes was carried out under conditions similar to those employed with membranes of higher eukaryotic organisms (Parodi & Leloir, 1979). The reaction was dependent on Mg^{2+} and was inhibited by an excess of disodium EDTA (results not shown). No mild acid-labile compound was formed when UDP-[^{14}C]Glc was used instead of GDP-[^{14}C]Man either in the presence or in the absence of exogenous liver Dol-*P*. The incubation of *C. fasciculata* membranes in the presence of $MgCl_2$ and GDP-[^{14}C]Man but without exogenous Dol-*P* produced two charged lipophilic substances as judged by DEAE-cellulose analytical column chromatography (Fig. 1*a*). Polyprenyl monophosphate and polyprenyl diphosphate derivatives can be separated by these columns (Quesada-Allue, 1981). Fig. 1(*a*) shows that 75% of the radioactivity co-chromatographed with liver Dol-*P*-[^{3}H]Glc. A more negatively charged compound was eluted with 10 mM-

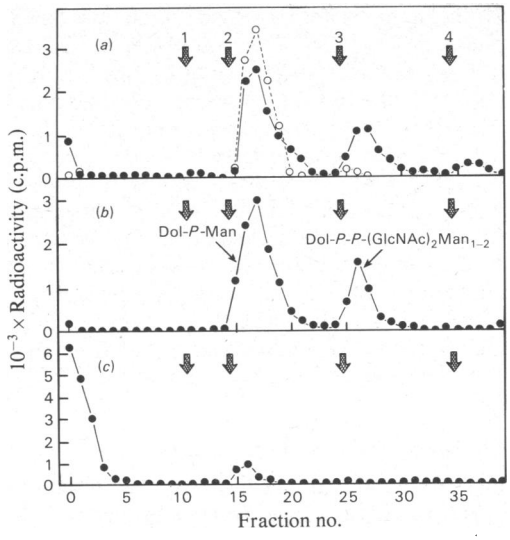

Fig. 1. *Anion-exchange chromatography of lipid derivatives*

(*a*) Lipophilic *C. fasciculata* substances labelled *in vitro* with [^{14}C]mannose (●) were dried under an N_2 stream and dissolved in 0.2ml of chloroform/methanol (3:2, v/v) containing purified liver Dol-*P*-[^{3}H]Glc (○). The sample was applied to a DEAE-cellulose column (acetate form) (0.6cm × 5.0cm) equilibrated with chloroform/methanol (3:2, v/v). The column was washed with the same solvent and the substances eluted with chloroform/methanol (1:1, v/v) (arrow 1) followed by a stepwise gradient of 0mM, 10mM- and 50mM-ammonium formate in chloroform/methanol/water (10:10:3, by vol.) as indicated by arrows 2, 3 and 4 respectively; 1ml fractions were collected. (*b*) As for (*a*), except that a mixture of insect Dol-*P*-[^{14}C]Man and Dol-*P*-*P*-(GlcNAc)$_2$[^{14}C]Man$_{1-2}$ was chromatographed. (*c*) Chromatography of lipophilic material from *C. fasciculata* labelled *in vivo*.

ammonium formate (Fig. 1*a*), exhibiting the same behaviour as insect Dol-*P*-*P*-(GlcNAc)$_2$ [^{14}C]-Man$_{1-2}$ (Fig. 1*b*). The identification of the more charged *C. fasciculata* compound as a lipid-*P*-*P*-(GlcNAc)$_2$Man$_{1-6}$ is not described in the present paper.

T.l.c. of mannolipids eluted without salt

The material from *C. fasciculata* ran as a single compound on t.l.c. developed with solvent A or other non-acidic solvents. The mobility corresponded to that of a polyprenyl monophosphate monosaccharide (result not shown) (Quesada-Allue *et al.*, 1975). Two substances appeared when the material eluted without salt from the DEAE-cellulose column was subjected to t.l.c. and developed with solvent B (Fig. 2*b*, lane 6). This solvent is useful

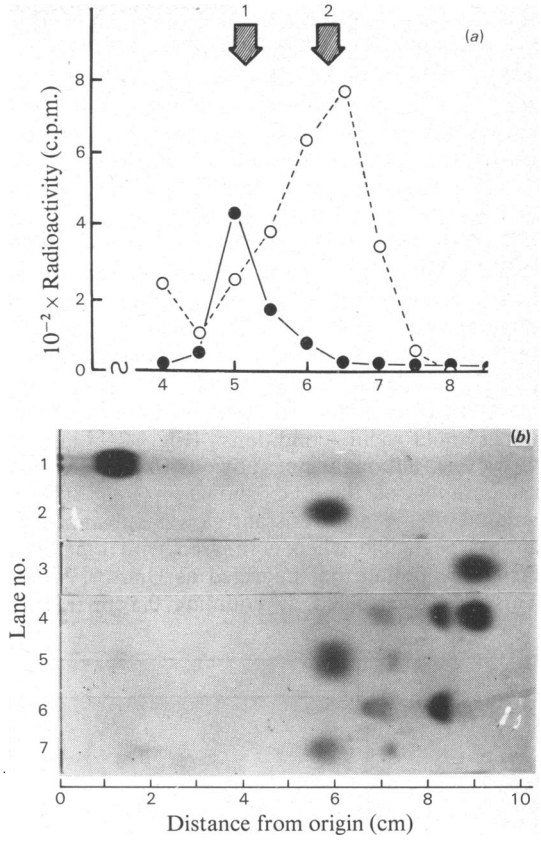

Fig. 2. *T.l.c. of mannolipids*

Fractions containing the *C. fasciculata* mannolipid eluted without salt from the column described in the legend to Fig. 1(*a*) were pooled, dried under an N_2 stream and the residue was dissolved in chloroform/methanol (3:2, v/v) and spotted on silica gel plates. Glass plates (0.25mm layer thickness) were used in (*a*) and plastic plates (0.20mm layer thickness) were used in (*b*). Differences in glycolipid mobilities were attributed mainly to the different binders and other plate-related factors. The chromatography was developed with solvent B at 19–21°C. (*a*) Mixture of *C. fasciculata* mannolipid (●) and liver Dol-*P*-[^{3}H]Glc (○). Standards of Und-*P*-[^{14}C]Gal and liver Dol-*P*-[^{14}C]Man were chromatographed in parallel lanes (arrows 1 and 2 respectively). Silica gel bands of 0.5cm were scraped off and counted for radioactivity. (*b*) As for (*a*), except that the protozoan mannolipid was chromatographed before (lane 6) and after (lane 7) mild acid hydrolysis. The mannolipids obtained from incubations supplemented with liver Dol-*P*- were chromatographed before (lane 4) and after (lane 5) mild acid hydrolysis. The following standards were chromatographed: [^{14}C]-glucosamine, [^{14}C]mannose and liver Dol-*P*-[^{14}C]-Man (lanes 1, 2 and 3 respectively). Autoradiography was carried out with a Kodak X-O Mat-S film.

to separate polyprenyl monophosphate monosaccharides having different chain lengths (Quesada-Allue, 1978; Quesada-Allue *et al.*, 1975). In this system, both non-saturated and α-saturated polyprenyl derivatives having the same chain length usually ran in the same position (Mentaberry, 1980).

The faster substance migrated the same distance as Und-*P*-[^{14}C]Gal from *Acetobacter xylinum* and differently from rat liver Dol-*P*-[^{3}H]Glc and Dol-*P*-[^{14}C]Man (Figs. 2*a* and 2*b*, lanes 3 and 6). Mild acid hydrolysis of this *C. fasciculata* mannolipid that migrated as Und-*P*-[^{14}C]Gal caused a complete release of [^{14}C]mannose in 30 min (Fig. 2*b*, lanes 5 and 7). It should be noted that the other slower mannolipid present in the sample was not so labile under mild acid conditions (Fig. 2*b*, lane 7). However, although not obvious from the autoradiography of the t.l.c. shown in Fig. 2(*b*), the amount of the acid-resistant slower substance was almost negligible when compared with that of the faster compound that migrated as Und-*P*-[^{14}C]Gal. This was ascertained by counting 0.5 cm fractions from silica gel (plate) in a scintillation counter. The addition of liver Dol-*P* in the presence of Triton X-100 to the incubation mixtures apparently enhanced 5–20-fold the amount of mannolipids formed. However, a new substance migrating as liver Dol-*P*-[^{14}C]Man was observed on the t.l.c. plate (Fig. 2*b*, lane 4). The substances that migrated as Dol-*P*-[^{14}C]Man and Und-*P*-[^{14}C]Gal appeared to be labile under mild acid conditions (Fig. 2*b*, lane 5). The experiments described below refer to the mannolipids from *C. fasciculata* synthesized in the absence of liver Dol-*P* and that were eluted from the DEAE-cellulose column without salt. As mentioned above, the bulk of label migrated as Und-*P*-[^{14}C]-Gal.

Gel filtration of C. fasciculata mannolipid

Figs. 3(*a*) and 3(b) show the profile of elution of the protozoan mannolipid from a Sephadex LH-20 column. It behaved in a similar way to that of bacterial Und-*P*-[^{14}C]Gal and ran slower than liver Dol-*P*-[^{3}H]Glc. Sodium deoxycholate forms

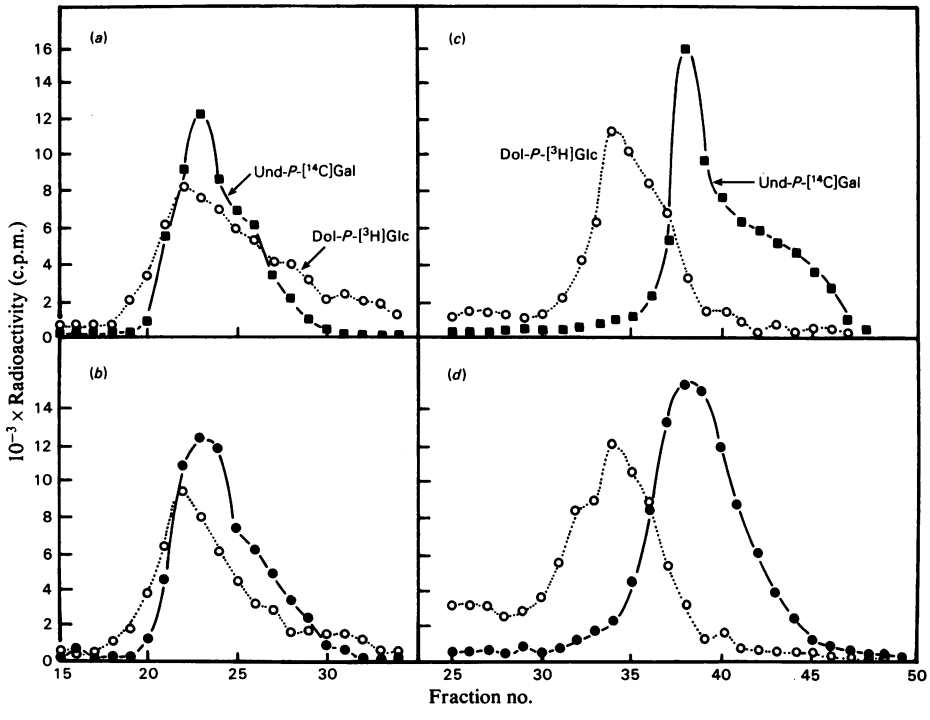

Fig. 3. *Gel filtration of the C. fasciculata mannolipid*

The lipid-linked saccharides were applied to a Sephadex LH-20 column (1.2 cm × 56.0 cm) equilibrated with chloroform/methanol/water (10:10:3, by vol.) (*a* and *b*) or to a Sephadex G-75 column (1.6 cm × 76.0 cm) equilibrated with 0.5% by weight (w/w) sodium deoxycholate in 0.1 M-Tris/HCl buffer, pH 7.7 (*c* and *d*). Elutions were performed with the same respective solutions; 0.8 ml fractions (*a* and *b*) or 2.0 ml (*c* and *d*) were collected. A mixture of Und-*P*-[^{14}C]Gal (■) and liver Dol-*P*-[^{3}H]Glc (○) was chromatographed in columns the results of which are depicted in (*a*) and (*c*). The protozoan mannolipid (●) purified as described in the legend to Fig. 1(*a*) was mixed with liver Dol-*P*-[^{3}H]Glc (○) and chromatographed (*b* and *d*).

inclusion compounds with fatty acids and polyprenols and the number of deoxycholate molecules combined depends on the chain length of the lipids. Gel filtration in Sephadex columns containing sodium deoxycholate has been used to measure the size of polyprenols from eukaryotic cells and the values obtained are in good agreement with those obtained by other methods such as mass spectrometry (Behrens *et al.*, 1971; Brett & Leloir, 1977; Parodi, 1977). Figs. 3(*c*) and 3(*d*) show that the *C. fasciculata* mannolipid was clearly separated from liver Dol-*P*-[^{3}H]Glc by gel filtration in a sodium deoxycholate-containing column. The protozoan compound behaved in the same way as bacterial Und-*P*-[^{14}C]Gal.

Synthesis in vivo of the protozoan mannolipid

When a [^{14}C]glucose pulse was given to exponential *C. fasciculata* cultures, radioactive label was incorporated into a substance that behaved on DEAE-cellulose chromatography as Dol-*P*-Man or Dol-*P*-Glc (Fig. 1*c*). The label was 95% released on mild acid hydrolysis and ran on paper chromatography with solvent C together with a mannose standard. The mannolipid formed *in vivo* was not distinguishable from that synthesized *in vitro* by t.l.c. with solvent B (results not shown).

Some properties of the C. fasciculata mannolipid synthesized in vitro

The mannolipid was resistant to mild alkaline methanolysis [0.1 M-KOH in methanol/toluene (3 : 1, v/v) for 30 min at 4°C]. The half life under mild acid treatment (0.01 M-HCl at 98°C in water) was about 9 min, similar to that of dolichol derivatives and different from that of α-unsaturated polyprenyl derivatives, which is 1–2 min. The resistance to a hot phenol treatment was similar to that of mammalian Dol-*P*-Man (Table 1). This treatment cleaves totally unsaturated polyprenyl monophosphate monosaccharides (García *et al.*, 1974). It may be assumed, therefore, that the protozoan compound probably had a saturated α-isoprene residue.

Table 1. *Hot phenol treatment of glycosylated polyprenyl derivatives*

The lipid-linked monosaccharides were dried under an N_2 stream and incubated in 50% (w/w) phenol for 60 min at 68°C. The samples were then ice-cooled and the upper (aqueous) and lower (phenolic) phases were washed and counted for radioactivity (García *et al.*, 1974). Human mammary-gland Dol-*P*-[^{14}C]Man was obtained as described by Quesada-Allue & Baldi (1981). Numbers in parentheses are the corresponding percentages before treatment.

Sample	Radioactivity (%) in: Aqueous phase	Phenolic phase
Rat liver Dol-*P*-[^{14}C]Man	6.0	94.0 (100)
Human mammary-gland Dol-*P*-[^{14}C]Man	11.3	88.7 (98.9)
Acetobacter xylinum Und-*P*-[^{14}C]Gal	43.0	56.9 (99.8)
C. fasciculata lipid-*P*-[^{14}C]Man	6.2	93.8 (100)

Discussion

The results here reported show that membranes derived from the protozoan *C. fasciculata* incubated with GDP-[^{14}C]Man, or intact cells of the same micro-organisms incubated with [^{14}C]glucose, synthesize a mannolipid behaving as if it was an α-saturated polyprenyl monophosphate mannose. The role of similar compounds as intermediates in sugar transfer in other eukaryotic cells is well known. In animal cells Dol-*P*-Man is the sugar donor of four of the nine mannose residues present in Dol-*P*-*P*-$(GlcNAc)_2Man_9Glc_3$ (Rearick *et al.*, 1981). The latter is the dolichol derivative normally involved in protein glycosylation. The synthesis of lipid-*P*-*P*-$(GlcNAc_2)Man_7$ *in vivo* in *C. fasciculata* has been described previously and evidence was presented indicating that in this protozoan the above mentioned glucose-free oligosaccharide was transferred to protein *in vivo* (Parodi *et al.*, 1981). It may be assumed, therefore, that the compound whose synthesis has been described here participates in the formation of lipid-*P*-*P*-$(GlcNAc)_2Man_7$. It must be noted, however, that in fungi Dol-*P*-Man may also transfer its sugar moiety to serine or threonine residues in proteins (Sharma *et al.*, 1974). Whether this reaction also occurs in *C. fasciculata* is unknown. It is noteworthy that the synthesis of a lipid-*P*-Glc could not be detected *in vivo* or *in vitro*. It is known that both in *S. cerevisiae* and mammalian cells Dol-*P*-Glc is the donor of the three glucose residues present in Dol-*P*-*P*-$(GlcNAc)_2Man_9Glc_3$ (Parodi, 1979; Staneloni *et al.*, 1980). The inability of *C. fasciculata* cells to synthesize Dol-*P*-Glc may explain the lack of formation of glucosylated Dol-*P*-*P* derivatives in cells incubated with [^{14}C]glucose (Parodi *et al.*, 1981).

Apparently there is a tendency in evolution towards the enlargement of the chain of polyprenols involved in sugar transfer. Thus bacterial, yeast, plant, insect and mammalian cells are known to have polyprenyl derivatives having 11, 17, 18, 18 and 17–20 isoprene units respectively (Behrens *et al.*, 1971; Jung & Tanner, 1973; Parodi, 1977; Brett & Leloir, 1977; Delmer *et al.*, 1978; Quesada-Allue, 1978; Adair & Keller, 1982). A free polyprenol having only 11 isoprene units, the first of which was saturated, has been described also in pig liver

(Mankowski *et al.*, 1976). No evidence has yet been presented indicating that this compound is involved in sugar transfer. On the contrary, a wealth of information points to the compound having 19 or 20 isoprene residues as that implicated in glycoprotein assembly in mammals (Behrens *et al.*, 1971; Parodi & Leloir, 1979).

A difference between bacterial and eukaryotic polyprenyl derivatives is that the latter have their α-isoprene unit saturated. *C. fasciculata*, one of the most primitive eukaryotes, has sugar-bound lipids of the same apparent size as the polyprenyl derivatives synthesized in bacteria, as judged by t.l.c. and by gel filtration in sodium deoxycholate-containing buffers. Given that the lipid moiety is novel and assuming that it could be an isoprenoid, the possibility of epoxidation, polysaturation or other modifications influencing its chromatographic properties and its binding to deoxycholate cannot be discarded. However, as with all eukaryotic polyprenols known to be involved in glycoprotein and polysaccharide formation, the *C. fasciculata* compound behaved as if it had an α-saturated isoprene unit. Hayes & Lucas (1980) have reported the only exception to this pattern: they described the formation in hen oviduct of an apparent polyprenyl monophosphate *N*-acetylglucosamine having the α-isoprene unit unsaturated in the same way as in bacteria. The chain length of the oviduct compound was similar to that of liver dolichol. Up to now no evidence that this novel compound participates in glycoconjugate assembly has been provided.

A compound with a size similar to that of liver Dol-*P*-Man was formed when liver Dol-*P* was added to the reaction mixtures containing *C. fasciculata* membranes. This showed that the lack of formation of large Dol-*P*-Man molecules was probably due to the absence of large Dol-*P* molecules in the protozoan membranes rather than to the inability of the GDP-Man-Dol-*P* mannosyltransferase to recognize such bulky polyprenyl phosphates.

Both the length of the polyprenol chain and the saturation of the α-isoprene unit of compounds known to be involved in sugar transfer might be, therefore, evolutive markers.

We thank Dr. J. J. Cazzulo and Mrs. Berta F. de Cazzulo from the Instituto Nacional de Diagnóstico e Investigación de la Enfermedad de Chagas 'Dr. Mario Fatala Chabén' (Buenos Aires) for growing the cells and Dr. Luis F. Leloir and all members of the Instituto de Investigaciones Bioquímicas 'Fundación Campomar' for continued advice and support. We are Career Investigators of the National Research Council (Argentina).

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