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The Mevalonate Pathway in the Bloodstream Form of *Trypanosoma brucei*

IDENTIFICATION OF DOLICHOLS CONTAINING 11 AND 12 ISOPRENE RESIDUES*

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Peter Löw‡§, and Gustav Dallner‡¶

From the ‡Department of Biochemistry, Stockholm University, 106 91 Stockholm, and ¶Clinical Research Center, Huddinge Hospital, Karolinska Institutet, Stockholm, Sweden

Satyajit Mayor‖, Steven Cohen, Brian T. Chait**, and Anant K. Menon‖**

*From the Laboratories of ‖Molecular Parasitology and **Mass Spectrometry, The Rockefeller University, New York, New York 10021*

The major surface antigen of the bloodstream form of *Trypanosoma brucei*, the variant surface glycoprotein, is attached to the plasma membrane via a glycosylphosphatidylinositol anchor. The biosynthesis of the glycosylphosphatidylinositol anchor, as well as the assembly of the asparagine-linked oligosaccharide chains found on the variant surface glycoproteins, involves polyisoprenoid lipids that act as sugar carriers. Preliminary observations (Menon, A. K., Schwarz, R. T., Mayor, and Cross, G. A. M. (1990) *J. Biol. Chem.* 265, 9033–9042) suggested that the sugar carriers in *T. brucei* were short-chain polyisoprenoids containing substantially fewer isoprene residues than polyisoprenols in mammalian cells. In this paper we describe metabolic labeling experiments with [^{3}H]mevalonate, as well as chromatographic and mass spectrometric analyses of products of the mevalonate pathway in *T. brucei*. We report that cells of the bloodstream form of *T. brucei* contain a limited spectrum of short chain dolichols and dolichol phosphates (11 and 12 isoprene residues). The total dolichol content was estimated to be 0.28 nmol/10^9 cells; the dolichyl phosphate content was 0.07 nmol/10^9 cells. The same spectrum of dolichol chain lengths was also found in a polar lipid that could be labeled with [^{3}H]mevalonate, [^{3}H]glucosamine, and [^{3}H]mannose, and which was characterized as $Man_5GlcNAc_2$-PP-dolichol. The most abundant product of the mevalonate pathway identified in *T. brucei* was cholesterol (140 nmol/10^9 cells). Ubiquinone (0.09 nmol/10^9 cells) with a solanesol side chain was also identified.

The mevalonate pathway, present in most living cells and organisms (Goldstein and Brown, 1990), generates a spectrum of isoprenoid compounds that are essential for a variety of cellular functions. The most detailed investigations of this pathway have so far been performed using mammalian cells, where many of the individual enzymes involved in the reaction sequence starting from acetate have been isolated and characterized (Spurgeon and Porter, 1981). The most abundant product of the mevalonate pathway is cholesterol, a constituent of membranes and blood lipoproteins, and the precursor for the synthesis of bile acids and steroids (Gaylor, 1981). The pathway also generates the dolichols, a family of polyisoprenoid compounds present in all membranes but in highly variable amounts (Chojnacki and Dallner, 1988); a partially distinct biosynthetic route gives rise to monophosphorylated dolichol, which is required for the asparagine-linked glycosylation of proteins (Struck and Lennarz, 1980). Ubiquinone, another end-product of the mevalonate pathway, is a component of the mitochondrial respiratory chain (Ernster, 1977). Its presence in all other cellular membranes as well may reflect its role as an endogenous antioxidant (Beyer *et al.*, 1987, Kalén *et al.*, 1987). Recently it has also been demonstrated that certain proteins are covalently modified by isoprenoid groups (farnesyl and geranyl-geranyl) (Farnsworth *et al.*, 1990, Rilling *et al.*, 1990). Isoprenylation is suggested to be one of the major factors in the activation of various growth regulating proteins and oncogenic products (Hancock *et al.*, 1989; Goldstein and Brown, 1990).

While studying the biosynthesis of the glycosylphosphatidylinositol (GPI)[1] membrane anchor of the variant surface glycoprotein of the parasitic protozoan *Trypanosoma brucei* (Menon *et al.*, 1990a, 1990b), we observed that trypanosome lipids tentatively identified as mannosyl-phosphorylpolyisoprenol and $GlcNAc_2$-PP-polyisoprenol by a number of criteria did not cochromatograph with standards derived from mammalian cells: in both cases the trypanosome lipids chromatographed more slowly than their mammalian counterparts on silica thin layer plates suggesting greater polarity (Menon *et al.*, 1990b). The family of dolichols in mammalian cells consist of lipids ranging from 15 to 23 isoprene residues, with the major species containing 18 isoprene residues. It had previously been reported (Parodi and Quesada-Allue, 1982; Quesada-Allue and Parodi, 1983) that polyisoprenol-linked monosaccharide and oligosaccharide structures in *Trypanosoma cruzi* and the trypanosomatid *Crithidia fasiculata* contain short chain α-saturated polyisoprenols (*i.e.* dolichols, esti-

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§ To whom correspondence should be addressed: Dept. of Biochemistry, Stockholm University, 106 91 Stockholm, Sweden. Tel. 46-8-158740; Fax: 46-8-153679.

[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; HPLC, high performance liquid chromatography; CMW, chloroform/methanol/water; HPTLC, high performance thin-layer chromatography; FABMS, fast atom bombardment mass spectrometry.

mated by gel filtration analyses to be approximately 13 isoprene units in *T. cruzi* and 11 isoprene residues in *C. fasciculata*). A comparison of these data with our observations suggested that the sugar-linked polyisoprenol species in *T. brucei* were short chain (≪18 isoprene residues) dolichols. In view of these preliminary observations we undertook a chemical analysis of polyisoprenoids in *T. brucei*. In this paper we report that cells of the mammalian bloodstream form of *T. brucei* possess a limited spectrum of dolichol species containing 11 and 12 isoprene residues. In the course of the analysis we also identified other products of the mevalonate pathway in *T. brucei*.

EXPERIMENTAL PROCEDURES

Chemicals—Polyprenol 11 was isolated from *Laurus nobilis*, polyprenol 12 from *Rhus typhina*, and polyprenol 15 from *Picea engelmanni*, as described earlier (Chojnacki *et al.*, 1987). The corresponding dolichols were prepared by the method of Manowski *et al.* (1976). Phosphorylation was performed according to Danilov and Chojnacki (1981). The identities of the individual polyprenols and dolichols and their phosphorylated derivatives were established by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. (*R,S*)-[2-^{3}H]mevalonate (>1 Ci/mmol) was prepared according to Keller (1986). D-[2-^{3}H]Mannose (10 Ci/mmol) and D-[6-^{3}H]glucosamine hydrochloride (35 Ci/mmol) were purchased from Amersham. All solvents were of analytical or HPLC grade.

Metabolic Labeling of Trypanosomes—Trypanosomes of the Molteno Institute Trypanozoon antigenic type 1.5 (variant clone 118) of *T. brucei* strain 427 were isolated from the blood of infected rats by centrifugation and passage through a column of DEAE-cellulose as previously described (Cross, 1975). For metabolic labeling experiments the DEAE step was omitted and buffy coat trypanosomes were used without further purification. Metabolic labeling was carried out at 37 °C in a shaking water bath as described by Mayor *et al.* (1990a): cells (5×10^8 cells/ml) were incubated with [^{3}H]mevalonate (100 μCi/ml) in RPMI 1640 medium supplemented as described (Mayor *et al.*, 1990a), and, at the end of the incubation the cells were centrifuged and subjected to extraction.

Extraction of Cells—Cell pellets were mixed with water, methanol, 60% (w/v) potassium hydroxide (2:2:1, v/v), and hydrolyzed by heating for 30 min at 100 °C. Methanol and chloroform were then added to generate a two-phase system, final ratio of chloroform/methanol/water (CMW) 3:2:1 (v/v). The upper phase was removed and the chloroform-rich lower phase was washed three times with theoretical "upper phase" (CMW, 3:48:47, v/v) (Folch *et al.*, 1957). The solvent was evaporated, and the residue was redissolved in CMW, 10:10:3 (v/v). Neutral nonsaponifiable lipids were separated from charged species on a DEAE-Sephadex A25 column equilibrated with CMW, 10:10:3 (v/v). The neutral fraction was eluted from the column with 10 volumes of CMW 10:10:3. Charged lipids were then eluted with CMW, 10:10:3, containing 0.2 M ammonium acetate. Chloroform and water were added to the fractions to give a CMW ratio of 3:2:1 (v/v). As before the upper phases were discarded; the lower phase from the charged lipid fraction was washed with theoretical "upper phase." The lower phases from both charged and neutral fractions were then evaporated and dissolved in a small amount of chloroform/methanol, 2:1 (v/v). Cells were also extracted without hydrolysis as described by Mayor *et al.* (1990a) (see below).

Thin Layer Chromatography—Thin layer chromatography of lipids was performed using either silica 60 TLC or Silica 50,000 HPTLC plates (Merck) with the solvent systems described below. Following chromatography, the plates were dried and scanned for radioactivity using a Berthold LB 2842 automatic scanner. Plates were also exposed to iodine vapor for detection of lipids. The solvent systems employed were system A, CMW (65:25:4, v/v); system B, CMW 4:4:1, v/v/v); and system C, toluene, ethylacetate (95:5, v/v).

Reversed-phase HPLC—Samples were analyzed on a Hewlett-Packard Hypersil C_{18} column (ODS 3 μm, 6 cm × 4.6 mm), using either a Shimadzu LC4A or an LC6A chromatograph system. The absorbance of the eluate was monitored at 210 nm. Fractions were collected and taken for liquid scintillation counting. Two different gradient elution systems were used, one for the neutral lipid fraction and one for the charged fraction. In the first case (neutral lipid analysis) solvent A was methanol, water (9:1, v/v) and solvent B was hexane, isopropanol, methanol (1:1:2, v/v). A linear gradient was run from 5 to 100% B over a period of 25 min; 100% B was then pumped through an additional 5 min. The flow rate was 1.5 ml/min. In the second system (charged lipid analysis) solvent A was methanol, water (9:1, v/v), and solvent B was hexane, isopropanol, methanol (1:1:1, v/v), both containing 20 mM phosphoric acid. Here a gradient from 25 to 80% B was run for 15 min followed by 80% B for an additional 5 min. Quantitation was based on area integration and internal standards (dolichol 15, dolichyl 15-P, [^{14}C]cholesterol, and ubiquinone 6) were used to compensate for sample losses.

Normal Phase HPLC—The individual polyisoprenols isolated from reversed-phase HPLC were dried and dissolved in 50 μl of hexane. The samples were analyzed on a silica column (Hibar, 5 μ, Merck) eluted with 10% diethyl ether in hexane (flow rate, 2 ml/min). The eluate was monitored at 210 nm.

Enzymatic Dephosphorylation—Dolichyl-P collected from reversed-phase HPLC was dephosphorylated as follows (Fujii *et al.*, 1982). Samples were dissolved in 400 μl of 5% octyl-β-D-glucoside, incubated overnight with potato acid phosphatase (Sigma), and extracted as described above.

Mass Spectrometry of Cholesterol, Dolichol, and Dolichyl Phosphate—Large-scale samples of isoprenoids were prepared from 5×10^{10} trypanosomes. The lipids were purified by reversed-phase HPLC and rechromatographed on the same system in order to achieve further purification.

All mass spectrometry was performed on a VG-7070 double-focussing mass spectrometer equipped to perform chemical ionization as well as fast atom bombardment mass spectrometry (FABMS). Dolichols were analyzed by negative chemical ionization mass spectrometry. The isolated dolichol samples (in CM, 2:1, v/v) were deposited onto the filament of a direct insertion probe. The probe was placed into the source of the mass spectrometer which was prepared for negative chemical ionization. The chemical ionization reagent gas was a 4:1 mixture of CH_4 and N_2O. The source was maintained at a temperature of 200 °C. The sample filament was separately heated to insure proper volatilization of the dolichols. Six to eight scans were averaged to improve the signal-to-noise ratio. Cholesterol samples were also analyzed by negative chemical ionization.

Dolichyl phosphate samples were analyzed using negative FABMS. The amphiphilic character of these compounds made FABMS an ideal method of analysis. FAB was performed with a capillaritron ion/atom gun mounted directly on the sample insertion probe (Phrasor Scientific, Duarte, CA). The samples were prepared as follows. Several microliters of HPLC-purified sample (~1 nanomole of dolichyl phosphate) were evaporated onto a stainless steel FAB sample tip. 1 μl of the FAB matrix, 3-nitrobenzyl alcohol, was then added to the tip, and the probe was introduced into the source of the mass spectrometer. The source was maintained at room temperature. Under FAB conditions, samples were bombarded by 10 keV of xenon atoms using the capillaritron probe discharge current of 5–10 μA. 10–16 FABMS scans were averaged to produce acceptable signal-to-noise ratios.

Extraction of Polar Lipids and Neutral Glycan Analysis—Trypanosomes were metabolically labeled with [^{3}H]glucosamine as described (Mayor *et al.*, 1990a) and subjected to sequential extraction. The labeled cells were first extracted with chloroform, methanol (CM, 2:1, v/v) and the partially delipidated cell residue was extracted with chloroform, methanol, water (CMW, 10:10:3, v/v/v). The CMW extract containing polar lipids was dried and the residue was partitioned between water and *n*-butanol. The polar lipids recovered in the butanol phase were dephosphorylated, deaminated and reduced as previously described (Mayor *et al.*, 1990a, 1990b) to generate neutral glycans. Desalted glycans were analyzed by anion-exchange chromatography on a Dionex basic chromatography system (Dionex Corp., Sunnyvale, CA). Separation was accomplished by gradient elution (100% solution A (0.1 M NaOH), 0% solution B (0.1 M NaOH, 0.5 M NaOAc) for 6 min after injection, then a linear increase of buffer B to 15% over a period of 30 min) using an HPIC AS6 column preceded by a guard column (HPIC AG6) filled with the same ion exchange matrix. The eluant was neutralized by an anion-exchange micromembrane suppressor (Dionex) with 0.1 M H_2SO_4 as the counter flow regenerant. Fractions (0.25 min) were collected directly into scintillation vials and taken for scintillation counting.

Analysis of the Lipid Moiety of Oligosaccharyl-PP-polyisoprenol—Oligosaccharyl-PP-polyisoprenol lipids from [^{3}H]-mevalonate labeled trypanosomes were dissolved in 90% methanol containing 0.1 M HCl and hydrolyzed for 1 h at 50 °C. Chloroform and water were added to give a ratio of CMW of 3/2/1 (v/v) and the labeled lipid moiety was

purified as described under "Extraction of Cells." The charged fraction after DEAE-Sephadex chromatography was analyzed by HPLC.

RESULTS

In order to identify lipids of the mevalonate pathway, cells were incubated with [^{3}H]mevalonate, and samples were withdrawn at different time points for analysis. The neutral lipid fraction obtained after alkaline hydrolysis and ion-exchange chromatography on DEAE-Sephadex contains sterols, oligosaccharides, and a portion of the ubiquinone, while the fraction purified as nonsaponifiable charged lipids contains polyisoprenyl phosphates (Behrens and Tábora, 1978). After an initial lag period, a continuously increasing level of radioactivity was recovered in both lipid fractions, indicating that [^{3}H]mevalonate was taken up and utilized for biosynthesis by the cells. In the following studies, a 90-min incubation period was used for labeling, since longer incubations occasionally resulted in decreased cell viability.

Dolichol and Cholesterol—The neutral lipid fraction obtained after alkaline hydrolysis of trypanosomes was analyzed by C_{18} reversed-phase HPLC. The chromatogram obtained by monitoring absorbance at 210 nm (Fig. 1*A*) shows a number of peaks including one cochromatographing with cholesterol (retention time ~8 min), and two peaks (retention times ~20 min and ~21 min) corresponding to polyisoprenols containing 11 and 12 isoprene units, respectively (see elution of standards in Fig. 1*B*). Quantitative determination indicated 33% polyisoprenol-11 and 67% polyisoprenol-12. No long chain (>12 isoprene residues) polyisoprenols were detected. Scintillation counting showed that both the cholesterol and polyisoprenol peaks were labeled by [^{3}H]mevalonate (Fig. 1*C*); a number of other labeled products (retention times 10–20 min) were also evident, and, although these products were not identified, they probably correspond to intermediate metabolites of the mevalonate pathway. Comparing the amount of radioactivity incorporated into the different [^{3}H]mevalonate-labeled products (Fig. 1*C*) with their relative abundance in the cell (Fig. 1*A*), the specific radioactivity incorporated into the polyisoprenols appeared higher than that in cholesterol, and, furthermore, the specific radioactivity of polyisoprenol 12 was lower than that of its 11-residue counterpart. The neutral [^{3}H]mevalonate-labeled lipid fraction was also resolved by thin layer chromatography on silica plates and the presence of cholesterol and polyisoprenol was confirmed by reference to authentic standards (data not shown).

Depending on the cell type, polyisoprenols may exist in an α-unsaturated form (polyprenol), and/or in an α-saturated form (dolichol). In order to characterize this aspect of the polyisoprenols isolated from *T. brucei*, the individual isoprenoids obtained by reversed-phase HPLC were chromatographed on a silica HPLC column capable of distinguishing α-saturated and α-unsaturated derivatives. As shown in Fig. 2, *A* and *C*, both polyisoprenol 11 and 12 eluted as single peaks with the same elution times as the α-saturated dolichol-11 and -12 standards. The identity of the reversed-phase-HPLC-purified polyisoprenoids was also confirmed by negative chemical ionization mass spectrometry. Ion peaks with masses corresponding to deprotonated dolichol-11 (Fig. 3*A*) and dolichol-12 (Fig. 3*B*) were obtained for the polyisoprenoid-11 and polyisoprenoid-12 material, respectively.

As stated earlier, a significant amount of material cochromatographed with a cholesterol standard on reversed-phase HPLC (see Fig. 1). Since the final products of the mevalonate pathway in several organisms are sterols other than cholesterol, this material was isolated by HPLC, analyzed by negative chemical ionization mass spectrometry and confirmed to be cholesterol (Fig. 4).

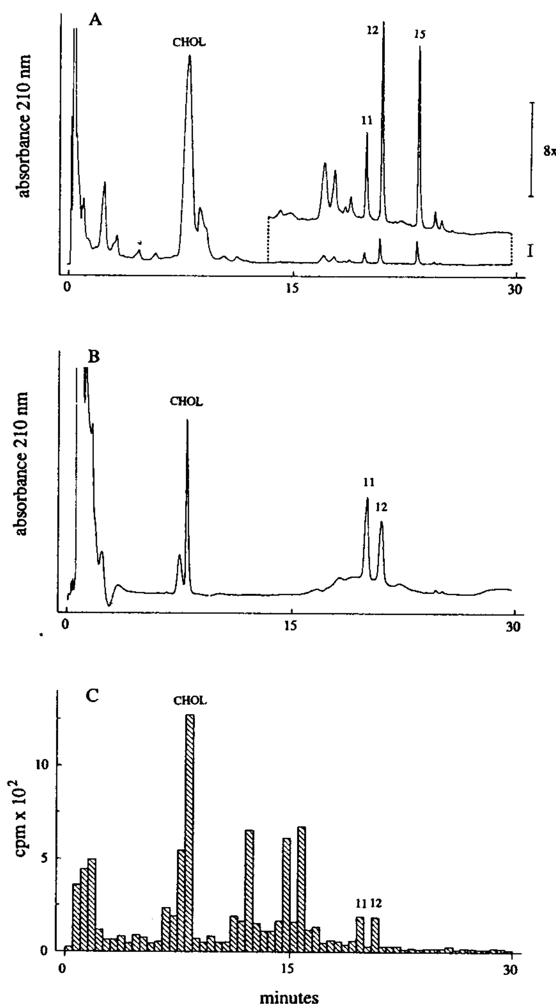

FIG. 1. **Reversed-phase HPLC analysis of the neutral lipid fraction from trypanosomes.** *A*, trypanosomes (5×10^9 cells) were extracted as described under "Experimental Procedures" and the neutral lipid fraction obtained after DEAE-Sephadex chromatography was analyzed by reversed-phase HPLC. The profile was obtained by monitoring the eluant at 210 nm. The *upper chromatogram* between elution times 13 and 30 min is an 8-fold enlargement of the *lower trace* in the same region. *B*, chromatography of cholesterol, dolichol-11 and dolichol-12 standards under conditions identical to those used for the analysis in *A*. *C*, trypanosomes (5×10^8 cells/ml) were labeled with [^{3}H]mevalonate (100 μCi/ml) for 1.5 h and analyzed as in *A*. *CHOL*, cholesterol; *11, 12*, dolichol 11 and 12; *15*, dolichol 15 internal standard.

Dolichyl Phosphate—The charged lipid fraction isolated after DEAE-Sephadex chromatography of base-hydrolyzed cells was also subjected to reversed-phase HPLC. Two dolichyl-phosphate peaks were identified (Fig. 5*A*), containing 11 and 12 isoprenoid units, respectively (see elution of standards in Fig. 5*B*). Again, roughly 33% of this phosphorylated lipid was in the form of dolichyl 11-P and 67% in the form of dolichyl 12-P. Both lipids could be labeled with [^{3}H]mevalonate (Fig. 5*C*). The specific radioactivity of the lipid with 11 isoprene units was found to be higher than that of the 12-residue counterpart (Fig. 5*C*). When the material eluting as dolichyl 12-P was isolated by HPLC, dephosphorylated enzymatically and rerun on a reversed-phase HPLC column using the system employed for the neutral lipid analysis, a single peak corresponding to dolichol-12 was obtained (data not shown).

The molecular mass of the material eluting as dolichyl 12-P was measured by fast-atom bombardment mass spectrom-

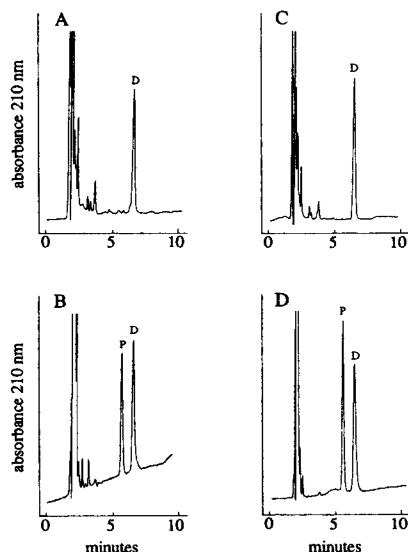

FIG. 2. **Identification of dolichols by silica HPLC.** The material chromatographing with the dolichol-11 and dolichol-12 standards on reversed-phase HPLC (see Fig. 1) was reanalyzed by HPLC using a silica column. *A*, analysis of material chromatographing as dolichol-11 (Fig. 1*A*). *B*, chromatography of polyprenol-11 (*P*) and dolichol-11 (*D*) standards. *C*, analysis of material chromatographing as dolichol-12 (Fig. 1*A*). *D*, chromatography of polyprenol-12 (*P*) and dolichol-12 (*D*) standards.

etry. The molecular mass obtained from this analysis (917 Da) indicated that this lipid was also α-saturated (Fig. 6).

Oligosaccharyl-PP-dolichol Species—Dolichyl phosphate participates as an intermediate in the biosynthesis of asparagine-linked oligosaccharide chains and GPI anchors. Consequently, it is present in the cell not only in the form of the monophosphate derivative, but also in association with sugar residues. We have previously shown that three labeled lipids can be resolved by thin layer chromatography of polar lipid extracts of trypanosomes metabolically labeled with [^{3}H]glucosamine (Mayor *et al.*, 1990b). Two of these (P2 and P3, Menon *et al.*, 1988; Mayor *et al.*, 1990b) were characterized as precursors of the variant surface glycoprotein GPI anchor, and [^{3}H]glucosamine labeling of these species was shown to be insensitive to tunicamycin. [^{3}H]Glucosamine labeling of the third lipid (referred to as P1) was tunicamycin-sensitive, and preliminary structural analyses showed that all the radioactivity could be released in water-soluble form after treatment with ice-cold aqueous hydrofluoric acid under conditions specific for cleavage of phosphodiester bonds (Mayor *et al.*, 1990b). P1 could also be labeled with [^{3}H]mannose (Menon *et al.*, 1988, see below). Taken together, these data suggested that P1 was a lipid-linked oligosaccharide involved in *N*-glycosylation.

Fig. 7*A* shows an anion-exchange HPLC analysis of neutral glycans generated from a polar lipid extract of [^{3}H]glucosamine-labeled trypanosomes. Peak III was identified as the P1 glycan after an identical analysis of material generated from TLC-purified [^{3}H]glucosamine-labeled P1 (data not shown). As shown in Fig. 7*A*, peak III cochromatographs with a characterized [^{14}C]Man$_5$GlcNAcGlcNAc-ol standard (Vijay and Perdew, 1980; structure illustrated in Fig. 7*B*). Bio-Gel P4 gel filtration analysis of the P1 glycan obtained after dephosphorylation and *N*-acetylation of TLC-purified [^{3}H] glucosamine-labeled P1 showed a single peak at 8.8 glucose units, which shifted to 9.0 glucose units after reduction (consistent with the structure illustrated in Fig. 7*B*). After treatment with jackbean α-mannosidase the reduced P1 glycan collapsed to 5.7 glucose units (consistent with Man$_1$-GlcNAcGlcNAc-ol) (data not shown). Furthermore, thin layer chromatography of permethylated and hydrolyzed [^{3}H]mannose-labeled P1 glycan showed three partially *O*-methylated mannose species (2,3,4,6-*O*-tetra-, 3,4,6-*O*-tri-, and 2,4-*O*-dimethyl mannose), again consistent with the Man$_5$ structure illustrated in Fig. 7*B* (data not shown).

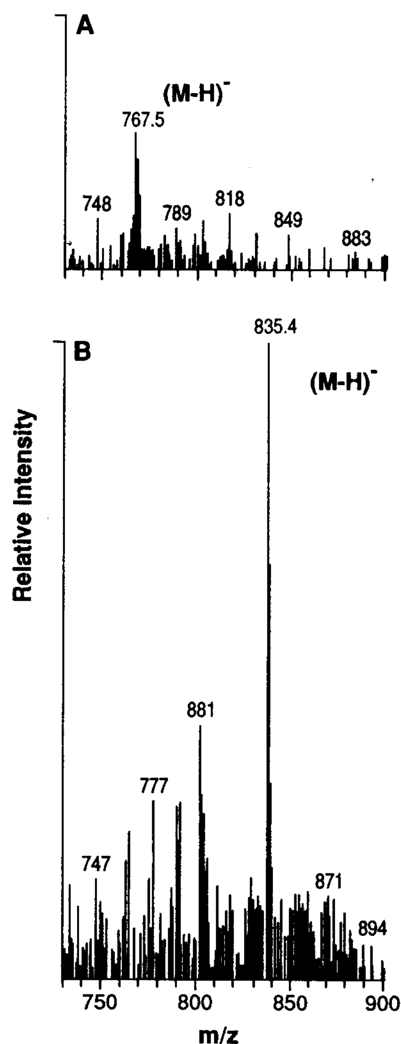

FIG. 3. **Negative chemical ionization mass spectrometric analysis of compounds having the same elution time on reversed-phase HPLC as dolichol-11 and dolichol-12 standards.** *A*, partial mass spectrum of the material having the same elution time as the dolichol-11 standard (see Fig. 1*A*). *B*, partial mass spectrum of the material having the same elution time as the dolichol-12 standard (see Fig. 1*A*). In each case, *M* designates the intact molecule, and *m/z* symbolizes the mass-to-charge ratio. The calculated molecular mass of the monoisotopic (^{12}C, ^{16}O, ^{1}H) component of dolichol-11 is 768.7 Da and dolichol 12 is 836.8 Da.

Polar lipid extracts of [^{3}H]mevalonate-labeled trypanosomes contained a single-labeled lipid that cochromatographed with [^{3}H]glucosamine-labeled P1 in two TLC systems (Fig. 8 and data not shown); the incorporation of radioactivity was tunicamycin-sensitive (data not shown). Release of the [^{3}H]mevalonate-labeled moiety by hydrolysis, and analysis of the released material by reversed-phase HPLC showed two labeled peaks corresponding to polyisoprenyl 11 and 12 phosphates (Fig. 9). The data indicate that the lipid moiety in P1 is a mixture of polyisoprenoids containing 11 and 12 isoprene residues.

Although P1 is the most prominent radiolabeled oligosac-

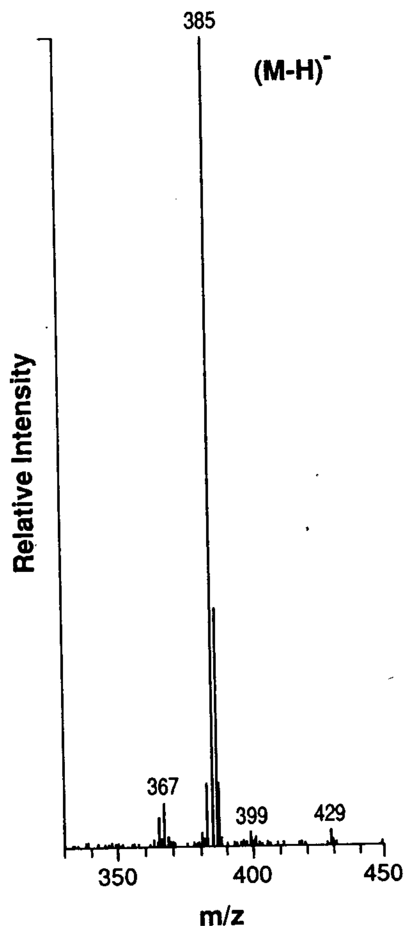

FIG. 4. **Negative chemical ionization mass spectrometric analysis of material having the same elution time on reversed-phase HPLC as the cholesterol standard.** The figure shows the partial mass spectrum of the material having the same elution time as cholesterol (Fig. 1*A*). The calculated monoisotopic mass for cholesterol is 386.4 Da. See the legend to Fig. 3 for additional details.

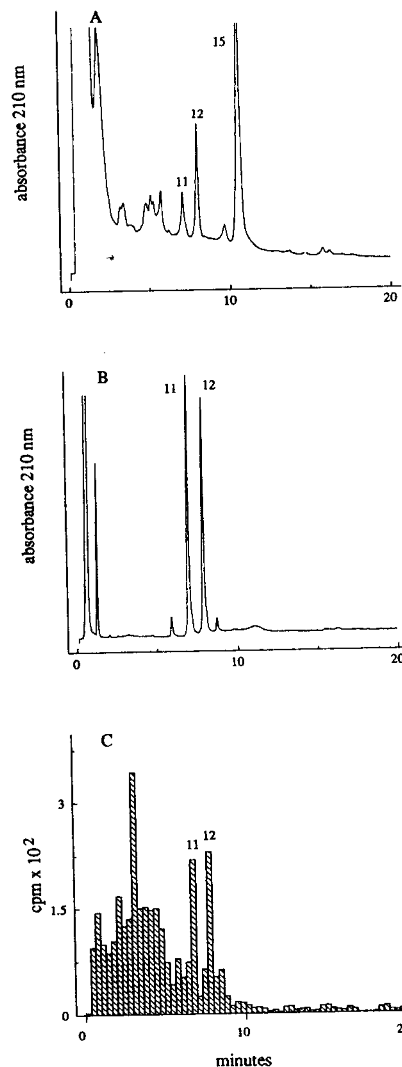

FIG. 5. **Reversed-phase HPLC analysis of the charged lipid fraction from trypanosomes.** Cells were labeled and processed as described in Fig. 1. Dolichyl-15-P was included as an internal standard. *A*, HPLC profile obtained by monitoring the eluant at 210 nm; *B*, chromatography of dolichyl-11-P and dolichyl-12-P standards under conditions identical to those used in *A*; *C*, HPLC analysis of the charged lipid fraction obtained from [^{3}H]mevalonate-labeled trypanosomes (see legend to Fig. 1*C*). *11*, *12*, and *15*, dolichyl-11-P, -12-P, and -15-P.

charyl-PP-dolichol species generated when trypanosomes are metabolically labeled with [^{3}H]glucosamine, larger structures (apparently $Man_{6-9}GlcNAc_2$-PP-dolichol) can also be detected in labeling experiments both *in vivo* (Mayor *et al.*, 1990b) and *in vitro* (Menon *et al.*, 1990b). It is not clear why these structures are not detected in the [^{3}H]mevalonate labeling experiment shown in Fig. 8. The prominence of P1 labeling may be due to limiting amounts of dolichol-P-mannose, the donor of the last 4 mannose residues in the $Man_9GlcNAc_2$-PP-dolichol structure (Kornfeld and Kornfeld, 1985).

Ubiquinone—Most cells capable of isoprenoid synthesis also synthesize ubiquinone. For this reason, lipids were extracted from trypanosomes without alkaline hydrolysis and investigated by HPLC (monitored at 275 nm, where ubiquinone absorbs specifically). Comparison with standards demonstrated that ubiquinone was present in only one form possessing a side-chain with 9 isoprenoid residues (data not shown). However, in contrast to the situation in most animal tissue, its level in *T. brucei* is less than that of dolichol (Table I).

Quantitation of the Different Lipid Species—The methods used here for identification of the products of the mevalonate pathway allowed quantitation of these compounds; the amounts were compared with those found in rat hepatocytes (Table I). Since the trypanosome is much smaller than the hepatocyte, the amounts of these lipids in *T. brucei* are about 100-fold less than in the hepatocytes. In other respects, the distribution pattern is similar. Cholesterol is the dominant polyisoprenoid lipid and the remainder account for only 1–3%. As in many other cells, about 20% of the dolichol is in phosphorylated form. Interestingly, ubiquinone in *T. brucei* is present in only a third of the molar amount of dolichol, whereas in hepatocytes the amount of this lipid exceeds that of dolichol.

DISCUSSION

In this paper we show that cells of the bloodstream form of *T. brucei* possess the complete mevalonate pathway (as they can make steroids and polyisoprenoids) but synthesize only a limited spectrum of short polyisoprenoids. In metabolic labeling experiments with [^{3}H]mevalonate, and in chemical analyses of bulk material, the polyisoprenoid spectrum in *T. brucei* was shown to consist of two α-saturated polyisoprenols (dolichols), containing 11 and 12 isoprene residues. These data may be compared with previous reports of short chain dolichols in *T. cruzi* and *C. fasciculata* (Parodi and Quesada-Allue,

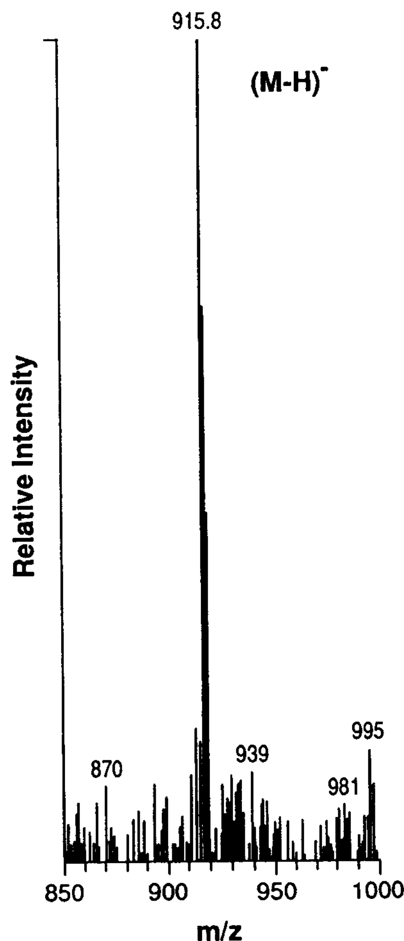

FIG. 6. **Fast-atom bombardment mass spectrometric analysis of material having the same elution time on reversed-phase HPLC as the dolichyl-12-P standard.** Material was obtained from the chromatographic analysis shown in Fig. 5*A* and analyzed by FABMS as described under "Experimental Procedures." The calculated monoisotopic mass for dolichyl-12-P is 916.7 Da. See the legend to Fig. 3 for additional details.

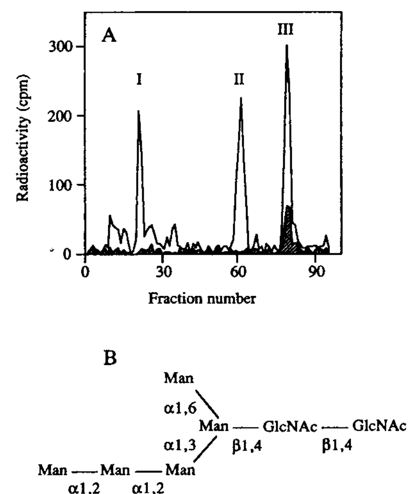

FIG. 7. **Anion-exchange HPLC analysis of neutral glycans generated from polar glycolipids extracted from [^{3}H]glucosamine-labeled trypanosomes.** A polar lipid extract was prepared from [^{3}H]glucosamine-labeled trypanosomes and neutral glycans were generated as described in "Experimental Procedures." Desalted glycans were analyzed by anion exchange HPLC (*A*). A ^{14}C-labeled glycan ($Man_5GlcNAcGlcNAc$-ol, see structure illustrated in *B*) was included in the analysis. Fractions were collected every 0.25 min and radioactivity was detected using dual channel counting. *Roman numerals* indicated at the *top* of the panel correspond to standards included in the run: *I*, 2,5-anhydromannitol; *II*, Manα1–2Manα1–6Manα1(2,5-anhydromannitol); *III*, $Man_5GlcNAcGlcNAc$-ol (see *B* for structure).

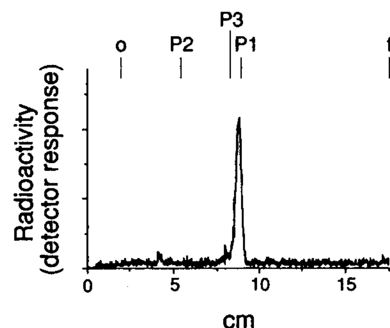

FIG. 8. **Thin-layer chromatography analysis of a polar lipid extract from [^{3}H]mevalonate-labeled trypanosomes.** Polar lipids (extracted as described in Fig. 7) from [^{3}H]mevalonate-labeled trypanosomes were chromatographed on silica TLC plates using a solvent system B. The positions of [^{3}H]glucosamine-labeled standards are indicated at the *top*. *P2* and *P3* are *GPI* anchor precursors (see Mayor *et al.*, 1990a,b), and *P1* is $Man_5GlcNAc_2$-PP-dolichol.

1982; Quesada-Allue and Parodi, 1983). However, unlike the analyses described in the present paper, these earlier reports relied on gel filtration analyses to estimate dolichol chain length. The dolichol spectrum in trypanosomatids is unusual in comparison with that found in other organisms analyzed and is in contrast to the broad spectrum long-chain dolichols (16–23 isoprene residues) synthesized by mammalian cells (Chojnacki and Dallner, 1988) and the short α-unsaturated polyprenols synthesized by bacteria (Hemming, 1981). The reasons for the different chain-length distributions in different organisms is unclear. Modifications of polyisoprenoid length are described in both *in vitro* and *in vivo* conditions. In *in vitro* systems, the length may be altered by changing the concentration of substrates such as mevalonate, isopentenyl pyrophosphate, or farnesyl pyrophosphate, in the incubation medium (Ekström *et al.*, 1987; Sagami *et al.*, 1989). The chain length pattern is also regulated by the amount of Mg^{2+} and various detergents (Matsuoka *et al.*, 1991), and if sterol carrier protein 2 is included (Ericsson *et al.*, 1991). In *in vivo* conditions longer polyisoprenes are enriched in rats treated with plasticizers (Edlund *et al.*, 1986), and in human hepatocellular cancer the short polyisoprenes are in higher concentration (Eggens *et al.*, 1988). The short dolichols are relatively enriched in liver upon treatment of rats with mevinolin (lovastatin).[2]

Studies with model membranes have established that dolichol has a profound effect on membrane stability and also increases phospholipid fatty acid fluidity and membrane permeability (Valtersson *et al.*, 1985; de Ropp *et al.*, 1985). In these studies, the effects of dolichol were found to be more pronounced with increasing number of isoprene units. However in spite of intensive investigations in recent years no definitive function for dolichol has been found.

We also identified two dolichyl phosphate species (with the same isoprenoid structure and distribution as free dolichol) in the charged lipid fraction from *T. brucei*. These accounted for about 20% of the total dolichol. Dolichyl phosphate participates in two types of glycosylation reactions in *T. brucei* and in mammalian cells, one of which is associated with the construction of asparagine-linked oligosaccharides via dolichyl-PP-oligosaccharide and dolichyl-P-monosaccharide intermediates; the second type, in which dolichyl-P-mannose is

[2] P. Low, unpublished data.

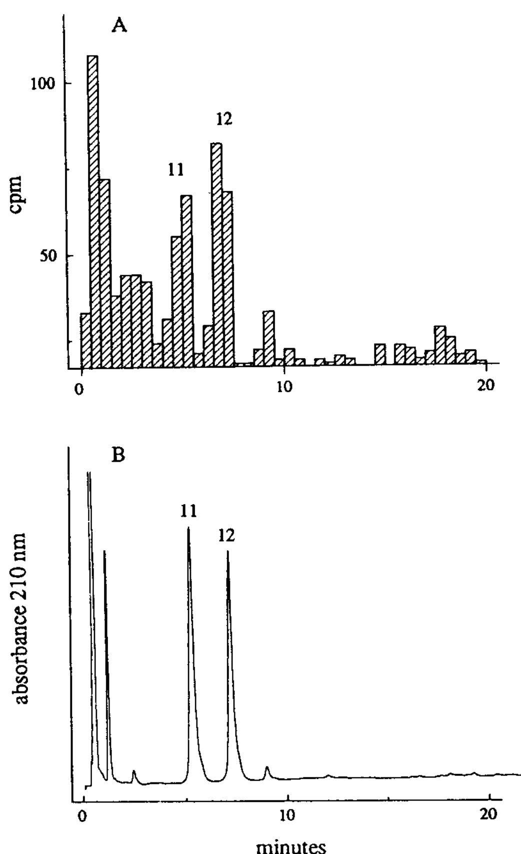

FIG. 9. **Reversed-phase HPLC analysis of the lipid moiety in [^{3}H]mevalonate-labeled P1.** Cells were labeled with [^{3}H]mevalonate and polar lipids were extracted and hydrolysed as described under "Experimental Procedures." Dolichyl phosphate-11 and -12 standards were added. *A*, radioactivity in fractions obtained every 0.5 min; *B*, HPLC profile obtained by monitoring the eluant at 210 nm.

TABLE I
Levels of polyisoprenoid lipids (nmol/10⁹ cells) in T. brucei and in rat hepatocytes

	T. brucei[a]	Hepatocytes[b]
Dolichol-11	0.09 ± 0.01	
Dolichol-12	0.19 ± 0.02	
Dolichol total	0.28 ± 0.02	91[c]
Dolichyl-11-P	0.025 ± 0.005	
Dolichyl-12-P	0.045 ± 0.007	
Dolichyl-P total	0.070 ± 0.009	18[c]
Ubiquinone-9	0.10 ± 0.02	410
Cholesterol	140 ± 20	14,000
Volume/cell	25[d,e]	4,940[d,f]

[a] Mean for four to six experiments ± S.E.
[b] Values from Åstrand *et al.* (1986).
[c] 17–22 isoprene units.
[d] μm^3.
[e] Personal communication with Paul Webster, Yale University.
[f] Weibel *et al.* (1969).

an obligatory intermediate in the transfer of mannose from GDP-mannose to Man_{0-2}GlcN-PI, is involved in the construction of GPI anchors (Menon *et al.*, 1990a). It is possible that two pools of dolichyl-P are present in trypanosome membranes, each separately serving the two types of glycosylation reactions. However, even if this were to be the case, it is expected that each pool would contain both 11- and 12-residue dolichyl-P forms since the specificity of sugar transport is not related to the chain length of the carrier (Löw *et al.*, 1986). This conclusion is justified by our finding that the most prominent dolichyl-PP-oligosaccharide ($Man_5GlcNAc_2$) species identified in *T. brucei* metabolically labeled with [^{3}H]mevalonate, [^{3}H]glucosamine, or [^{3}H]mannose contains both 11 and 12 isoprenoid residues. Also, dolichyl phosphates (17–22 isoprene units) from pig liver can be used as substrates for glycosyl transferases in *T. brucei* (Menon *et al.*, 1990a), demonstrating that the transferases are not specific for short dolichols.

The main sterol product of the mevalonate pathway in *T. brucei* was identified as cholesterol. The presence of cholesterol is important from two points of view. First, the effect of cholesterol on cellular membranes is opposite to that of dolichol, *i.e.*, it increases stability and decreases permeability (Bloch, 1985). Secondly, cholesterol is the precursor for a number of functionally active metabolites in animal tissues and the large amount of this lipid present in trypanosomes raises the question as to what extent this organism produces steroid-like metabolites.

One can hypothesize as to what extent external lipids products of the mevalonate pathways are taken up by *T. brucei* growing in the bloodstream. This may be possible in the case of cholesterol, given the presence of an low density lipoprotein receptor in *T. brucei* (Coppens *et al.*, 1988) resembling that found in various animal cells (Goldstein *et al.*, 1985). However, uptake cannot be a major factor in supplying dolichol and ubiquinone to trypanosomes. These lipids are found in the blood in very low concentrations only (Elmberger *et al.*, 1988, 1989).

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