OZONOLYTIC CLEAVAGE OF AUTHENTIC AND PANCREATIC DOLICHYL MANNOPYRANOSYL PHOSPHATES: DETERMINATION OF SUGAR CONFIGURATION IN THE FRAGMENTS WITH α- AND β-MANNOSIDASES[1]

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SUMMARY. Exposure of authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate (I) or calf pancreas dolichyl [^{14}C]mannopyranosyl phosphate (II) to ozone at -70° in pentane followed by treatment with triphenylphosphine gave water-soluble fragments in 65-95% yield. The radioactive products obtained were similar; the major fragment had a mobility on tlc greater than that of mannose but lower than that of citronellyl β-D-mannopyranosyl phosphate. The electrophoretic behavior of the fragments indicated that they possessed intact phosphodiester linkages. α-Mannosidase released [^{14}C]mannose from the fragments of I but not from the fragments of II; however, the latter were susceptible to β-mannosidase indicating that the pancreatic mannolipid contains a β-linked mannosyl residue.

The enzymatic transfer of mannose from GDP-mannose to dolichyl phosphate has been demonstrated with particulate preparations from a wide variety of animal tissues (1), and evidence is now accumulating which suggests that the resulting dolichyl mannopyranosyl phosphate functions as a mannosyl donor in the formation of certain glycoproteins (2-4). As these glycoproteins contain both α- and β-linked mannosyl residues, the question of the anomeric configuration in the dolichyl derivative arises.

Ozonolytic scission of the double bonds within the isoprenyl portion of authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate or calf pancreatic dolichyl [^{14}C]mannopyranosyl phosphate yielded water-soluble fragments in which

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the phosphodiester linkage between the [^{14}C]mannose and the degraded lipid moiety was intact. Specific mannosidases were employed to establish the anomeric configuration of the mannosyl residues in these fragments.

MATERIALS AND METHODS

Chloroform-methanol extracts of calf pancreatic, rough microsomes which have been incubated with GDP-[^{14}C]mannose contain a single radioactive lipid that has been identified as dolichyl [^{14}C]mannopyranosyl phosphate (5). Such extracts prepared as previously described (5) were used for ozonolysis without further purification after removal of water by the repeated addition and evaporation of toluene. Chemically synthesized dolichyl α-D-[^{14}C]manno-pyranosyl phosphate (6) and citronellyl β-D-mannopyranosyl phosphate (7) were obtained from Dr. C. D. Warren, Laboratory for Carbohydrate Research, Massachusetts General Hospital, Boston, MA, 02114.

An ozone generator similar to that described by Beroza and Bierl (8) was purchased from Supelco, Inc., Bellefonte, PA, 16823. Oxygen or nitrogen, dried by passage through silica gel and Linde 5A molecular sieve, was conducted through the apparatus at a flow rate of approximately 5 ml/min. For conversion of the [^{14}C]mannolipids to ozonides, the solvent (chloroform-methanol, 2:1, v/v) was removed by evaporation under N_2, and the residues were suspended in freshly distilled pentane, cooled in an acetone-dry ice bath and flushed with nitrogen. The suspension was then exposed to ozone until a starch-potassium iodide solution through which the effluent gas from the reaction vessel was passed indicated the presence of excess ozone (3-6 min). Subsequently, the mixtures were flushed with nitrogen for 5 min, warmed to room temperature, reduced with triphenylphosphine for 15 min (8), and extracted with water. The pentane phases were discarded, and the aqueous extracts were washed four times with pentane to remove traces of triphenylphosphine.

For thin layer chromatography, Merck precoated plates of Silica Gel G (0.25 mm thick, without fluorescence indicator, Brinkmann Instruments, Inc.,

Westbury, NY, 11590) were developed with chloroform-methanol-water (10:10:3, by vol). Radioactive components were located by autoradiography with Kodak No-Screen medical X-ray film (NS-2T); mannose and citronellyl β-D-mannopyranosyl phosphate were detected with anisaldehyde reagent (9).

Electrophoresis was performed on Whatman 3 MM paper (H. Reeve Angel and Co., Clifton, NJ, 07014) at 10 V/cm for 2.5 hr in a buffer consisting of 50 *mM* ethanolamine adjusted to pH 7.6 with HCl. Whatman No. 1 paper was employed for descending paper chromatography with ethyl acetate-pyridine-water (8:2:1, by vol). Radioactive compounds were located with a Packard Radiochromatogram Scanner, Model 7201 (Packard Instrument Co., Inc., Downers Grove, IL, 60515); α-D-mannopyranosyl phosphate (Sigma Chemical Co., St. Louis, MO, 63178) and mannose were detected with periodate-benzidine (10).

α-Mannosidase (EC 3.2.1.24) was purified from jack beans by the method of Snaith and Levvy (11) modified to include a protamine precipitation prior to treatment of the preparation with pyridine. Hen oviduct β-mannosidase (EC 3.2.1.25) was obtained by the procedure of Sukeno *et al.* (12) from a partially purified preparation supplied by Dr. A. L. Tarentino, New York State Department of Health, Division of Laboratories and Research, Albany, NY, 12201. Mannosidase activity was estimated at 37° in 1 ml of 50 *mM* sodium acetate buffer, pH 4.5, containing either 5 *mM* *p*-nitrophenyl α-D-mannopyranoside (Sigma Chemical Co., St. Louis, MO, 63178) or 5 *mM* *p*-nitrophenyl β-D-mannopyranoside (Research Products International Corp., Elk Grove Village, IL, 60007). The reaction was terminated by the addition of 1 ml 0.5 *M* sodium carbonate buffer, pH 10.0, and the liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm ($\varepsilon = 1.77 \times 10^{4} M^{-1} cm^{-1}$). One unit of enzyme is the amount that releases 1 μmole of *p*-nitrophenol per min under the conditions of the assay. One unit of the jack bean α-mannosidase preparation contained less than 2×10^{-6} units of β-mannosidase activity; one unit of hen oviduct β-mannosidase had less than 5×10^{-3} units of α-mannosidase activity.

An Intertechnique Liquid Scintillation Spectrophotometer, Model SL30

(Intertechnique, Dover, NJ, 07801), was employed to measure the radioactivity of liquid samples in 10 ml portions of Aquasol (New England Nuclear Corp., Boston, MA, 02118).

RESULTS AND DISCUSSION

In dolichol, the isoprenyl residue at the hydroxyl end of the molecule is saturated (1). Accordingly, ozonolytic attack of the double bonds within the isoprenyl portion of dolichyl [^{14}C]mannopyranosyl phosphate followed by reduction of the ozonide would be expected to produce a radioactive fragment with the following structure:

$$O{=}CH{-}CH_2{-}CH_2{-}CH(CH_3){-}CH_2{-}CH_2{-}(PO_4^-){-}\text{mannose}$$

This fragment would be similar in size and polarity to citronellyl mannopyranosyl phosphate (the diisoprenyl analogue of dolichyl mannopyranosyl phosphate) and would, therefore, be water-soluble and susceptible to cleavage by the appropriate mannosidase.

When ozonides of authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate and calf pancreatic dolichyl [^{14}C]mannopyranosyl phosphate were reduced with triphenylphosphine between 65 and 95% of the radioactivity originally associated with the mannolipids could be recovered in a water-soluble form.

These radioactive products were compared with the starting compounds and with authentic citronellyl β-D-mannopyranosyl phosphate by thin layer chromatography (Fig. 1). Authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate (Fig. 1A), like the pancreatic mannolipid (Fig. 1C) migrated with an R_f value of 0.90. Ozonolysis of the authentic compound gave several radioactive fragments (Fig. 1B): one major product (R_f = 0.53) and several minor components (R_f = 0.56 - 0.64). A similar pattern was observed with the fragments from the pancreatic dolichyl [^{14}C]mannopyranosyl phosphate (Fig. 1D): one major radioactive component (R_f = 0.53) and several minor fragments (R_f = 0.57 - 0.69). In both cases, the major ^{14}C-labeled fragment migrated slightly behind citronellyl β-D-mannopyranosyl phosphate (R_f = 0.61) but ahead of D-mannose (R_f = 0.41) and thus, with respect to polarity, corresponded to the expected

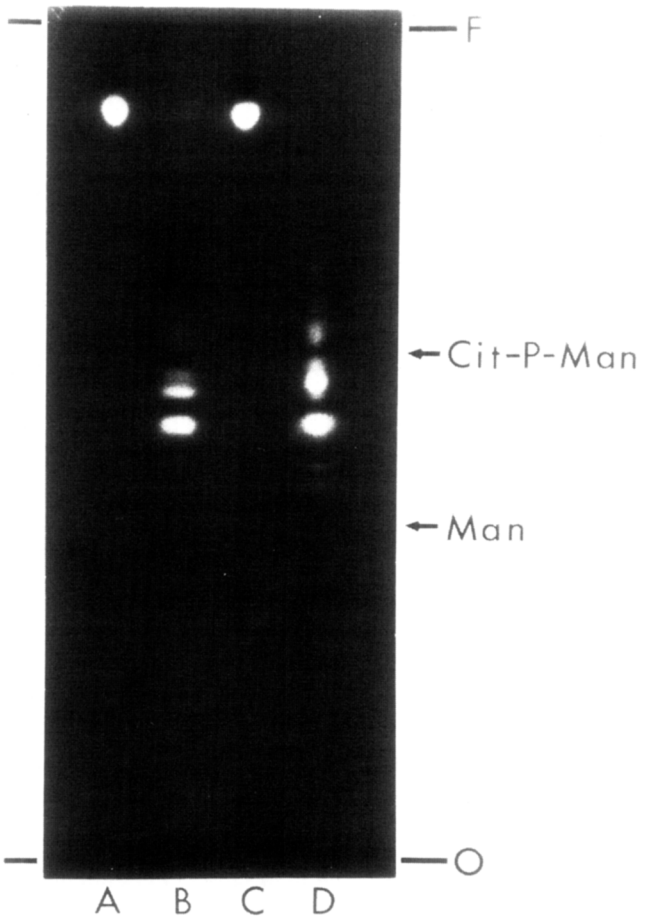

FIGURE 1. Thin layer chromatography of the [^{14}C]mannolipids and their radioactive ozonolysis fragments: A, authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate; B, water-soluble fragments derived from A; C, pancreatic dolichyl [^{14}C]mannopyranosyl phosphate; D, water-soluble fragments derived from C. O, origin; F, front; *MAN*, D-mannose; *Cit-P-Man*, citronellyl β-D-mannopyranosyl phosphate.

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fragment. It is not known whether the less polar fragments resulted from incomplete reaction of the parent molecule (ozonization or reduction) or from the occurrence of saturated isoprenyl residues adjacent to the α-residue in the starting compound.

At pH values above the pK_2 of phosphate, the expected ozonolysis fragment, a phosphodiester, would bear half the charge exhibited by a phosphomonoester

such as α-D-mannopyranosyl phosphate. During electrophoresis at pH 7.6, the water-soluble products from both the authentic (Fig. 2A) and the pancreatic (Fig. 3A) dolichyl [^{14}C]mannopyranosyl phosphate migrated approximately half the distance traveled by α-D-mannopyranosyl phosphate suggesting that the

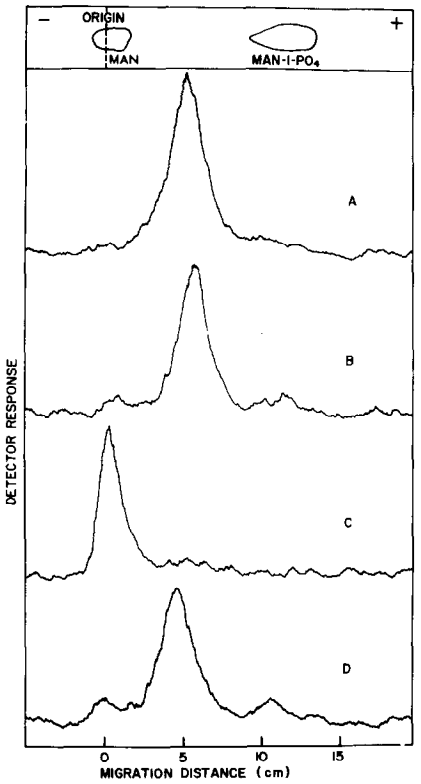

FIGURE 2. Action of α- and β-mannosidases on the ozonolytic fragments from authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate. Each reaction mixture contained 10 µl of the aqueous extract from the ozonolysis procedure, 10 µl of 100 *mM* sodium acetate buffer, pH 5.0, supplemented with 30 *mM* β-mercaptoethanol, and 10 µl of either (A,B) 50 *mM* acetate buffer, pH 5.0, (C) 50 *mM* sodium acetate buffer, pH 5.0, containing 0.01 unit of jack bean α-mannosidase or (D) 10 *mM* potassium phosphate buffer, pH 7.1, containing 0.01 unit of hen oviduct β-mannosidase. Mixtures B, C and D were incubated at 30° for 48 hr and then subjected to electrophoresis; mixture A was subjected to electrophoresis without incubation at 30°. *MAN*, D-mannose; *MAN-1-PO$_4$*, α-D-mannopyranosyl phosphate. β-Mercaptoethanol was included in the reaction mixtures to suppress the formation of the radioactive material which migrated near α-D-mannopyranosyl phosphate in samples that were incubated for 48 hr (cf. A and B or D). This material presumably arose by the oxidation of the aldehydic groups in the fragments (products of triphenylphosphine reduction of ozonides) to carboxylic acid functions.

phosphodiester link between the [^{14}C]mannosyl residue and the apolar portion of the molecule remained intact throughout the degradative procedure.

The anomeric configuration of the [^{14}C]mannosyl residues in each set of fragments could be investigated by use of jack bean α-mannosidase and hen

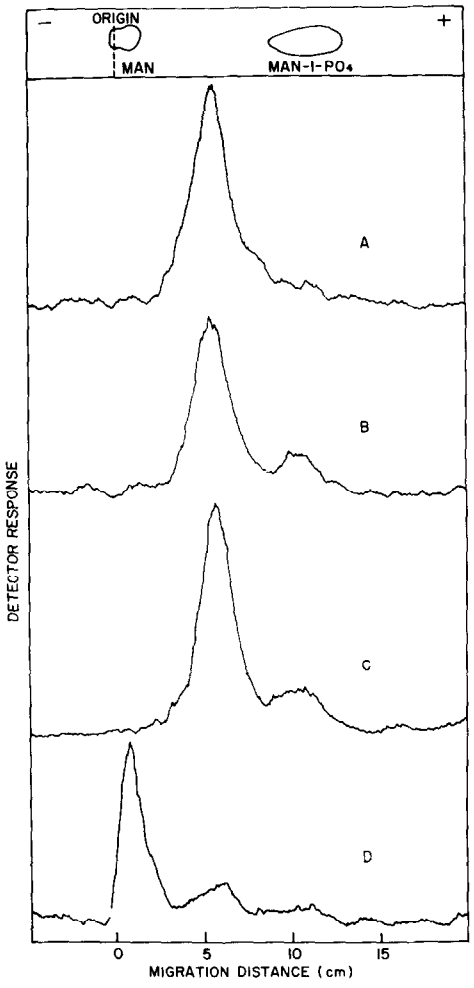

FIGURE 3. Treatment of the ozonolytic fragments from pancreatic dolichyl [^{14}C]mannopyranosyl phosphate with α- and β-mannosidases. Reaction mixtures were prepared and processed as described in the legend to Fig. 2 with the exception that the 100 *mM* sodium acetate buffer, pH 5.0, was supplemented with 0.3% (w/v) Triton X-100 in addition to 30 *mM* β-mercaptoethanol. The detergent was necessary because pancreatic lipids formed emulsions in the aqueous extracts of the ozonolysis mixtures. The abbreviations were the same as those used in Fig. 2.

oviduct β-mannosidase since preliminary experiments showed that the enzymes cleaved the α and β anomers of citronellyl D-mannopyranosyl phosphate, respectively (6,13). Incubation of the products obtained from authentic dolichyl α-D-[^{14}C]mannopyranosyl phosphate with α-mannosidase yielded a radioactive compound which did not migrate during electrophoresis (Fig. 2C) and which behaved as D-mannose upon paper chromatography. Negligible release of mannose was observed during the same interval in similar reaction mixtures containing β-mannosidase (Fig. 2D) or no mannosidase (Fig. 2B). In contrast, the radioactivity associated with the fragments from the pancreatic mannolipid was converted to a neutral form by the action of β-mannosidase (Fig. 3D); the released compound moved with D-mannose during paper chromatography. No release was observed in the α-mannosidase-treated sample (Fig. 3C) or in the control lacking enzyme (Fig. 3B).

These data like those from alkali decomposition studies (13) indicate that the mannosyl residue in the pancreatic lipid occurs as the β-anomer and imply that anomeric inversion takes place during the enzymatic transfer of mannose from GDP-α-mannose to dolichyl phosphate. An anomeric inversion also occurs in the formation of dolichyl β-D-glucopyranosyl phosphate from UDP-α-glucose by liver microsomes (14). In contrast, the enzymatic synthesis of P^1-2-acetamido-2-deoxy-D-glucopyranosyl P^2-dolichyl pyrophosphate presumably proceeds without inversion (15).

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