PROPERTIES OF THE REACTION OF GDP-MANNOSE WITH ENDOGENOUS POLYISOPRENYLPHOSPHATES OF LIVER MEMBRANES

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SUMMARY: Two labeled mannolipids were formed when a rat liver membrane fraction was incubated with GDP-[^{14}C]mannose: dolichyl mannosyl phosphate (DMP) and a compound with the same R_f as mannosyl retinyl phosphate, in ratio 20:1. When the reaction was carried out at pH 6.3, DMP was practically the only mannolipid formed. The uptake of mannose into DMP was tested for its dependence on membrane-enzyme concentration: at enzyme concentrations of <0.8 mg/ml, DMP formation was near-linear with time (over a 75-min period). Under these conditions, mannose uptake into DMP increased with increasing enzyme concentration. Transfer of mannose from DMP to endogenous acceptors (dolichyl pyrophosphate oligosaccharides and protein) was 40% in presence of GDP-mannose and 85% in its absence, thus showing a near 1:1 molar transfer.

Polyisoprenols, in the form of phosphate esters, are now well established as acceptors of sugar nucleotides when sugar is transferred to oligosaccharides and glycoproteins. Mammalian systems utilize dolichyl phosphate (DP), which accepts mannose from GDP-mannose to form dolichyl mannosyl phosphate (DMP) (1). Retinol can function similarly as a sugar acceptor, as shown *in vivo* (2,3), or with retinyl phosphate *in vitro* (4). The product of this reaction was identified as mannosyl retinyl phosphate (4).

Several investigators (3,5,6) have found that the uptake of sugar into DMP continues only for very short periods (generally 5 min or less); then, after reaching a peak, sugar incorporated in lipid declines rapidly. This decline is caused not only by the action of hydrolases, but also by transfer of sugar from DMP to endogenous acceptors--dolichyl oligosaccharides (DO) and glycoprotein (GP) (7). Vessey and Zakim (8) used ATP or AMP to inhibit pyrophosphatase and thus obtained uptake of mannose into lipid

and DO and GP acceptors for longer periods (8 min).

This report demonstrates that, with much lower enzyme concentrations than heretofore reported, the reaction can be made to continue for 40 min in linear fashion. Under these conditions, transfer of mannose from DMP (in absence of GDP-mannose) to DO and GP acceptors was over 85%.

METHODS

Minced rat livers were homogenized in 3 vol of buffer (50 mM Tris [pH 7.6], 5 mM $MgSO_4$, 25 mM KCl, 5 mM EDTA and 0.25 M sucrose) and centrifuged at 20,000 × *g* for 20 min. The supernatant was decanted and 6-ml portions of it were layered over a discontinuous sucrose gradient (2.5 ml of 0.5 M sucrose layered over 3.0 ml of 1.8 M sucrose in the homogenizing buffer). After centrifugation at 105,000 × *g* for 4 hr, the membrane fraction at the interface was collected and stored at -30°--a temperature which maintains its activity for at least 3 mon. Before each incubation, the thawed fraction was repelleted in homogenizing buffer at 105,000 × *g* for 60 min. The pellet was resuspended by gentle homogenization. Incubations were done at 37° with conditions described under the figures. The reaction was stopped with 3 ml of cold 7% trichloroacetic acid (TCA). The control consisted of incubation mix without enzyme. Trypsin (2 mg) was added as carrier protein. The precipitate was centrifuged and the pellet resuspended in 7% TCA and recentrifuged. To the resulting pellet was added 2 ml of chloroform-methanol (2:1, by vol) and 0.2 vol of 0.9% saline; the suspension was then mixed. Three phases resulted: the upper, aqueous phase was discarded; the lower, lipid phase was placed in a scintillation vial for radioactivity determination after removal of solvent; and the interphase material, containing DO and GP (9), was re-extracted with chloroform-methanol (2:1) after addition of saline, as above. The resulting lower phase was added to the lipid fraction. The upper phase was again discarded and the interphase material was solubilized in 1 ml of 0.2 N NaOH at 50° for 15 hr, neutralized, and counted in Triton X-100 scintillator solution. Counting was done with a Beckman LSC 250 liquid scintillation counter. GDP-[^{14}C]mannose (sp. act., 245 μCi/μmole) was obtained from New England Nuclear Corp., Boston, MA; GDP-mannose from C.F. Boehringer, Mannheim, Germany; Zonyl A from E.I. du Pont de Nemours Co., Wilmington, DE.

RESULTS AND DISCUSSION

As previously reported from our laboratory (4), two labeled mannolipids in ratio 20:1 were detectable by thin-layer chromatography (TLC) when GDP-[^{14}C]mannose was incubated with the rat liver membrane fraction described above. The more abundant mannolipid was identified as DMP (4); the other had an R_f on TLC

identical to that of authentic MRP (prepared enzymatically from retinyl phosphate and GDP-mannose) (4). We have now determined (Fig. 1) that DMP formation has a broad pH optimum (6.0-6.8), while the MRP-like compound displays a sharp peak at pH 6.5. Therefore, all reactions described in the figures were performed

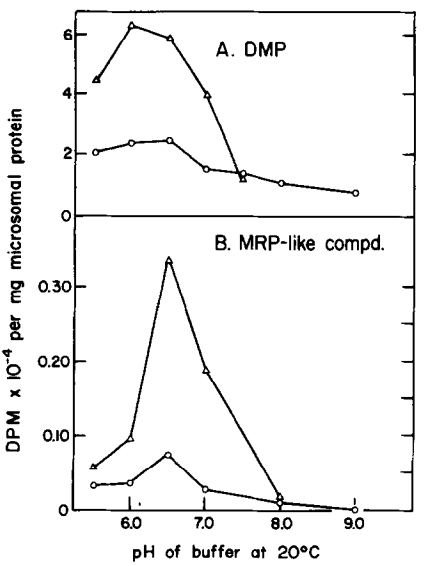

Fig. 1. Effect of pH and Mn^{++} on the formation of DMP and MRP-like compound upon reaction of GDP-[^{14}C]mannose and endogenous membrane acceptors. Assays for each data point contained 0.216 mg of membrane protein in 0.15 ml homogenizing buffer, to which were added the following compounds (to give the indicated concentrations) in a final volume of 0.30 ml: sodium cacodylate (pH 5.5-7.0) or Tris-HCl (pH 7.5-9.0) buffers, 50 mM; NaF, 3.0 mM; Zonyl A (adjusted to pH 7.0), 0.075%; GDP-[^{14}C]mannose, 4.05 µM (0.2 µCi); ATP, 1.8 mM (premixed with equimolar $MgCl_2$); $MnCl_2$, 8 mM (Δ) *or* $MgCl_2$, 1.8 mM (O). After 60 min, the reaction was stopped by addition of 5 vol of cold chloroform-methanol (2:1). Water was added to make the aqueous phase 0.6 ml. Trypsin (2 mg) was added as carrier protein. Samples were mixed and centrifuged, causing the appearance of three phases. The upper (aqueous) phase was discarded; the lower (organic) phase and interphase were washed with 0.2 vol of 0.9% saline. The organic phase was then concentrated to 25 µl and applied to a silica gel G thin-layer plate under N_2 gas. The plate was developed in chloroform-methanol-water 65:25:4 in an N_2-flushed tank in dim light. After drying, the plate was exposed to Kodak NS2T X-ray film for 2 wk. DMP and MRP spots were located (R_f 0.5 and 0.25, respectively) and scraped from the plate into counting vials; the silica gel was heated to 50° for 2 hr in 2 ml of 0.2 M HCl and then neutralized. The released radioactivity was determined after addition of 15 ml Triton X-100 scintillator.

at pH 6.3 (with the exception of the experiment in Fig. 2, which was done at pH 7.0), with DMP virtually the only mannolipid formed (>97%).

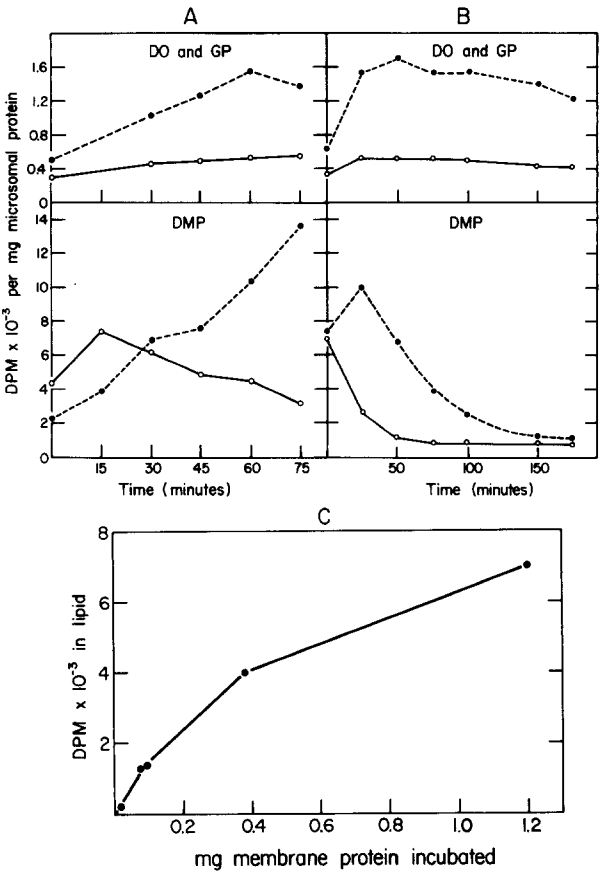

Fig. 2. The uptake, over time, of radioactivity into DMP, DO and GP, relative to membrane protein concentration. The assay for each data point contained membrane protein in 0.10 ml of homogenizing buffer, to which were added the following compounds (to give the indicated concentrations) in a final volume of 0.2 ml: 30 mM Tris-HCl (pH 7.0), 10 mM $MnCl_2$, 2.5 mM EDTA (pH 7.0), 0.91 mM ATP, 0.075% Zonyl A, and 0.1 μCi of 3.14 μM GDP-[^{14}C]mannose. (The homogenizing buffer for experiment B is described under METHODS; the same buffer, but without EDTA, was used for experiment A.) The reactions were stopped by the addition of 3 ml of cold 7% TCA and processed as described in METHODS. Membrane protein concentrations per 0.20 ml incubation: A, 0.122 mg (●---●); 0.660 mg (O——O). B, 1.46 mg (O——O); 0.38 mg (●---●). C, The dependence of uptake of radioactivity into lipid on membrane protein concentration. The incubation conditions were identical to those described for Fig. 2B, except that incubation lasted 25 min with the indicated amounts of protein per 0.20 ml. The amount of radioactivity incorporated into lipid at zero time (Fig. 2B) was substracted from each value as background.

We studied the effect of membrane-enzyme concentration on formation of DMP. Specific enzyme activity actually decreased with increasing concentration of the fraction. A similar anomaly was reported by Martin and Thorne (3), though not explicitly recognized. However, by keeping the enzyme concentration low enough (<0.8 mg protein/ml), we observed a nearly linear increase in labeled mannose uptake into lipid over 75 min (Fig. 2A). Uptake also increased with increasing concentration of the incubated membrane protein (Fig. 2C) when determined by 25-min incubations.

ATP was essential for this reaction (10), presumably to protect GDP-mannose from hydrolysis (8). We found that NaF provided an additional protective effect (Fig. 3) and led to a linear in-

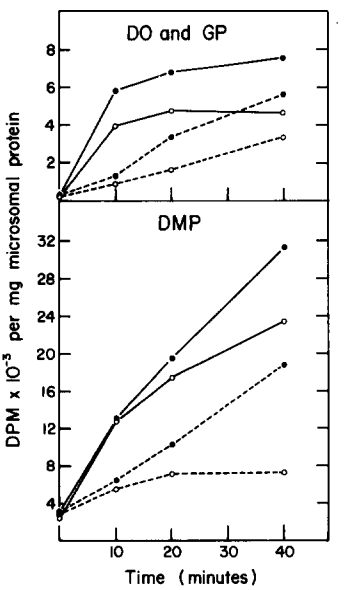

Fig. 3. The effects of NaF and ATP on [^{14}C]mannose uptake into DMP, DO and GP. The incubation for each data point contained 0.153 mg of membrane protein in 0.10 ml of homogenizing buffer, to which were added the following compounds (to give the indicated concentrations) in a final volume of 0.2 ml: 60 mM sodium cacodylate (pH 6.3), 10 mM $MnCl_2$, 0.075% Zonyl A (pH 6.5), and 0.2 μCi of 4.05 μM GDP-[^{14}C]mannose. Other additions were: None (O---O); 1.82 mM ATP (O——O); 3 mM NaF (●---●); 3 mM NaF and 1.82 mM ATP (●——●). The reactions were stopped by the addition of 3 ml of cold 7% TCA and processed as described in METHODS.

crease in mannose uptake into DMP, which lasted at least 40 min. The optimum concentration of GDP-mannose was found to be 3.25 μM--similar to that described by Waechter *et al.* (5)--and showed a K_m of 0.49 μM.

Transfer of mannose from DMP to endogenous acceptors (DO and GP) has not been reported to exceed 20% (8). Under our conditions of low enzyme concentration, however, a 40% transfer occurred in the first 20 min (Fig. 3) when GDP-[^{14}C]mannose was present throughout the reacton (to replenish the [^{14}C]mannose in DMP during mannose transfer to DO and GP acceptors). Furthermore, if the [^{14}C]DMP was made first, and GDP-[^{14}C]mannose then removed, the transfer reached 85% upon reincubation. A nearly 1:1 molar transfer of mannose occurred then, from lipid to DO and GP (Fig. 4). This almost equivalent loss of [^{14}C]mannose from lipid and incorporation of [^{14}C]mannose into acceptors was observed during the first 10 min of incubation (Fig. 4). Independent tests revealed that, prior to reincubation, the washed pellet contained 98% of the normally extractable DMP. Because of the near-100% transfer, one can conclude that the observed loss of radioactive mannose from DMP and the simultaneous gain in DO and GP are not merely fortuitous: they must indicate a true transfer.

This reaction, as opposed to that of mannolipid formation, was not stimulated by ATP. On the other hand, both Mn^{++} and F^- more than tripled the transfer from lipid to acceptors. The decline in label in acceptors over time (Fig. 5) with the presence (but not the absence) of Mn^{++} is of interest: glycoprotein mannosidases are known to be stimulated by this ion (11).

Contrary to the findings of Vessey and Zakim (8), we have found that the reaction between GDP-mannose and endogenous DP to

produce DMP is linear with time and therefore zero-order, *i.e.*, so long as the membrane-enzyme concentration remains low (<0.8 mg protein/ml). Hence endogenous DP is not a limiting factor in the reaction. The reaction rate (1.32 pmoles mannose incorporated into DMP/min/mg membrane protein) resembled that reported by Vessey and Zakim (7.6 pmoles/8 min/mg protein). The total 40-min uptake of mannose into lipid and acceptors was 66 pmoles/mg pro-

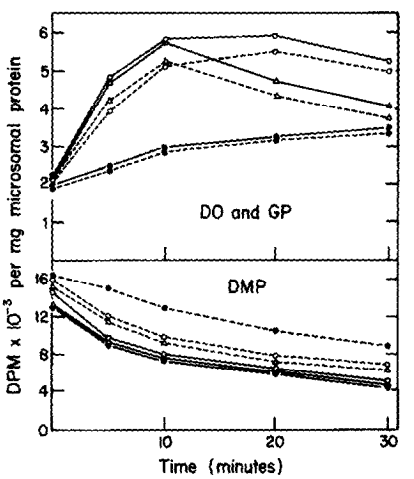

Fig. 4. The transfer of [^{14}C]mannose from DMP to DO and GP acceptors. Incubation I: The formation of lipid-[^{14}C]mannose. The incubation contained 6.70 mg of membrane protein suspended in 1.0 ml of homogenizing buffer, to which were added the following compounds (to give the indicated concentrations) in a final volume of 2.0 ml: 50 mM sodium cacodylate (pH 6.3), 1.8 mM ATP (pH 7.0, premixed with equimolar $MgCl_2$), 8 mM $MnCl_2$, 0.075% Zonyl A (pH 6.5), and 2.0 μCi of 4.05 μM GDP-[^{14}C]mannose. The reaction was incubated for 10 min and then diluted with 11 ml of cold homogenizing buffer containing 4.05 μM nonradioactive GDP-mannose, and the membranes spun down at 900 × *g* for 25 min. The pellet (4.31 mg protein) was resuspended in 1.25 ml of the homogenizing buffer.

Incubation II: The transfer of [^{14}C]mannose from [^{14}C]DMP to DO and GP. The incubation system contained 0.25 ml (0.863 mg protein) of the preincubated membrane suspension to which were added the following compounds (to give the indicated concentrations) in a final volume of 0.5 ml: 50 mM sodium cacodylate (pH 6.3), 0.075% Zonyl A (pH 6.5), and either--A, no addition (●---●); B, 1.82 mM ATP (pH 7.0, premixed with equimolar $MgCl_2$) (●——●); C, 8 mM $MnCl_2$ (Δ---Δ); D, 1.82 mM ATP (pH 7.0, premixed with equimolar $MgCl_2$) and 8 mM $MnCl_2$ (Δ——Δ); E, 1.82 mM ATP (pH 7.0, premixed with equimolar $MgCl_2$), 8 mM $MnCl_2$ and 3 mM NaF (O——O); or F, 8 mM $MnCl_2$ and 3 mM NaF (O---O). At the times indicated, 0.1 ml was taken out of the incubation, precipitated in 3.0 ml of cold 7% TCA, and processed as described in METHODS.

tein, however, with no sign of leveling off (Fig. 3). Vessey and Zakim (8) determined the pool of DP (and therefore the limit of uptake) to be 7.6 pmoles/mg microsomal protein. Martin and Thorne (3), on the other hand, estimated the total amount of dolichol in a liver microsomal fraction to be 58 pmoles/mg protein, a figure comparable to our value for the available dolichol reacting as DP.

The question of why low enzyme concentration leads to a proportionately greater reaction remains unanswered. One clue appears in Fig. 2, which shows a rapid disappearance of DMP at high enzyme concentrations. Possibly, under these conditions, the substrates or products become available to hydrolytic enzymes, which no doubt contaminate the crude membrane fraction used as a source of enzyme.

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