Rat liver microsomes catalyse mannosyl transfer from GDP-D-mannose to retinyl phosphate with high efficiency in the absence of detergents

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In the absence of detergent, the transfer of mannose from GDP-mannose to rat liver microsomal vesicles was highly stimulated by exogenous retinyl phosphate in incubations containing bovine serum albumin, as measured in a filter binding assay. Under these conditions 65% of mannose 6-phosphatase activity was latent. The transfer process was linear with time up to 5 min and with protein concentration up to 1.5 mg/0.2 ml. It was also temperature-dependent. The microsomal uptake of mannose was highly dependent on retinyl phosphate and was saturable against increasing amounts of retinyl phosphate, a concentration of 15 μM giving half-maximal transfer. The uptake system was also saturated by increasing concentrations of GDP-mannose, with an apparent K_m of 18 μM . Neither exogenous dolichyl phosphate nor non-phosphorylated retinoids were active in this process in the absence of detergent. Phosphatidylethanolamine and synthetic dipalmitoylglycerophosphocholine were also without activity. Several water-soluble organic phosphates (1.5 mM), such as phenyl phosphate, 4-nitrophenyl phosphate, phosphoserine and phosphocholine, did not inhibit the retinyl phosphate-stimulated mannosyl transfer to microsomes. This mannosyl-transfer activity was highest in microsomes and marginal in mitochondria, plasma and nuclear membranes. It was specific for mannose residues from GDP-mannose and did not occur with UDP-[^3H]galactose, UDP- or GDP-[^{14}C]glucose, UDP-*N*-acetyl[^{14}C]glucosamine and UDP-*N*-acetyl[^{14}C]galactosamine, all at 24 μM . The mannosyl transfer was inhibited 85% by 3 mM-EDTA and 93% by 0.8 mM-amphotycin. At 2 min, 90% of the radioactivity retained on the filter could be extracted with chloroform/methanol (2:1, v/v) and mainly co-migrated with retinyl phosphate mannose by t.l.c. This mannosylphospholipid was shown to bind to immunoglobulin G fraction of anti-(vitamin A) serum and was displaced by a large excess of retinoic acid, thus confirming the presence of the β -ionone ring in the mannosylphospholipid. The amount of retinyl phosphate mannose formed in the bovine serum albumin/retinyl phosphate incubation is about 100-fold greater than in incubations containing 0.5% Triton X-100. In contrast with the lack of activity as a mannosyl acceptor for exogenous dolichyl phosphate in the present assay system, endogenous dolichyl phosphate clearly functions as an acceptor. Moreover in the same incubations a mannosylphospholipid with chromatographic properties of retinyl phosphate mannose was also synthesized from endogenous lipid acceptor. The biosynthesis of this mannosylphospholipid (retinyl phosphate mannose) was optimal at MnCl_2 concentrations between 5 and 10 mM and could not be detected below 0.6 mM- MnCl_2 , when synthesis of dolichyl phosphate mannose from endogenous dolichyl phosphate was about 80% of optimal synthesis. Under optimal conditions (5 mM- MnCl_2) endogenous retinyl phosphate mannose represented about 20% of dolichyl phosphate mannose at 15 min of incubation at 37°C.

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Abbreviations used: medium A, 50 mM-Tris/HCl

(pH 7.6), containing 5 mM- MgCl_2 , 25 mM-KCl and 0.25 M-sucrose; Dol-P, dolichyl phosphate; Dol-P-Man, dolichyl phosphate mannose; Ret-P, retinyl phosphate; Ret-P-Man, retinyl phosphate mannose.

A molecular function for vitamin A in mannosyl-transfer reactions of mammalian membranes has been supported by previous work (De Luca, 1977). Although Ret-*P* was shown to be synthesized by intestinal cells *in vitro* (Frot-Coutaz *et al.*, 1976) and Ret-*P*-Man was purified from rat liver and intestine (Barr & De Luca, 1974; Masushige *et al.*, 1978), it is still unclear whether and how these derivatives of vitamin A play a role in the expression of the biological activity of the vitamin. Particularly, Ret-*P* has been considered to be a poor substrate for a GDP-mannose:dolichyl phosphate mannosyl-transferase in cell-free membranes, because of less mannosyl transfer to Ret-*P* than to Dol-*P* in the presence of the detergent (Lennarz, 1980). However, it has been reported that mannose incorporation into Ret-*P*-Man *in vivo* is even more efficient than into Dol-*P*-Man (De Luca, 1977; Sato *et al.*, 1978) and mannose incorporation into lipid extracts of vitamin A-deficient hamster liver was greatly enhanced by the vitamin treatment (De Luca *et al.*, 1975). In the present paper, we report an extremely efficient transfer of mannose to Ret-*P* by cell-free membranes in the absence of detergents, 100-fold higher than in the presence of Triton X-100 (Shidoji *et al.*, 1981); we characterize the mannosyl lipid by a specific anti-(vitamin A) antiserum and present a simple filter assay for Ret-*P*-Man in the absence of Triton X-100.

Materials and methods

Materials

Bovine serum albumin, phenyl phosphate, retinyl palmitate (type IV), *O*-phospho-L-serine, L- α -phosphatidylcholine (type III-E), L- α -phosphatidylethanolamine (type V), Dol-*P* (grade III), phosphocholine, GDP-mannose, UDP-galactose and UDP-glucose, UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine were all obtained from Sigma. Dipalmitoyl-L- α -glycerophosphocholine was obtained from Calbiochem; 4-nitrophenyl phosphate from BDH Chemicals, Poole, Dorset, U.K.; amphomycin from Bristol Myers; retinyl phosphate was synthesized by Carol S. Silverman-Jones (Bhat *et al.*, 1980); millipore filters were obtained from Millipore, Boston, MA, U.S.A.

Radioactive materials

The following products were obtained from New England Nuclear Corp., Boston, MA, U.S.A.: GDP-[^3H]mannose (sp. radioactivity 10 Ci/mmol); GDP-[^{14}C]mannose (0.2 Ci/mmol); UDP-[^3H]galactose (14.5 Ci/mmol); UDP-[^{14}C]glucose (0.24 Ci/mmol), GDP-[^{14}C]glucose (240 Ci/mmol); UDP-*N*-D-[^{14}C]glucosamine (49 Ci/mol) and UDP-*N*-acetyl-D-[^{14}C]galactosamine (47 Ci/mol).

Preparation of rat liver microsomal vesicles

Normal male rats, Osborne-Mendel strain (weighing 150–200 g), starved overnight, were killed by bleeding under light diethyl ether anaesthesia. The livers were homogenized with 2 vol. of medium A by a glass/Teflon homogenizer. After centrifugation at 2500 *g* for 20 min, the resultant supernatant was centrifuged at 105 000 *g* for 60 min. The pellet was resuspended in medium A. This crude microsomal preparation was used for most assays for mannosyl-transfer reactions. The mannosyl-transfer activity of this preparation did not change after 1 month of storage in liquid N₂. Subcellular fractionation of rat liver was done by the method of Fleischer & Kervina (1974).

Filter assay for mannosyl-transfer reaction

The standard incubation was conducted as follows: 0.4 μCi of GDP-[^3H]mannose (or 0.1 μCi of GDP-[^{14}C]mannose) and 10 μg (150 μM) of Ret-*P* in 99% methanol were transferred to test tubes. After removing the solvent under an N₂ stream, 4 mg of bovine serum albumin/ml, GDP-D-mannose (24 μM final concn.), 30 mM-Tris/HCl buffer (pH 8), 2.5 mM-MnCl₂, 8 mM-NaF, 2 mM-ATP, 5 mM-AMP and 0.9 mg of the microsomal protein were added in a final volume of 200 μl . The mixture was incubated at 37°C for 2 min. The reaction was stopped by addition of 1 ml of ice-cold medium A and immediately poured on the MF-millipore (HA, 0.45 μm) on the filter manifold (Millipore), allowing processing of several samples. The filter was washed with an additional 1 ml of medium A to remove unbound radioactive materials. The radioactivity retained on the filter was measured in 10 ml of Aquafuor (New England Nuclear); counting efficiency for ^3H was 30% and for ^{14}C was 70%.

Protein A–Sephacel column chromatography

After 2 min incubation of GDP-[^3H]mannose (0.1 μCi) with 0.15 mM-Ret-*P* in the bovine serum albumin/rat liver microsomal system, the radioactive compounds on the Millipore filter were extracted with 5 ml of chloroform/methanol (2:1, v/v) twice. The resultant extract was applied to DEAE-Sephacel, equilibrated with 99% methanol, to remove the excess unreacted Ret-*P* (Sasak *et al.*, 1979). After removing the solvent under an N₂ stream, the 10 mM-ammonium acetate eluate was incubated with anti-(vitamin A) rabbit antiserum (0.5 ml) (a generous gift from Dr. George H. Wirtz, Department of Biochemistry, West Virginia University Medical Center, Morgantown, WV, U.S.A.) in the ice-box for 20 min. A portion of the mixture was applied to a column (0.8 cm $\times$ 1.5 cm) of Protein A–Sephacel CL-4B (Pharmacia, Uppsala, Sweden), equilibrated with 30 mM-Tris/HCl buffer, pH 8.0, containing 0.1% Triton X-100. After washing the

gel with 5 vol. of the starting buffer, the radioactivity retained on the column was eluted with 10 μ M-retinoic acid in the buffer. As a control experiment, non-immune rabbit serum (GIBCO, Grand Island, NY, U.S.A.) was used, instead of anti-(vitamin A) serum. Fractions (10 drops) were collected and radioactivity in each fraction was measured in 10 ml of Aquafleur.

Ret-P-Man and Dol-P-Man synthesis from endogenous lipid acceptors

Rat liver microsomes were prepared as usual, except that homogenization was conducted in saline (0.9% NaCl) at 0°C, instead of medium A, to avoid $MgCl_2$ present in medium A at 5 mM concentration. The microsomes were immediately suspended in saline and stored in liquid N_2 until used. After thawing, the equivalent of 1 mg of microsomal protein was used in the same bovine serum albumin-containing incubation as described below, except that 0.2 μ Ci of GDP-[^{14}C]mannose (5 μ M final concn.) was used with $MnCl_2$ at the following concentrations (mM): 0, (a); 0.025; (b); 0.125, (c); 0.625, (d); 2.5, (e); 5, (f); 10, (g); 50, (h). Incubations were run in duplicate at 37°C and stopped at 15 min by addition of 5 vol. (1 ml) of chloroform/methanol (2:1, v/v) to yield two phases. The lower phase, containing about 35% of total Ret-P-Man, was used for t.l.c. in chloroform/methanol/water (45:35:6, by vol.). The silica gel (0.5 cm sections) was collected into counting vials and radioactivity was determined after addition of 0.25 ml of methanol and 10 ml of Betafluor (National Diagnostics, Sommerville, NJ, U.S.A.). Fluorography was carried out on a Kodak made film XR-5 and an exposure of 4 days was used after spraying with Enhance (New England Nuclear).

Other procedures

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Retinyl phosphate was measured spectrophotometrically on the basis of $A_{1cm, 325}^{1\%}$ (in methanol) = 1440. T.l.c. of the lipid extract from the microsomal vesicles was done as previously reported (Sasak *et al.*, 1979). Mannose 6-phosphatase was measured as a marker enzyme of microsomal membrane (Hanover & Lennarz, 1979), in the presence or the absence of Triton X-100.

Results

Mannosyl transfer from GDP-mannose to microsomal vesicles

Time course and temperature dependence (Fig. 1). In the presence of 0.15 mM-Ret-P at 37°C, the

Fig. 1. Time course of mannosyl transfer by rat liver microsomal vesicles

GDP-[3H]mannose (0.4 μ Ci, 24 μ M) was incubated with 0.92 mg of rat liver microsomal protein at 0°C (----) or 37°C (—) in the presence (○) or absence (●) of 10 μ g (0.150 mM) of Ret-P, for the indicated times. Radioactivity retained on the filter was plotted against the incubation time. The details of the filter assay for the mannosyl-transfer reaction are described in the Materials and methods section. Each point represents the average of two incubations.

transfer of mannose from GDP-mannose to microsomes proceeded linearly for about 5 min and then showed a slower increase until 30 min of incubation. By this time, 47% of the initial radioactivity had been transferred to the membrane. At 0°C, there was an obviously smaller rate of transfer, which proceeded linearly for at least 30 min. In the absence of Ret-P, less than 2% of the transfer activity was detectable at either 0°C or 37°C. In subsequent experiments, the initial rate of this transfer reaction was measured by 2 min incubation at 37°C.

Protein dependence. Fig. 2 shows a linear protein dependence for the transfer process up to approx. 1.5 mg of protein per 0.2 ml of the incubation mixture. At higher protein concentrations no further increase in initial (2 min) transfer rate was observed. There was no increase in the mannosyl transfer in the absence of Ret-P up to 5 mg of microsomal

Fig. 2. Protein-concentration dependence of mannosyl transfer in rat liver microsomes

The amounts of rat liver microsomal protein were varied up to 4.6 mg/incubation in the presence (○) or absence (●) of 10 μ g (0.150 mM) of Ret-*P* and in the presence of 8.3 μ g (0.150 mM) of retinol (□). The mixture was incubated at 37°C for 2 min. Each point represents an average of duplicate incubations.

Fig. 3. Ret-*P*-concentration dependence of mannosyl transfer

GDP-[14 C]mannose (0.4 μ Ci, 24 μ M) was incubated with 0.9 mg of rat liver microsomal protein at various concentrations of Ret-*P* (○) or Dol-*P* (●) at 37°C for 2 min. Each point represents an average of duplicate incubations.

protein/0.2 ml (Fig. 2). This mannosyl-transfer reaction was further characterized by using 0.9 mg of the protein per incubation.

Ret-*P*-dependence. The microsomal uptake of mannose was highly dependent on Ret-*P* and was saturable against increasing amounts of Ret-*P*, a concentration of 15 μ M giving half-maximal transfer (Fig. 3). However, this mannosyl transfer was not observed when exogenous Dol-*P* replaced Ret-*P* in the incubation mixture up to 0.3 mM (Fig. 3). Moreover, retinol (Fig. 2), retinyl palmitate, retinoic acid, synthetic dipalmitoylglycerophosphocholine and *Escherichia coli* phosphatidylethanolamine were all without effect when assayed at the same concentrations as Ret-*P* (Table 1). Exceptionally, egg-yolk phosphatidylcholine had 38% of the activity of Ret-*P* (Table 1).

GDP-mannose specificity. The transfer of mannose from GDP-mannose to microsomal membranes was a saturable process with increasing amounts of the sugar nucleotide. Lineweaver-Burk plots gave a K_m value of 18 μ M for GDP-mannose (Fig. 4). This transfer process is highly specific for GDP-mannose (0.520 nmol/min per mg of protein was transferred); other sugar nucleotides, such as UDP-[3 H]galactose, UDP-[14 C]glucose, GDP-[14 C]glucose, UDP-[14 C]-GlcNAc and UDP-[14 C]GalNAc all failed to function as substrates at 24 μ M.

Cofactors and inhibitors. The reaction required bovine serum albumin. Optimal bovine serum albumin concentration was between 2 and 5 mg/ml (results not shown). It was greatly enhanced also by bivalent manganese (Table 2). Exogenous ATP and AMP stimulated the transfer reaction (Table 2). This transfer was inhibited by 3 mM-EDTA and by 0.8 mM-amphotycin (Table 3). However, phenyl phosphate, an exogenous acceptor of mannose (Kato *et al.*, 1980) or other water-soluble organic phosphate such as 4-nitrophenyl phosphate, phosphocholine and phosphoserine did not inhibit the Ret-*P*-mediated mannosyl transfer at 10 times the concentration of Ret-*P*.

Subcellular distribution. Catalysing activity for this mannosyl-transfer process was highest in microsomes (Fig. 5). The activity in other fractions was parallel to the level of mannose 6-phosphatase in each fraction.

Demonstration of Ret-*P*-Man as a product of the mannosyl-transfer process

T.l.c. After 2 min incubation, 90% of the radioactivity retained on the filter paper was extracted by 5 ml of chloroform/methanol (2:1, v/v), in three consecutive extractions. T.l.c. analysis of this lipid extract showed the majority of the radioactivity to chromatograph as Ret-*P*-Man (Fig. 6). Radioactivity was also found in the areas of mannose (R_F 0.25) and mannose 1-phosphate (R_F 0). These

Table 1. *Study of different lipids*
Results for mannosyl transfer are means $\pm$ S.D.

Lipids	Amount (μ g)	Mannosyl transfer (c.p.m./2 min per 0.92 mg of protein)	Proportion of control value (%)
Experiment I			
Ret-P	10	54 900 $\pm$ 6700	100.0
Retinyl palmitate	13.2	6900 $\pm$ 1000	12.7
Phosphatidylcholine (type III-E, egg yolk)	20.0	21 000 $\pm$ 2000	38.3
Phosphatidylethanolamine (type V, <i>E. coli</i>)	20.0	6700 $\pm$ 700	12.3
Experiment II			
Ret-P	10.0	60 400 $\pm$ 3400	100.0
None	—	4900 $\pm$ 330	8.1
Dipalmitoylglycerophosphocholine	20.0	5400 $\pm$ 280	9.1
Retinoic acid	8.0	5200 $\pm$ 110	8.6

Fig. 4. *Effect of GDP-mannose concentration on the mannosyl-transfer reaction in rat liver microsomal vesicles*

The incubation was conducted at 37°C for 2 min as described in the Materials and methods section with various amounts of GDP-mannose up to 80 μ M in the presence of 10 μ g (0.15 mm) of Ret-P. The inset shows data from the main Figure plotted double-reciprocally. Each point represents the average of duplicate incubations.

peaks are usually derived from breakdown of Ret-P-Man on t.l.c. (Sasak *et al.*, 1979). A radioactive product behaving as dolichyl phosphate mannose (R_F 0.9) constituted about 5% of the total lipid extractable radioactivity (Fig. 6). Analyses of the lipid extract at 30 min incubation showed a great increase in Ret-P-Man but not in Dol-P-Man formation (results not shown).

Interaction with anti-(vitamin A) serum

Anti-(retinoic acid) rabbit antiserum, a generous gift from Dr. George Wirtz is specific for the

β -ionone ring and cannot distinguish between terminal functional groups such as the alcoholic group in retinol, the carboxy group in retinoic acid, the aldehyde in retinal and the phosphate in Ret-P (Wirtz, 1981). As a further proof that the major mannosylated product has a β -ionone ring in its molecule, interaction of the total lipid-extractable radioactive compounds with anti-(vitamin A) serum was studied. As shown in Fig. 7(a), 70% of the radioactivity eluted from DEAE-Sephacel (see in the Materials and methods section) was bound to the immunoglobulin of anti-(vitamin A) serum and retained by the Protein A-Sepharose column. This radioactivity could be eluted by 10 μ M-retinoic acid in the elution buffer. Non-immune rabbit serum failed to interact with the mannolipid (Fig. 7b).

Different effects of Triton X-100 on two mannosyl-transfer reactions

Exogenous Dol-P was unable to increase the radioactivity retained on the filter in the bovine serum albumin/rat liver microsome system (Fig. 3). Therefore we investigated the possibility that the filter assay might miss Dol-P-Man, since microsomal membranes from mammalian livers had been reported to have the enzyme activity catalysing the formation of Dol-P-Man from GDP-mannose and exogenous Dol-P (Behrens *et al.*, 1971; Richards & Hemming, 1972). The formation of mannolipids was followed by t.l.c. analysis of the lipid extracts after incubation in the bovine serum albumin/microsome system with or without detergent. In the absence of detergent Dol-P-Man was synthesized, but no stimulation of Dol-P-Man synthesis by exogenous Dol-P was detected on t.l.c. (Fig. 8c). On the other hand exogenous Ret-P stimulated Ret-P-Man formation (Fig. 8a), consistent with the results of the filter assay (Fig. 3). In contrast, 0.5% Triton X-100 in the bovine serum

Table 2. *Cofactors for mannosyl transfer to microsomes*
The two values refer to duplicate experiments.

	^3H radioactivity (c.p.m./2 min per 0.92 mg of protein)	Proportion of transfer in complete mixture (%)
Complete mixture (see the text)	95 700	100.0
	87 600	
—bovine serum albumin	22 700	25.0
	23 000	
— MnCl_2	12 700	14.5
	13 900	
— NaF	70 900	73.5
	63 900	
—ATP	62 300	57.0
	42 200	
—AMP	63 100	51.7
	31 700

Fig. 5. *Subcellular distribution of mannosyl-transfer activities in rat liver*

Incubations were conducted as described in the Materials and methods section in the presence (open columns) or absence (filled columns) of 0.15 mM Ret-P. The bars represent $\pm$ S.D. from experiments run in triplicate. Abbreviations: Mc, microsomal; Mt, mitochondrial; Nc, nuclear; PM, plasma membranes. Microsomal contaminations are 11.5% in Mt, 23.8% in Nc and 29.0% in PM from mannose 6-phosphatase activity, expressed as percentages of microsomes (100%). Ret-P-Man synthesis is 12% in Mt, 17.7% in Nc and 27.7% in PM.

Fig. 6. *T.l.c. of the lipid extract of the radioactive materials retained on the filter from incubation with $\text{GDP}-[^3\text{H}]\text{mannose}$*

$\text{GDP}-[^3\text{H}]\text{mannose}$ (0.4 μCi) was incubated with 0.9 mg of rat liver microsomal protein in the presence of 10 μg (0.15 mM) of Ret-P. After incubation for 2 min, the mixture was filtered through an MF-millipore filter (0.45 μm). A portion (0.05 ml from 0.2 ml) of the chloroform/methanol (2:1, v/v) extract from the filter was applied to silica-gel 60 F-254 (E. Merck) with a developing solvent of chloroform methanol/water (45:35:6, by vol.). The black spot shows the position of Ret-P used as internal standard and visually detected by using a u.v. lamp.

Table 3. *Effect of inhibitors of Ret-P-mediated mannosyl transfer to rat liver microsomes*Results for ^3H radioactivity are means $\pm$ S.D.

	^3H radioactivity (c.p.m./2 min per 0.92 mg of protein)	Proportion of control value (%)
Control	66 000 $\pm$ 13 200	100
EDTA (3 mM)	9700 $\pm$ 800	14
Amphotycin (0.8 mM)	4400 $\pm$ 300	6

Fig. 7. *Protein A-Sepharose column chromatography of the ^3H -labelled mannolipids incubated with anti-(vitamin A) antiserum (a) and non-immune rabbit serum (b)*

After 2 min incubation of GDP-[^3H]mannose with 0.15 mM-Ret-P in the bovine serum albumin/microsome system, the mannolipids retained on the filter were extracted by chloroform/methanol (2:1, v/v) and applied to a DEAE-Sephacel column to remove excess Ret-P. After removing the solvent, 10 mM-ammonium acetate eluate was incubated with anti-(vitamin A) serum or control rabbit serum. A portion of the incubated mixture was applied to Protein A-Sepharose column, and after washing the gel with the starting buffer, peak II radioactivity was eluted with 10 μM -retinoic acid in the buffer. Recovery of the radioactivity from the column was 110% (a) and 97% (b). Each fraction was equivalent to 10 drops.

Fig. 8. *T.l.c. profiles of lipid extracts from the incubations with or without Triton X-100*

After 2 min incubations of GDP-[^3H]mannose in the bovine serum albumin/microsome systems in the presence of 0.15 mM-Ret-P (a and b) or 0.15 mM-Dol-P (c and d) with (b and d) or without 0.5% Triton X-100 (a and c), 20 vol. of chloroform/methanol (2:1, v/v) were added to stop the reaction. After removing the insoluble materials by centrifugation, the solvent was flash-evaporated. The residues were resolubilized in 100 μl of methanol, and 70 μl out of this methanolic solution was applied to the t.l.c. plate with chloroform/methanol/water (45/35/6, by vol.) as developing solvent. The black spot shows the position of Ret-P.

albumin/microsome incubation mixture permitted enhancement of Dol-P-Man synthesis in the presence of exogenous Dol-P (Fig. 8d), with little

Fig. 9. Linear representation (A) and fluorography (B) of $MnCl_2$ dependence of Ret-P-Man and Dol-P-Man synthesis from endogenous lipid acceptors

Incubations were carried out as described in the Materials and methods section. $MnCl_2$ concentrations (mM) were: 0, (a); 0.025, (b); 0.125, (c) 0.625, (d); 2.5, (e); 5, (f); 10, (g); 50, (h). The positions of mannose, Ret-P-Man and Dol-P-Man are shown. The solvent front was at 14 cm and the solvent was chloroform/methanol/water (45/35/6, by vol.). The lower phase was used for t.l.c.

stimulation of Ret-P-Man synthesis by exogenous Ret-P (Fig. 8b). The apparent difference in the profiles of Fig. 6 and Fig. 8 is due to the larger amount of mannose present in the chloroform/methanol extract (Fig. 8) compared with the relatively small amount retained on the filter (Fig. 6).

Synthesis of Ret-P-Man and Dol-P-Man from endogenous lipid acceptors: differential dependence on bivalent manganese

In as much as the bovine serum albumin-based assay system has greatly increased the amount of Ret-P-Man synthesized from exogenous Ret-P compared with the Triton X-100-based assay, we looked for Ret-P-Man and Dol-P-Man synthesis from endogenous lipid acceptors. Fig. 9(B) shows the t.l.c. patterns of the lipid extracts obtained from incubations containing from 0 (a) to 50 mM- $MnCl_2$ (h). Ret-P-Man synthesis was not detectable in the presence of 0, 0.025 and 0.125 mM- $MnCl_2$ (Figs. 9A and 9B), even though Dol-P-Man synthesis was

about 80% of maximum. Ret-P-Man synthesis could be detected at 0.6 mM and became evident at 2.5 mM- $MnCl_2$, with optimal synthesis obtained at 5 and 10 mM- $MnCl_2$ (Fig. 9B, f and g). Inhibition of both Ret-P-Man and Dol-P-Man synthesis was found at 50 mM- $MnCl_2$ (Fig. 9A). Fig. 9(B) shows the autoradiogram of the parallel and identical set of incubations.

Discussion

The present study has shown that the transfer of mannose from GDP-mannose to Ret-P occurs with a very high efficiency in incubations of rat liver microsomal vesicles, in the presence of bovine serum albumin and without detergent. In this work, we have utilized Millipore filters (0.45 μ m) for the separation of microsomal particles from the medium. Although no information is available about particle size of our microsomal preparation, there was no difference between 0.2 μ m (Nilsson *et al.*, 1973) and

0.45 μm filter in terms of trapping activity for Ret-*P*-Man formed in the microsomal vesicles (results not shown). Since no Ret-*P*-Man was found in the filtrate by t.l.c. analysis, it is obvious that the filter assay recovers most of the Ret-*P*-Man formed by microsomes.

Bovine serum albumin was used in these studies because of the carrier function for this protein for lipophilic compounds (Blaner & Churchich, 1980). The mechanism of bovine serum albumin stimulation for mannosyl-transfer reaction is still not clear, but it can be considered that the protein is aiding the solubilization of Ret-*P* into the buffer, and enhancing the transfer of Ret-*P* from the inner wall of the test tube to the microsomal vesicles, without destruction of the membrane, which would occur if detergents were used as solubilizing agents. In contrast exogenous Dol-*P* is not available to the enzyme under these conditions.

The sugar transfer to microsomes (mostly accounted for by Ret-*P*-Man synthesis at 2 min of incubation in the presence of exogenous Ret-*P*) was highly specific for Ret-*P* and for GDP-mannose as a sugar donor in this assay system. Neither non-phosphorylated retinoids nor phospholipids were able to stimulate the mannosyl-transfer process, suggesting that the reaction requires both the retinol and the phosphate moiety in the Ret-*P* molecule. Of interest is the observation that egg-yolk phosphatidylcholine apparently enhanced the binding of radioactivity to the filter (Table 1). Inasmuch as the radioactivity on the filter from incubations containing egg-yolk phosphatidylcholine was not due to Ret-*P*-Man and stayed at the origin on t.l.c. (results not shown), we speculate that GDP-mannose is entrapped by egg-yolk-phosphatidylcholine liposomes and transferred to microsomal vesicles through a fusion complex. In any event, egg-yolk phosphatidylcholine, as well as other phospholipids (0.15 mM), was unable to stimulate Ret-*P*-Man or Dol-*P*-Man synthesis. Ret-*P* does not increase the transfer of radioactivity to the filter from UDP-[^{14}C]glucose, UDP-[^3H]galactose, GDP-[^{14}C]glucose, UDP-*N*-acetyl[^{14}C]glucosamine and UDP-*N*-acetyl[^{14}C]galactosamine, consistent with previous results (Bergman *et al.*, 1978).

More recently, concentrations of endogenous GDP-mannose have been reported to be in the range of 50–100 μM in rat liver (Akamatsu & Hasegawa, 1980) or cultured hepatocytes (Howe *et al.*, 1980). Therefore the K_m (18 μM) for GDP-mannose of the Ret-*P*-mediated transfer process is less than the physiological concentration of the sugar nucleotide, suggesting the possibility that Ret-*P* may be physiologically active in trapping mannose as Ret-*P*-Man in microsomal membrane from GDP-mannose, the substrate synthesized by a cytosolic enzyme (Coates *et al.*, 1980). This would explain the decrease in the

incorporation of mannose into glycoconjugates of liver from vitamin A-depleted rodents (De Luca *et al.*, 1975) and the decrease in the amount of mannose covalently bound to glycoconjugates of liver of vitamin A-deficient animals (De Luca *et al.*, 1975; Adhikari & Vakil, 1980), particularly since Ret-*P*-Man has been shown to act as a donor of mannose in protein glycosylation (Rosso *et al.*, 1977; Frot-Coutaz *et al.*, 1979; Sasak & De Luca, 1980).

The poor solubility of exogenous Dol-*P* in the aqueous environment of the bovine serum albumin-based incubation explains why exogenous Dol-*P* is a poorer substrate in this mannosyl-transfer reaction, in contrast with previous reports (Tkacz *et al.*, 1974; Wedgwood *et al.*, 1974; Kean, 1977). It should be emphasized that these previous findings are indeed consistent with ours that retinyl phosphate mannosyltransferase in rat liver microsomes displays a 10-times higher K_m for GDP-mannose and a 5–6-fold lower V_{max} than dolichyl phosphate mannosyltransferase in the presence of Triton X-100 (Shidoji *et al.*, 1981). However, we now find that native (undisrupted) microsomal membranes, with about 65% latency in mannose 6-phosphatase activity (results not shown), catalysed Ret-*P*-Man synthesis with high efficiency, and Fig. 8 shows clearly that the detergent stimulated the formation of Dol-*P*-Man, but it markedly lowered Ret-*P*-Man synthesis in the bovine serum albumin/Ret-*P* incubations. We have not excluded the possibility that the detergent might inhibit the formation of a putative bovine serum albumin–Ret-*P* complex, which may be necessary for the reaction, whereas Triton X-100 is necessary for the solubilization of the highly hydrophobic Dol-*P*, which may not be available to the enzyme in the bovine serum albumin incubation.

The amount of Ret-*P*-Man formed increased from 5 pmol/min per mg of protein in the presence of the detergent (Shidoji *et al.*, 1981) to 520 pmol/min per mg of protein in the absence of the detergent. However, the K_m for GDP-mannose was found to be similar (18 μM) to that (13 μM) found in the presence of detergent (Shidoji *et al.*, 1981). Smith *et al.* (1979) have reported an inhibitory effect of Triton X-100 on Ret-*P*-Man synthesis in smooth- and rough-endoplasmic-reticular membranes as the detergent to protein ratio increased from 1 to 5. However, their optimal transfer (0.48 pmol/min per mg of protein) is about 10-times less than the rate obtained by Shidoji *et al.* (1981) in the presence of 0.5% detergent and 1000 times less than that reported here in the bovine serum albumin/Ret-*P* incubations.

Finally Fig. 9 shows that Ret-*P*-Man and Dol-*P*-Man are both synthesized in the bovine serum albumin incubation from endogenous acceptor lipids.

The synthesis of Ret-*P*-Man appears to have an absolute requirement for MnCl_2 with optimal concentrations between 5 and 10 mM. In contrast, the amount of Dol-*P*-Man accumulated at 15 min of incubation in the absence of MnCl_2 was 80% of the maximum obtained at 5–10 mM (Fig. 9). At 5 mM- MnCl_2 , the amount of Dol-*P*-Man accumulated after 15 min per mg of microsomal protein is about 10-fold greater than the amount of Ret-*P*-Man found in the lower phase. Since about 60% of Ret-*P*-Man partitions in the upper phase, it can be calculated that approx. 1 ng (2.7 pmol) of endogenous Ret-*P* and 20 ng (13.3 pmol) of endogenous Dol-*P* per mg of microsomal protein are mannosylated under the conditions of the assay. The lack of activity of exogenous Dol-*P* to function as an acceptor of mannose from GDP-mannose is probably due to its poor solubility under these conditions.

Therefore these studies allow us to conclude that both Ret-*P*-Man and Dol-*P*-Man are synthesized by rat liver microsomal membranes from endogenous acceptor lipids, that Ret-*P*-Man synthesis has an absolute requirement for MnCl_2 and that Ret-*P*-Man synthesis can be greatly enhanced in the bovine serum albumin/Ret-*P* incubation system, possibly allowing the further definition of the role of Ret-*P* in mannosyl-transfer reactions in future studies.

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