Synthesis of retinyl phosphate mannose *in vitro*

Non-enzymic breakdown and reversibility

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Hamster liver microsomal membranes catalyse the synthesis of retinyl phosphate mannose (Ret-*P*-Man) from GDP-mannose and exogenous retinyl phosphate (Ret-*P*). We have previously shown that maximal Ret-*P*-Man synthesis occurs *in vitro* at 20–30 min, followed by a subsequent loss of mannose from Ret-*P*-Man, suggestive of an intermediary function of Ret-*P*-Man and/or Ret-*P*-Man breakdown [Shidoji, Silverman-Jones & De Luca (1982) *Biochem. J.* **208**, 865–868; Creek, Morre, Silverman-Jones, Shidoji & De Luca (1983) *Biochem. J.* **210**, 541–547]. To monitor Ret-*P*-Man synthesis and breakdown carefully, we developed a chromatographic system in which mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose are separated in a single analysis on a Mono Q column eluted with a gradient of NaCl. Using this chromatographic system, we have determined that 80–90% of the Ret-*P*-Man made *in vitro* by hamster liver membranes in 30 min is recovered with the membranes upon centrifugation. Subsequent incubation of Ret-*P*-Man-loaded membranes at 37°C results in a non-enzymic breakdown of Ret-*P*-Man to β -mannopyranosyl phosphate and anhydroretinol. However, incubation of the Ret-*P*-Man-loaded hamster liver membranes with GDP, but not GMP, ADP, CDP or UDP, results in a loss of mannose from Ret-*P*-Man and the formation of GDP-mannose and Ret-*P*. These results demonstrate that Ret-*P*-Man synthesized *in vitro* is subject to non-enzymic breakdown to β -mannopyranosyl phosphate and anhydroretinol and that the GDP-mannose:retinyl phosphate mannosyltransferase reaction is reversible.

Although the exact molecular mechanism of vitamin A action remains to be elucidated, several studies are consistent with an involvement of the vitamin in the assembly of the carbohydrate moieties on glycoproteins (De Luca, 1977; Wolf *et al.*, 1979). Livers from vitamin A-deficient animals exhibit a decrease in their ability to incorporate mannose into glycolipids and glycoproteins (De Luca *et al.*, 1975), and under conditions of vitamin A excess incorporation of mannose but not of galactose into liver glycoconjugates is increased

(Hassell *et al.*, 1978). More specifically, synthesis and mannosylation of the glycoproteins α_{2u} -globulin (Haars & Pitot, 1979) and α_1 -macroglobulin (Wolf *et al.*, 1979) are decreased under conditions of vitamin A deficiency. The identification of retinyl phosphate (Ret-*P*) in hamster intestine (Frot-Coutaz *et al.*, 1976) and retinyl phosphate mannose (Ret-*P*-Man) in liver (Barr & De Luca, 1974; Masushige *et al.*, 1978) support the proposed function of vitamin A in mannosylation.

Studies conducted *in vitro* have demonstrated that liver microsomal membranes are active in the transfer of mannose from GDP-mannose to both endogenous (De Luca *et al.*, 1982; Creek *et al.*, 1983) and exogenous Ret-*P* (Rosso *et al.*, 1975; De Luca *et al.*, 1977, 1982; Shidoji & De Luca, 1981; Creek *et al.*, 1983). The mannosyltransferase responsible for Ret-*P*-Man synthesis (Bergman *et al.*, 1978; Smith *et al.*, 1979; De Luca *et al.*, 1983),

Abbreviations used: medium A, 50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-MgCl₂, 25 mM-KCl and 0.25 M-sucrose; Ret-*P*, retinyl phosphate; Ret-*P*-Man, retinyl phosphate mannose.

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as well as most of the endogenous Ret-*P* available for glycosylation *in vitro* (Creek *et al.*, 1983), have been localized to the endoplasmic reticulum, suggestive of a specific function of Ret-*P* or Ret-*P*-Man in this subcellular compartment. Curiously, the kinetics of formation of Ret-*P*-Man *in vitro* from GDP-mannose and exogenous Ret-*P* have shown that maximal Ret-*P*-Man synthesis occurs at 20–30 min, followed by a subsequent decline in Ret-*P*-Man (Shidoji *et al.*, 1982; Creek *et al.*, 1983). This observation could be explained by an intermediary function of Ret-*P*-Man or Ret-*P*-Man breakdown. We demonstrate in the present paper that Ret-*P*-Man made *in vitro* is subject to a non-enzymic breakdown to β -D-mannopyranosyl phosphate and anhydroretinol. However, in the presence of GDP the loss of mannose from Ret-*P*-Man results in the formation of GDP-mannose and Ret-*P*, thereby demonstrating that the GDP-mannose:retinyl phosphate mannosyltransferase reaction is reversible.

Materials and methods

Materials

Bovine serum albumin, phosphodiesterase 1 from *Crotalus atrox*, alkaline phosphatase from *Escherichia coli*, GDP-mannose, ATP, ADP, AMP, UDP, CDP and GDP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. GDP-[U- 14 C]mannose (sp. radioactivity 269 mCi/mmol), [2- 3 H]mannose (sp. radioactivity 27.2 Ci/mmol) and all-*trans*-[1- 3 H]retinol (sp. radioactivity 13.6 Ci/mmol) were from New England Nuclear Corp., Boston, MA, U.S.A. Ret-*P* and [1- 3 H]Ret-*P* (6520 c.p.m./ μ g), synthesized as previously described (Bhat *et al.*, 1980), were prepared by Microbiological Associates, Bethesda, MD, U.S.A. α -Mannosidase from *Canavalia ensiformis* and Pronase from *Streptomyces griseus* were from Boehringer, Mannheim, West Germany. β -D-Mannose 1-phosphate and D-mannose 2-phosphate standards were kindly given by Dr. Christopher D. Warren, Harvard Medical School, Boston, MA, U.S.A. Precoated silica-gel plates (silica gel 60 F-254) from Merck, Darmstadt, West Germany, were obtained from Brinkman, Westbury, NY, U.S.A.

Preparation of microsomal membranes

Microsomal membranes were prepared from normal male Syrian-golden-hamster livers as described previously (Shidoji & De Luca, 1981). The membranes were resuspended in medium A, to a protein concentration of 30–40 mg/ml, and stored at -80°C until use.

Synthesis of Ret-*P*-Man *in vitro* from exogenous Ret-*P*

The incubation conditions used for Ret-*P*-Man synthesis were patterned from those described previously (Shidoji & De Luca, 1981), which maximize the formation of Ret-*P*-Man from exogenous Ret-*P*. GDP-[14 C]mannose (1 μ Ci, final concentration 18.5 μ M) and 10 μ g of Ret-*P* (0.136 mM) in 99% (v/v) methanol were transferred to test tubes, and the solvent was removed under a stream of N_2 . To this was added 30 mM-Tris/HCl buffer, pH 7.8, containing 2.5 mM-MnCl₂, 8 mM-NaF, 2 mM-ATP, 5 mM-AMP, bovine serum albumin (4 mg/ml) and 2 mg of microsomal protein in a final volume of 200 μ l. The mixture was incubated at 37°C for the times indicated and the incubation stopped by the addition of 1.0 ml of chloroform/methanol (2:1, v/v). The phases were separated by low-speed centrifugation, the upper phase was removed and used for analysis of [14 C]-mannose formation by anion-exchange chromatography and the lower phase was used for analysis of Ret-*P*-Man formation by h.p.l.c. (see below). The insoluble protein residue that collected at the interface was extracted twice with 1.0 ml of chloroform/methanol (2:1, v/v), three times with 1.0 ml of water and three times with chloroform/methanol/water (10:10:3, by vol.). The protein residue was dried with a stream of N_2 and resuspended in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 20 mM-CaCl₂ and 10 mg of Pronase. The digest was incubated at 37°C for 14 h, and 14 C radioactivity present in 50 μ l of the digest was determined in 10.0 ml of Hydrofluor (National Diagnostic, Somerville, NJ, U.S.A.).

Radioactivity in the upper phase that behaved neutral by anion-exchange chromatography was used as a measure of the [14 C]mannose present at various times of incubation. Upper phase (100 μ l) was added to water (0.9 ml) and added to a column (0.5 cm $\times$ 3.0 cm) of AG1-X2 (200–400 mesh; Cl⁻ form) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated in water. The column was washed with 5.0 ml of water, 1.0 ml fractions were collected and radioactivity was determined in 10 ml of Hydrofluor.

Incubation conditions for studies of the reversibility of Ret-*P*-Man synthesis

GDP-[14 C]mannose (0.2 μ Ci) and Ret-*P* (7.5 μ g) in 99% (v/v) methanol were added to Beckman polyallomer Airfuge tubes. After removal of solvent under a stream of N_2 , 30 mM-Tris/HCl buffer, pH 7.8, containing 2.5 mM-MnCl₂, 8 mM-NaF, 2 mM-ATP, 5 mM-AMP, 20 μ M-GDP-mannose, bovine serum albumin (4 mg/ml) and 1.5 mg of membrane protein was added in final volume of 150 μ l.

The mixture was incubated at 37°C for 30 min, and the membranes were pelleted by centrifugation for 5 min in a Beckman air-driven ultracentrifuge run at 0.2 MPa (30 lbf/in²) above atmospheric pressure. Approx. 80–90% of the Ret-*P*-Man made *in vitro* is recovered with the membranes (see Table 1). The supernatant was removed, and the membranes were washed by resuspension in 150 μ l of medium A and pelleted as described above. The wash was removed and the membranes were resuspended in 150 μ l of the original incubation mixture without GDP-[¹⁴C]mannose and Ret-*P* and in the presence or in the absence of various nucleotides (final concentration 2 mM) as described in the Figures and Tables. The incubation was now continued at 37°C for the indicated periods of time and terminated by the addition of the incubation mixture to 850 μ l of methanol/water (17:3, v/v). The mixture was extracted and the protein residue was removed by low-speed centrifugation. The amounts of mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose in the supernatant were quantified by h.p.l.c. analysis as described below.

H.p.l.c. analysis

The h.p.l.c. analyses were conducted on an Altex model 100A programmable liquid-chromatography system and radioactivity was monitored by a Flow-One model HS radioactivity flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.). The column used was the Mono Q HR 5/5 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Samples were introduced to the column in up to 1.0 ml of methanol/water (7:3, v/v). The column was then eluted at a flow rate of 1.0 ml/min with a linear gradient of 0.4–300 mM NaCl in methanol/water (7:3, v/v) over a 30 min period. Typical retention times for mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose standards are 2.3, 8.3, 11.3 and 16.3 min respectively (see Fig. 2).

Ret-*P*-Man and Ret-*P* were quantified by using an h.p.l.c. program designed to separate mannose, dolichyl phosphate mannose, Ret-*P*-Man and Ret-*P* on a Mono Q column eluted with a gradient of ammonium acetate in 99% (v/v) methanol (Creek *et al.*, 1985).

Other procedures

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The concentration of Ret-*P* was determined spectrophotometrically at 325 nm in 99% (v/v) methanol on the basis of $A_{325}^{1\%} = 1440$. α -D-[U-¹⁴C]Mannopyranosyl phosphate standard was generated by enzymic cleavage of GDP-[U-¹⁴C]mannose with *Crotalus atrox* phosphodiesterase 1 (15 munits) in 100 mM-Tris/HCl buffer, pH 8.8, at

37°C for 30 min. α -Mannosidase digests were conducted at 37°C for 13 h in 50 μ l of 0.05 M-sodium citrate/phosphate buffer, pH 4.5, containing 1 unit of α -mannosidase.

Results

Kinetics of Ret-*P*-Man synthesis from exogenous Ret-*P*

Microsomal membranes isolated from hamster liver were highly active in the synthesis of Ret-*P*-Man from GDP-[¹⁴C]mannose and exogenous Ret-*P*. Ret-*P*-Man synthesis proceeded rapidly for 30 min, after which time the amount of Ret-*P*-Man began to decline (Fig. 1). As shown in Fig. 1, the loss of [¹⁴C]mannose from Ret-*P*-Man did not result in a corresponding increase in [¹⁴C]mannose associated with the protein residue, and furthermore the loss of [¹⁴C]mannose from Ret-*P*-Man could not be quantitatively recovered as free [¹⁴C]mannose. These results suggested that the [¹⁴C]mannose loss from Ret-*P*-Man is not due to the breakdown of Ret-*P*-Man to [¹⁴C]mannose and is not the result of a transfer of [¹⁴C]mannose to endogenous glycoproteins acceptors in the hamster liver membranes.

Fig. 1. Time course of Ret-*P*-Man synthesis and breakdown

Hamster liver membranes were incubated in the presence of GDP-[¹⁴C]mannose (1.0 μ Ci) and exogenous Ret-*P* (10 μ g) in the conditions described in the Materials and methods section. At the times indicated, the amounts of [¹⁴C]mannose incorporated into Ret-*P*-Man (●), mannose (○) and protein residue (■) were determined as described in the Materials and methods section. The results shown are representative of those obtained in two similar experiments.

Development of an h.p.l.c. procedure for the separation of mannose, Ret-P-Man, mannose phosphate and GDP-mannose

In order to characterize and elucidate the product(s) of Ret-*P*-Man breakdown *in vitro* it was necessary to develop a chromatographic system in which we could determine mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose in a single analysis. We found that these compounds could be separated on an anion-exchange Mono Q column eluted with a linear gradient of NaCl (Fig. 2).

Reversibility of Ret-P-Man synthesis in vitro

We previously showed that the synthesis of Ret-*P*-Man *in vitro* from GDP-mannose and exogenous Ret-*P* by liver membranes could be measured in a filter binding assay (Shidoji & De Luca, 1981). This method suggested that Ret-*P*-Man synthesized *in vitro* was associated with the membranes. Table 1 demonstrates that 83% of the total Ret-*P*-Man synthesized at 30 min by hamster liver microsomal membranes from GDP-[¹⁴C]mannose and exogenous Ret-*P* is recovered with membranes

Fig. 2. Separation of mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose by h.p.l.c. on a Mono Q column. Standard ¹⁴C-labelled mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose (1000 c.p.m.) were applied to a Mono Q column and eluted with a programmed gradient of NaCl as described in the Materials and methods section.

upon centrifugation. Furthermore, the membrane pellet was essentially free of GDP-mannose and mannose phosphate. These findings have allowed us to follow precisely the subsequent fate of Ret-*P*-Man after its synthesis *in vitro*.

In the experiments presented in Fig. 3 hamster liver membranes were incubated for 30 min in the presence of GDP-[¹⁴C]mannose and exogenous Ret-*P*, and the membranes were obtained by centrifugation and then incubated again for various times in the absence (Fig. 3a) or in the presence (Fig. 3b) of GDP. At the times indicated the incubation mixture was analysed by chromatography on the Mono Q column. As shown in Fig. 3(a), the loss of [¹⁴C]mannose from Ret-*P*-Man resulted primarily in the formation of a compound with the retention time of mannose phosphate. Strikingly, if GDP was added during the second incubation, the loss of mannose from Ret-*P*-Man resulted in a rapid increase in radioactivity recovered at the retention time of GDP-mannose (Fig. 3b). The loss of mannose from Ret-*P*-Man to form GDP-mannose was nucleotide-specific. GDP was an active acceptor of mannose from Ret-*P*-Man to form GDP-mannose, but GMP, UDP, CDP and ADP were inactive (Table 2).

To characterize further the product formed from

Table 1. Distribution between membranes and supernatant of Ret-*P*-Man synthesized *in vitro*

Hamster liver membranes were incubated with GDP-[¹⁴C]mannose and exogenous Ret-*P* in Beckman Airfuge tubes in the reaction mixture described in the Materials and methods section. After 30 min the membranes were pelleted by centrifugation, and the supernatant was removed (supernatant 1) and added to 850 μl of 85% (v/v) methanol. The membranes were then resuspended in medium A (150 μl), pelleted a second time, and the supernatant was removed as above (supernatant 2). The membrane pellet was then extracted with 1.0 ml of 70% (v/v) methanol. The supernatants and the pellet extract (40 μl) were analysed on the Mono Q h.p.l.c. system as described in the Materials and methods section. A total of 191 000 c.p.m. was recovered as Ret-*P*-Man, 135 500 c.p.m. as mannose phosphate and 67 200 c.p.m. as GDP-mannose. The results are representative of those obtained in two similar experiments.

Fraction	Percentage of compound found in each fraction		
	Ret- <i>P</i> -Man	Mannose phosphate	GDP-mannose
Supernatant 1	16	79	95
Supernatant 2	1	15	0
Pellet	83	6	5

Fig. 3. Time course of the loss of mannose from Ret-P-Man in the absence and in the presence of GDP

Hamster liver membranes were incubated with GDP-[¹⁴C]mannose and exogenous Ret-P in Beckman Airfuge tubes in the incubation mixture described in the Materials and methods section. After 30 min the membranes were pelleted by centrifugation, washed with medium A, and resuspended in the original incubation mixture without GDP-[¹⁴C]mannose and Ret-P (a) or also containing 2 mM-GDP (b). The incubation continued at 37°C and at the times indicated the reactions were terminated by addition of 850 μl of 85% (v/v) methanol. The mixture was extracted and 40 μl (6000–8000 c.p.m.) was analysed on the Mono Q h.p.l.c. system. The data are expressed as percentages of the total radioactivity co-migrating with retention times of standard Ret-P-Man (●), mannose phosphate (■) and GDP-mannose (○). Over 90% of the radioactivity applied co-migrated with retention times of the standards, and none was detected as free mannose. The results are representative of those obtained in four similar experiments.

Ret-P-Man in incubation mixtures containing GDP, we isolated the material and compared its properties with those of authentic GDP-mannose. Like standard GDP-mannose, the compound was

Table 2. Effect of various nucleotides on the loss of mannose from Ret-P-Man to form a compound with the chromatographic properties of GDP-mannose

The initial 30 min incubation and centrifugation were conducted as described in Fig. 3 legend. The second incubations were at 37°C for 60 min in the standard incubation medium without ATP but in the absence or in the presence of the indicated nucleotides at a final concentration of 2 mM. The reactions were then stopped and a portion (5000–7000 c.p.m.) of each incubation mixture was analysed on the Mono Q h.p.l.c. system as described in the Materials and methods section. The data are presented as percentages of the total radioactivity co-migrating with a retention time of standard GDP-mannose. The results shown are representative of those obtained in two similar experiments.

Radioactivity migrating as GDP-mannose	
Nucleotide	(% of total)
None	5.2
GDP	33.4
GMP	1.3
ADP	7.8
CDP	6.0
UDP	6.4

Table 3. Effect of various nucleotides on the loss of mannose from Ret-P-Man to form Ret-P

Hamster liver membranes were incubated with [³H]Ret-P (7.5 μg) and GDP-mannose (100 μM) in Beckman Airfuge tubes in the reaction mixture described in the Materials and methods section. After 30 min the membranes were collected by centrifugation. The membranes were resuspended in incubation mixture (without Ret-P) containing 20 μM-GDP-mannose in the presence or in the absence of 2 mM-nucleotide and incubated for 60 min at 37°C. The incubation was then added to methanol and the entire extract analysed on the Mono Q h.p.l.c. system. The data are expressed as percentages of the sum of the radioactivity (2000–2500 c.p.m.) co-migrating with retention times of standard Ret-P-Man and Ret-P. Anhydro[³H]-retinol produced by the non-enzymic breakdown of [³H]Ret-P-Man was eluted in the void volume of the Mono Q column and represented approx. 50% of the applied radioactivity. The results shown are representative of those obtained in two similar experiments.

Nucleotide	Percentage of sum of radioactivity	
	Migrating as Ret-P-Man	Migrating as Ret-P
None	75	25
GDP	25	75
GMP	71	29
UDP	73	27

cleaved to mannose by α -mannosidase digestion, and furthermore it was capable of donating mannose to Ret-*P* to form Ret-*P*-Man in incubations with liver membranes (results not shown).

By using [^3H]Ret-*P* we could also demonstrate that, in incubations containing GDP but not GMP or UDP, the mannose lost from Ret-*P*-Man results in the formation of Ret-*P* (Table 3). These results demonstrate that, in the presence of GDP, GDP-mannose and Ret-*P* are formed from Ret-*P*-Man as the result of a reversal of the enzymic reaction catalysing Ret-*P*-Man synthesis.

Characterization of the products of Ret-*P*-Man breakdown

As shown in Fig. 3(a), incubation of Ret-*P*-Man in the presence of hamster liver membranes results in the loss of mannose from Ret-*P*-Man to form a compound with the chromatographic characteristics of mannose phosphate. However, this break-

Fig. 4. Time course of Ret-*P*-Man breakdown in the absence of hamster liver membranes

Ret-*P*-Man was purified by h.p.l.c. from incubation mixtures containing hamster liver membranes, GDP-[^{14}C]mannose and exogenous Ret-*P*, the incubation system used being as described in the Materials and methods section. Ret-*P*-Man (30000 c.p.m.) was added to Beckman Airfuge tubes, the Ret-*P*-Man was resuspended in the incubation mixture described in the Materials and methods section in the absence of hamster liver membranes, and the tubes were incubated at 37°C. At the times indicated methanol (350 μl) was added to each incubation mixture and 100 μl was analysed on the Mono Q chromatographic system. The data are expressed as percentages of total radioactivity co-migrating with retention times of standard Ret-*P*-Man (●) or mannose phosphate (■). No radioactivity was detected with retention times of standard mannose or GDP-mannose. The results shown are representative of those obtained in two similar experiments.

down of Ret-*P*-Man is non-enzymic. When purified Ret-*P*-Man is added to the standard incubation mixture, in the absence of hamster liver membranes, and incubated at 37°C for various times, a rapid breakdown of Ret-*P*-Man to a compound with the retention time of standard α -D-mannose 1-phosphate on the Mono Q h.p.l.c. system results (Fig. 4). Although both α -D-mannose 1-phosphate and the Ret-*P*-Man breakdown product were sensitive to alkaline phosphatase, the breakdown product was insensitive to α -mannosidase digestion, whereas standard α -D-mannose 1-phosphate was sensitive (results not shown). On t.l.c. the Ret-*P*-Man breakdown product co-migrated with standard β -D-mannopyranosyl phosphate but not standard α -D-mannopyranosyl phosphate or mannose 2-phosphate (Fig. 5). Since the anomeric configuration of Ret-*P*-Man synthesized *in vitro* by liver membranes is β -D-mannosyl retinyl phosphate (Rosso *et al.*, 1976), these findings strongly suggest that the Ret-*P*-Man breakdown product is β -D-mannopyranosyl phosphate.

To follow the fate of the retinol moiety during the breakdown of Ret-*P*-Man, preparative amounts of Ret-*P*-Man were purified, then incubated at 37°C for various times in the absence of hamster liver membranes, and the absorbance

Fig. 5. T.l.c. of Ret-*P*-Man breakdown product. Approx. 5000 c.p.m. of standard [^{14}C] α -D-mannose 1-phosphate (a) or Ret-*P*-Man breakdown product (b) as well as 10 μg of standard β -D-mannopyranosyl phosphate and mannose 2-phosphate were spotted on a thin-layer plate of silica gel. The plate was developed in chloroform/methanol/water (10:10:3, by vol.) and exposed for 4 days to Kodak XR-5 film. The positions of the unlabelled sugar phosphates, located by spraying with 50% (v/v) H_2SO_4 followed by heating at 100°C, are indicated by the arrows. The results are representative of those obtained in three similar experiments.

Fig. 6. Absorption spectrum of Ret-*P*-Man and Ret-*P* before and after incubation at 37°C in the absence of hamster liver membranes

Ret-*P*-Man (7.4 µg) was purified by h.p.l.c. from 12 incubation mixtures each containing hamster liver membranes (1.5 mg of protein), [³H]Ret-*P* (7.5 µg) and GDP-mannose (100 µM). Ret-*P*-Man (a) or Ret-*P* (b) (1.0 µg) was then added to glass tubes, and the retinoids were incubated at 37°C in the standard incubation mixture (200 µl) in the absence of membranes for 0 min (1), 90 min (2) or 150 min (3). At these times each reaction mixture was extracted with methanol (800 µl), a precipitate removed by low-speed centrifugation, and the absorption spectrum recorded against a blank of an identical incubation without Ret-*P* or Ret-*P*-Man. The results shown are representative of those obtained in two similar experiments.

spectrum was recorded. As shown in Fig. 6(a) the Ret-*P*-Man undergoes an absorption-spectral change from its characteristic maximum at 325 nm to absorption maxima at 346, 368 and 388 nm, which are characteristic of the hydrocarbon anhydroretinol (Frot-Coutaz *et al.*, 1976). Under identical incubation conditions, Ret-*P* is stable and does not undergo this spectral change (Fig. 6b). Therefore, under the incubation conditions generally used to study its synthesis *in vitro*, Ret-*P*-Man is subject to a non-enzymic breakdown to β-mannopyranosyl phosphate and anhydroretinol.

Discussion

The development of a chromatographic system that rapidly separates mannose, Ret-*P*-Man, mannose phosphate and GDP-mannose has enabled us to study the enzymic synthesis and subsequent fate

of Ret-*P*-Man made *in vitro*. This study has allowed the following conclusions: (1) Ret-*P*-Man made *in vitro* from GDP-mannose and exogenous Ret-*P* in the presence of hamster liver membranes is associated with the membranes; (2) Ret-*P*-Man incubated at 37°C in an aqueous medium is subject to a rapid non-enzymic cleavage to β-mannopyranosyl phosphate and anhydroretinol; (3) in the presence of GDP the enzymic reaction that formed Ret-*P*-Man reverses to form GDP-mannose.

Previously we showed that the transfer of mannose from GDP-mannose to Ret-*P* in incubations containing liver membranes could be measured by a filtration assay (Shidoji & De Luca, 1981). This suggested that Ret-*P*-Man made *in vitro* was associated with the membrane vesicles. In the present study we have demonstrated that 80–90% of the Ret-*P*-Man made *in vitro* by hamster liver membranes from GDP-mannose and Ret-*P* is

recovered with the membranes upon centrifugation. The nature of the association between Ret-*P*-Man and the membranes is unknown.

A major challenge in working with Ret-*P* and Ret-*P*-Man is their relative instability. Previous studies have shown that mild acid or u.v. light will rapidly break down Ret-*P*, although this compound appears to be quite stable when incubated in Tris buffer at pH 8.0 (De Luca *et al.*, 1983). Ret-*P*-Man loses its characteristic absorbance maximum at 325 nm when exposed to u.v. light, and is also subject to breakdown on thin layers of silica gel (Sasak & De Luca, 1980; De Luca *et al.*, 1983). In the present work we show that Ret-*P*-Man is unstable in an aqueous medium at 37°C and undergoes a non-enzymic cleavage to β -mannopyranosyl phosphate and anhydroretinol. The β -configuration is expected, since Rosso *et al.* (1976) showed that Ret-*P*-Man formed by rat liver membranes is β -D-mannopyranosyl retinyl phosphate. Previous kinetic studies of the formation of Ret-*P*-Man *in vitro* showed maximal Ret-*P*-Man synthesis at 20–30 min, followed by a loss of mannose from Ret-*P*-Man (Shidoji *et al.*, 1982; Creek *et al.*, 1983). It is now clear that this loss is by and large due to the instability of Ret-*P*-Man and is not the result of Ret-*P*-Man acting in a rapid intermediary fashion.

An intriguing finding of the present study is the demonstration of the reversibility of the enzymic synthesis of Ret-*P*-Man. The evidence for reversibility is at least threefold. First, in the presence of GDP, but not in its absence, a loss of mannose from Ret-*P*-Man resulted in the formation of GDP-mannose and Ret-*P*. Secondly, the loss of mannose from Ret-*P*-Man to form GDP-mannose was nucleotide-specific in that it occurred in the presence of GDP but not of other nucleoside diphosphates or GMP. Finally, the products of the reversal reaction had characteristics identical with those of authentic GDP-mannose and Ret-*P*. The reversal of Ret-*P*-Man synthesis is not unique to the GDP-mannose:retinyl phosphate mannosyl-transferase present in hamster liver membranes, as reversibility could also be demonstrated in incubations with rat liver membranes (K. E. Creek, C. S. Silverman-Jones & L. M. De Luca, unpublished work).

Our results on the reversibility of Ret-*P*-Man synthesis are similar to previous studies showing that the enzymic synthesis of dolichyl phosphate mannose from GDP-mannose and dolichyl phosphate is freely reversible in the presence of GDP (Richards & Hemming, 1972; Baynes *et al.*, 1973; Martin & Thorne, 1974) and points to the similarities of the two reactions. Haselbeck & Tanner (1982) have provided evidence that this reversal allows a dolichyl phosphate-mediated

mannosyl transfer through liposomal membranes, and proposed that dolichols may play a role in GDP-mannose translocation through membranes.

The finding that the enzymic synthesis of Ret-*P*-Man is reversible raises some interesting possibilities as to the biological significance of this reversal and a role it may play in the molecular function of the vitamin. Several previous studies indicate that, under conditions of vitamin A deficiency, mannose incorporation into glycoproteins is impaired (De Luca *et al.*, 1975; Wolf *et al.*, 1979), but the exact point of the block is unknown. The synthesis of asparagine-linked oligosaccharides on glycoproteins involves the assembly in the endoplasmic reticulum of the oligosaccharide Glc₃Man₆GlcNAc₂ on the lipid carrier dolichyl pyrophosphate (for review see Hubbard & Ivatt, 1981). The first two *N*-acetylglucosamine residues as well as the first five mannose residues are added directly from the nucleotide sugars UDP-*N*-acetylglucosamine and GDP-mannose to yield Man₅GlcNAc₂-*P*-*P*-dolichol. The next four mannose residues as well as the glucose residues are then added from dolichyl phosphate mannose and dolichyl phosphate glucose respectively. Studies on the topology of lipid-linked oligosaccharide synthesis indicate that the Man₅GlcNAc₂-*P*-*P*-dolichol is assembled on the cytoplasmic surface of the endoplasmic reticulum and then translocated to the luminal side, where it is completed to the Glc₃Man₆GlcNAc₂-*P*-*P*-dolichol to serve as the donor in protein glycosylation (Snider *et al.*, 1980; Hanover & Lennarz, 1981, 1982; Snider & Robbins, 1982; Snider & Rogers, 1984). A possible point in this pathway where vitamin A may play a role was suggested by the finding that vitamin A deficiency causes an accumulation of Man₅GlcNAc₂-*P*-*P*-dolichol (Rosso *et al.*, 1981). This suggests that under conditions of vitamin A deficiency GDP-mannose is not available to react with dolichyl phosphate to form the dolichyl phosphate mannose, which is necessary for the addition of the final four mannose residues on growing lipid-linked oligosaccharide chains. Since GDP-mannose synthesis occurs in the cytosol (Coates *et al.*, 1980), some mechanism to transport GDP-mannose into the lumen of the endoplasmic reticulum during lipid-linked oligosaccharide synthesis must exist. One might speculate that Ret-*P*-Man or a similar compound could serve such a carrier function and that the reversal of the reaction catalysing Ret-*P*-Man synthesis may provide an intraluminal pool of GDP-mannose to act as mannose donor to form the dolichyl phosphate mannose necessary for lipid-linked oligosaccharide chain elongation. In support of this scheme is our observation that dolichyl phosphate accumulates in membranes isolated

from vitamin A-deficient hamster livers (De Luca *et al.*, 1982), possibly as a consequence of a lack of translocation of GDP-mannose across the endoplasmic reticulum under conditions of vitamin A deficiency.

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