Synthesis of retinyl phosphate mannose and dolichyl phosphate mannose from endogenous and exogenous retinyl phosphate and dolichyl phosphate in microsomal fraction

Specific decrease in endogenous retinyl phosphate mannose synthesis in vitamin A deficiency

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Rat liver microsomal fraction synthesized Ret-*P*-Man (retinyl phosphate mannose) and Dol-*P*-Man (dolichyl phosphate mannose) from endogenous Ret-*P* (retinyl phosphate) and Dol-*P* (dolichyl phosphate). Ret-*P*-Man synthesis displayed an absolute requirement for a bivalent cation, and also Dol-*P*-Man synthesis was stimulated by bivalent metal ions. Mn^{2+} and Co^{2+} were the most active, with maximum synthesis of Ret-*P*-Man occurring at 5–10 mM; Mg^{2+} was also active, but at higher concentrations. At 5 mM- Mn^{2+} the amount of endogenous Ret-*P* mannosylated in incubation mixtures containing 5 μ M-GDP-mannose in 15 min at 37°C was approx. 3 pmol/mg of protein. In the same assays about 7–10 pmol of endogenous Dol-*P* was mannosylated. Bivalent-cation requirement for Ret-*P*-Man synthesis from exogenous Ret-*P* showed maximum synthesis at 2.5 mM- Mn^{2+} or $-Co^{2+}$. In addition to Ret-*P*-Man and Dol-*P*-Man, a mannosylated lipid co-chromatographing with undecaprenyl phosphate mannose was detected. Triton X-100 (0.5%) abolished Ret-*P*-Man synthesis from endogenous Ret-*P* and caused a 99% inhibition of Ret-*P*-Man synthesis from exogenous Ret-*P*. The presence of detergent (0.5%) also inhibited Dol-*P*-Man synthesis from endogenous Dol-*P* and altered the requirement for Mn^{2+} . Microsomal fraction from Syrian golden hamsters was also active in Ret-*P*-Man and Dol-*P*-Man synthesis from endogenous Ret-*P* and Dol-*P*. At 5 mM- Mn^{2+} about 2.5 pmol of endogenous Ret-*P* and 3.7 pmol of endogenous Dol-*P* were mannosylated from GDP-mannose per mg of protein in 15 min at 37°C. On the other hand, microsomal fraction from vitamin A-deficient hamsters contained 1.2 pmol of Ret-*P* and 14.1 pmol of Dol-*P* available for mannosylation. Since GDP-mannose:Ret-*P* and GDP-mannose:Dol-*P* mannosyltransferase activities were not affected, depletion of vitamin A must affect Ret-*P* and Dol-*P* pools in opposite ways.

We have previously reported that bovine serum albumin, in the absence of detergent, stimulates the synthesis of Ret-*P*-Man in rat liver microsomal fraction (referred to below simply as microsomes) from GDP-mannose and exogenous Ret-*P* (Shidoji & De Luca, 1981).

It was therefore deemed important to investigate in detail the biosynthesis of Ret-*P*-Man from

Abbreviations used: Dol-*P*, dolichyl phosphate; Dol-*P*-Man, dolichyl phosphate mannose; Ret-*P*, retinyl phosphate; Ret-*P*-Man, retinyl phosphate mannose; Man, mannose; GlcNAc₂, chitobiose.

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endogenous Ret-*P* present in the microsomal membrane, by using the assay in the presence of bovine serum albumin. In addition, it was decided to study how the condition of vitamin A deficiency affects the pool of endogenous Ret-*P* and also that of endogenous Dol-*P*, because vitamin A deficiency *in vivo* decreases mannosylated lipid and mannoprotein biosynthesis in hamster liver (De Luca *et al.*, 1975) and allows the accumulation of Dol-*P*-*P*-GlcNAc₂-Man₅ in rat liver (Rosso *et al.*, 1981).

Materials and methods

Materials

Bovine serum albumin (globulin-free, crystallized, freeze-dried), Dol-*P* (grade III) and GDP-mannose

were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ret-*P* was synthesized as previously described (Bhat *et al.*, 1980); Millipore filters were obtained from Millipore, Boston, MA, U.S.A. The following radioactive products were obtained from New England Nuclear Corp., Boston, MA, U.S.A.: GDP-[³H]mannose (sp. radioactivity 10 Ci/mmol) and GDP-[¹⁴C]mannose (0.2 Ci/mmol). 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 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 saline (0.9% NaCl) in a glass/Teflon homogenizer. After centrifugation of the homogenate at 2500 *g* for 20 min, the resulting supernatant was centrifuged at 105 000 *g* for 60 min. The pellet was resuspended in a small volume of saline and stored in liquid N₂ until used. The incubation mixture contained: 0.4 μCi of GDP-[³H]mannose (or 0.1 or 0.2 μCi of GDP-[¹⁴C]mannose), 4 mg of bovine serum albumin/ml, GDP-D-mannose (24 μM final concn.), 30 mM-Tris/HCl buffer, pH 8, 5 mM-MnCl₂ or as stated, 8 mM-NaF, 2 mM-ATP, 5 mM-AMP and about 1 mg of the microsomal protein in a final volume of 200 μl. After incubation for appropriate time intervals, the lipids were extracted by the following procedure, which yields two phases.

To the mixture was added 5 vol. (1 ml) of chloroform/methanol (2:1, v/v), the tube was stirred and two phases were allowed to separate by low-speed centrifugation. Under these extraction conditions approx. 98% of Dol-*P*-Man is recovered in the lower phase, whereas Ret-*P*-Man partitions about 40–50% in the lower phase and 60–50% in the upper phase.

Alternatively 15 vol. (3 ml) of chloroform/methanol (2:1, v/v) was added to yield a monophasic extract.

Extracts were dried under a stream of N₂ and immediately dissolved in suitable volumes of chloroform/methanol (2:1, v/v) containing 10 μg of synthetic Ret-*P* ready for application on thin layers of silica gel. Chromatography was usually performed in chloroform/methanol/water (45:35:6, by vol.) (solvent A) on two identical plates. One plate was used for determination of radioactivity, the other one for fluorography. Sections (0.5 cm) of silica gel were collected into counting vials and radioactivity was determined after addition of 0.25 ml of methanol and 10 ml of Betafluor (National Diagnostics, Somerville, NJ, U.S.A.). The two bands obtained on occasion in the area of Ret-*P*-Man were both counted as Ret-*P*-Man, since variability in the relative proportions of the two compounds was

noted. Such variability may be due to enzymic or non-enzymic isomerization. Fluorography was performed with Kodak film XR-5 after it had been sprayed with En³Hance (New England Nuclear), by following a procedure developed by Bonner & Laskey (1974).

Preparation of vitamin A-depleted Syrian golden hamsters

Vitamin A-depleted male Syrian golden hamsters were prepared essentially as previously described (De Luca *et al.*, 1975), in accordance with the procedure of Rogers *et al.* (1974), by placing the mothers on a vitamin A-free diet at birth of the experimental animals. These were weaned on a vitamin A-deficient diet at about 21 days. At 40 days hamsters prepared in this way began to show signs of vitamin A deficiency, such as eye lesions and diminished growth rate. At about this stage of deficiency hamsters were anaesthetized with diethyl-ether and killed by bleeding from the neck. Livers were removed and microsomes were prepared as described for rat liver microsomes. Microsomes from normal male Syrian golden hamsters were prepared in the same way.

Other procedures

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Ret-*P* was measured spectrophotometrically on the basis of $A_{1\text{cm}, 325}^{1\%}(\text{in methanol}) = 1440$. Retinyl palmitate was measured by the high-pressure liquid-chromatography procedure of Bhat *et al.* (1980).

Results

*Comparison of bovine serum albumin and Triton X-100 incubations for Ret-*P*-Man synthesis from exogenous Ret-*P**

Fig. 1 compares the rates of synthesis of Ret-*P*-Man from GDP-mannose and exogenous Ret-*P* in incubation mixtures containing 0.5% Triton X-100 or bovine serum albumin as the dispersal agent for Ret-*P*. A 200-fold increase in the incubations in the presence of bovine serum albumin compared with those in the presence of Triton X-100 is obtained. Therefore the biosynthesis of Ret-*P*-Man and Dol-*P*-Man from endogenous acceptor lipids was studied in the bovine serum albumin system.

Fig. 2(a) shows the time course of the synthesis of Ret-*P*-Man and Dol-*P*-Man from endogenous acceptors at 5 mM-MnCl₂. Fig. 2(b) shows the fluorography of an identical thin-layer chromatograph showing a double spot in the area of Ret-*P*-Man, as previously reported for Ret-*P*-Man synthesized from exogenous Ret-*P* (Rosso *et al.*, 1975). In addition to Dol-*P*-Man, a labelled mannolipid is also seen at a

Fig. 1. Time course of Ret-P-Man synthesis by rat liver microsomes in incubations in the presence of bovine serum albumin (●) and Triton X-100 (■)

GDP-[^3H]mannose ($0.4\mu\text{Ci}$, $24\mu\text{M}$) was incubated with 0.92 mg of rat liver microsomal protein at 37°C in the presence of 4 mg of bovine serum albumin/ml as described in the Materials and methods section or 0.5% Triton X-100 under identical conditions, including $10\mu\text{g}$ of Ret-P. Ret-P-Man was measured by the filter assay as described previously for the bovine serum albumin system (Shidoji & De Luca, 1981) and from the lower phase of the extraction with 5 vol. of chloroform/methanol ($2:1$, v/v).

slightly lower R_F . This compound co-chromatographs with standard undecaprenyl phosphate mannose, as shown in Fig. 2(c). Fig. 2(d) shows that Ret-P-Man partitions between the lower and the upper phase in the extraction procedure as used in these studies, where the reaction mixture (0.2 ml) is extracted with 1 ml of chloroform/methanol ($2:1$, v/v); nearly all the Dol-P-Man is found in the lower phase, as shown in Fig. 2(d). At 5 mM-MnCl_2 the ratio of Ret-P-Man to Dol-P-Man is about $1:4.2$, and at 10 mM-MnCl_2 $1:3$ (Expt. 1, Table 1). In Expt. 2 of Table 1 the upper phase was also examined, demonstrating that about 50% of Ret-P-Man partitions in the upper phase and that the actual ratio of Ret-P-Man to Dol-P-Man synthesized at 15 min of incubation is $1:3$ at 5 mM-MnCl_2 and $1:2.3$ at 10 mM-MnCl_2 . The amounts of Ret-P-Man and Dol-P-Man and their ratios are similar to these values when calculated for monophasic extracts

obtained by adding 15 vol. (3 ml) of chloroform/methanol ($2:1$, v/v) to the incubation mixture. The main products formed at 15 min are Ret-P-Man and Dol-P-Man with a ratio of $1:3$ at 5 mM-MnCl_2 (results not shown).

Inclusion of 0.5% Triton X-100 in the bovine serum albumin incubation system maintains Dol-P-Man synthesis, albeit of about 50% of that in the incubation with bovine serum albumin alone, but completely inhibits Ret-P-Man synthesis from endogenous acceptor Ret-P (Figs. 3a and 3b). It should be noted that the detergent also inhibits the synthesis of the undecaprenyl phosphate mannose-like compound, though it greatly stimulates the synthesis of material A co-migrating with standard Dol-P-P-GlcNAc $_2$ -Man (Fig. 3b).

It was decided to compare bivalent cations for their ability to stimulate Ret-P-Man and Dol-P-Man synthesis. Fig. 4(a) shows the Mn^{2+} , Co^{2+} and Mg^{2+} -dependence of Ret-P-Man synthesis from endogenous Ret-P. A maximum between 5 and 10 mM with strong inhibition at 50 mM was found with MnCl_2 and CoCl_2 ; MgCl_2 also stimulated Ret-P-Man synthesis, but its optimal concentration was 10 mM and little inhibition was observed at 50 mM . These metal ions also stimulated Dol-P-Man synthesis by about 2 -fold, but without a sharp maximum; however, optimal conditions were about 5 mM (Fig. 4b). The fluorographs for the t.l.c. profiles of the lower-phase extracts of incubation mixtures containing MnCl_2 (Fig. 4c), CoCl_2 (Fig. 4d) and MgCl_2 (Fig. 4e) are also shown (for only a few concentrations) and represent duplicate plates identical with those utilized for radioactivity measurement in Fig. 4(a). The metal ions also appeared to stimulate the liberation of mannose. Occasionally a doublet of bands is found opposite the Dol-P-P-GlcNAc $_2$ -Man marker. Similar results were obtained when the analyses were conducted on monophasic extracts (Fig. 5a). Fe^{2+} , Cu^{2+} , Ca^{2+} , Zn^{2+} were without activity (results not shown).

Triton X-100 at 0.5% completely inhibited Ret-P-Man synthesis from endogenous acceptors, as shown in Fig. 3. The same inhibitory effect is seen in the range of concentration of MnCl_2 0 – 50 mM (Figs. 6a and 6b). However, Dol-P-Man synthesis in the presence of Triton X-100 shows an absolute requirement for MnCl_2 (2.5 mM maximum). Fig. 6(b) also shows the stimulatory effect of MnCl_2 on the biosynthesis of a compound migrating as Dol-P-P-GlcNAc $_2$ -Man. The curve for the MnCl_2 requirement for the synthesis of Dol-P-Man from exogenous Dol-P is shown in Fig. 7, with maximum at 2.5 mM .

In the presence of exogenous Ret-P (0.15 mM) Ret-P-Man synthesis (2 min at 37°C) was highest at 2.5 mM-MnCl_2 or CoCl_2 and at 10 mM-MgCl_2 (Fig. 8a). As for the studies on Ret-P-Man synthesis from

Fig. 2. Time course of the synthesis of Ret-P-Man (O) and Dol-P-Man (●) from endogenous Ret-P and Dol-P of rat liver microsomes

(a) GDP-[^{14}C]mannose ($0.2\mu\text{Ci}$; $5\mu\text{M}$) was incubated with 1.0mg of rat liver microsomes as described in the Materials and methods section at 37°C for the indicated times. Lower phases of the extracts were applied to $20\text{cm} \times 20\text{cm}$ Merck t.l.c. plates, which were chromatographed in solvent A, and radioactivity was measured as described in the Materials and methods section. (b) A duplicate t.l.c. plate of (a) was utilized for fluorography. Exposure time was 14 days. A mixture (2000c.p.m.) of Ret-P-Man and Dol-P-Man was applied in the column marked St. The positions of standard Dol-P-Man, undecaprenyl phosphate mannose (C_{55} -P-Man), Ret-P-Man, mannose and GDP-mannose are indicated. Only the 5 min, 30 min and 90 min time points are shown. (c) Fluorography of t.l.c. plate of mannolipids from incubations with and without exogenous undecaprenyl phosphate. Incubations were identical with those described for (a) except that the incubation mixture in lane II contained standard undecaprenyl phosphate ($25\mu\text{g}$) solubilized in 0.5% Triton X-100 and incubation had proceeded for 15 min at 37°C . For the incubation mixture chromatographed in lane I incubation had proceeded for 30 min at 37°C . Similar amounts of radioactivity (10000c.p.m.) were used for lanes I and II on a $5\text{cm} \times 20\text{cm}$ plate developed as described in the text. Film was exposed for 3 days. (d) Fluorography of t.l.c. plates of lower-phase and upper-phase extracts. Incubation mixture and conditions were the same as in (a). The lower-phase extract was dried under N_2 , immediately dissolved in $60\mu\text{l}$ of 99% (v/v) methanol containing $10\mu\text{g}$ of standard Ret-P and applied to the plate. The upper-phase extract was dried under N_2 , resuspended in $60\mu\text{l}$ of chloroform/methanol ($2:1$, v/v), and $20\mu\text{l}$ of 99% methanol containing $10\mu\text{g}$ of standard Ret-P was added. Samples were applied to a $5\text{cm} \times 20\text{cm}$ t.l.c. plate of silica gel and developed as usual along with the extract of an incubation mixture containing exogenous Ret-P (marked St) to show the position of Ret-P-Man. The amount of radioactivity in the area of Ret-P-Man in the upper-phase extract was 440c.p.m. and that in the lower-phase extract 350c.p.m. , whereas that of Dol-P-Man in the lower-phase extract was 2160c.p.m. giving an approximate ratio of $1:3.6$. The film was exposed for 5 days. The positions of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

Table 1. Measurement of Ret-P-Man/Dol-P-Man ratios in rat liver microsomes as measured from the two-phase and the monophasic extraction procedures

For full experimental details see the text. Expt. 1. Rat liver microsomes were incubated in the usual incubation system to assay for endogenous Ret-*P* and Dol-*P* in quadruplicates at 5 mM- and 10mM-MnCl₂. The procedure described for the 'lower-phase extraction' was utilized to measure Ret-*P*-Man and Dol-*P*-Man. Expt. 2. The same incubation systems as for Expt. 1 was used. Both upper and lower phases were dried and processed in the same way for Ret-*P*-Man and Dol-*P*-Man determination. Incubations were done in duplicate at 5 mM- and 10mM-MnCl₂.

[MnCl ₂] (mM)	Ret- <i>P</i> -Man synthesis				Dol- <i>P</i> -Man synthesis				Ret- <i>P</i> -Man Dol- <i>P</i> -Man ratio
	(c.p.m./15 min per mg of protein)		(pmol)		(c.p.m./15 min per mg of protein)		(pmol)		
Expt. 1, lower phase									
5	{ 640 710 680 770 }	Mean	700	2.1	{ 3320 2870 2610 3070 }	Mean	2960	8.8	0.23
10	{ 490 540 470 450 }	Mean	490	1.47	{ 1340 1610 1260 1260 }	Mean	1400	4.2	0.35
Expt. 2, upper and lower phases									
5	Upper	{ 440 570 }	500	1.5	{ 60 60 }		60	0.18	
	Lower	{ 665 535 }	600	1.8	{ 3250 3450 }		3350	10.0	
	Total		1100	3.3			3410	10.23	0.32
10	Upper	{ 370 610 }	490	1.47	{ 95 70 }		80	0.24	
	Lower	{ 550 490 }	520	1.56	{ 2370 2050 }		2210	6.63	
	Total		1010	3.03			2290	6.87	0.44

Fig. 3. Time course of the synthesis of Ret-P-Man (○) and Dol-P-Man (●) from endogenous acceptors by rat liver microsomes in the presence of Triton X-100

(a) The same conditions as described for Fig. 2(a) were used except that 0.5% Triton X-100 was included in the incubation mixture. (b) Fluorography of the t.l.c. plate of the experiment shown in (a). Purified standard Ret-P-Man was applied in the column marked St. Positions of standard Dol-P-Man, undecaprenyl phosphate mannose (C₅₅-P-Man), Ret-P-Man and Dol-P-P-GlcNAc₂-Man are shown. Film was exposed for 32 days. Only the 2 min, 10 min and 90min time points are shown. The positions of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

Fig. 4. Studies on the bivalent-cation-dependence of the synthesis of Ret-P-Man and Dol-P-Man from endogenous acceptors by rat liver microsomes

(a) Bivalent-cation-dependence of Ret-P-Man synthesis from endogenous Ret-P in rat liver microsomes. The same conditions as described for Fig. 2(a) were used, except for stopping the reaction at 15 min. The effects of MnCl₂ (●), MgCl₂ (○) and CoCl₂ (△) are shown. The lower-phase extract was used for the assay. (b) Bivalent-cation effect on Dol-P-Man synthesis from endogenous Dol-P in rat liver microsomes. The effects of MnCl₂ (●), MgCl₂ (○) and CoCl₂ (△) on Dol-P-Man synthesis are shown. The values were obtained from the same experiments as in (a). (c) Fluorography of Mn²⁺-dependence study. The positions of standard Dol-P-Man, undecaprenyl phosphate mannose (C₅₅-P-Man), Ret-P-Man and mannose are shown. The film was exposed for 14 days. Only concentrations of 0 mM-, 5 mM- and 50 mM-MnCl₂ are shown. (d) Fluorography of Co²⁺-dependence study. The film was exposed for 14 days. Concentrations of 0.025 mM-, 5 mM-, 10 mM- and 50 mM-CoCl₂ are shown. (e) Fluorography of Mg²⁺-dependence study. The film was exposed for 5 weeks. Concentrations of 0.621 mM-, 5 mM- and 50 mM-MgCl₂ are shown. The position of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

endogenous Ret-P, optimal MgCl₂ concentration was at 10 mM, and Ret-P-Man synthesis was still very high (65% of maximum) at 50 mM-MgCl₂, whereas CoCl₂ and MnCl₂ completely inhibited at 50 mM. Fluorographs of t.l.c. profiles (representative points only are shown) of the lower-phase extract of incubation mixture containing MnCl₂ (Fig. 8b), CoCl₂ (Fig. 8c) and MgCl₂ (Fig. 8d) are shown for comparison. Here again, as for Figs. 4 and 9(d), a multiplicity of bands is observed with *R_F* close to that of Dol-P-P-GlcNAc₂-Man.

Studies on hamster liver in normal and vitamin A-depleted animals

A remarkable similarity between the rat and the hamster liver microsomes was observed for Mn²⁺-dependence of Ret-P-Man synthesis from endogenous Ret-P (Fig. 9a). As for the rat, MnCl₂ constituted an absolute requirement for Ret-P-Man synthesis, but not for Dol-P-Man synthesis, by hamster liver microsomes in the bovine serum albumin incubation system. However, the ratio of

Ret-*P*-Man to Dol-*P*-Man in the lower-phase extract was greater (0.3:1) with the hamster preparation than that (0.23:1) with the rat preparation. Vitamin A-depleted and normal hamster liver microsomes

Fig. 5. Dependence on Mn^{2+} and Co^{2+} of Ret-*P*-Man synthesis by rat liver microsomes as measured in monophasic extracts

Incubation mixtures were the same as described for Fig. 2(a) except that either $MnCl_2$ (●) or $CoCl_2$ (△) was used. The monophasic extraction procedure was used for the assay. The positions of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

Fig. 6. Effect of Triton X-100 on Mn^{2+} -dependence of Ret-*P*-Man (○) and Dol-*P*-Man (●) synthesis from endogenous acceptors by rat liver microsomes

The incubation system to test 'endogenous lipid acceptors' (see the Materials and methods section) was used except that 0.5% Triton X-100 was included. Duplicate t.l.c. plates were used: one (a) for scraping and counting of radioactivity, the other (b) for fluorography; only concentrations of 0 mM-, 5 mM- and 50 mM- $MnCl_2$ are shown. The film was exposed for 2 weeks. The positions of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

were compared for their ability to synthesize Ret-*P*-Man and Dol-*P*-Man from endogenous lipids. Fig. 9(b) shows a maximum (15 min at 37°C) for

Fig. 7. Mn^{2+} -dependence of Dol-*P*-Man synthesis from exogenous Dol-*P* by rat liver microsomes

The incubation system contained: exogenous Dol-*P* (0.15 mM) dissolved in Triton X-100 (final concn. 0.5%), 278 μ g of rat liver microsomal protein suspended in 5 μ l of 0.9% NaCl and GDP-[¹⁴C]mannose (0.2 μ Ci; 5 μ M) in addition to the usual ingredients as specified in the Materials and methods section. Incubation proceeded for 2 min at 37°C. The reaction was stopped with 3 ml of chloroform/methanol (2:1, v/v), to which 0.6 ml of distilled water was added. The mixture was centrifuged at 1000 *g* for 5 min. The lower phase was washed with 0.6 ml of methanol/water (1:1, v/v) and re-centrifuged. The lower phase was dried and the radioactivity measured in Econofluor.

Fig. 8. Bivalent-cation requirement for the synthesis of Ret-P-Man from exogenous Ret-P by rat liver microsomes (a) Linear expression of Mn²⁺ (●), Mg²⁺ (○) and Co²⁺ (Δ) dependence of Ret-P-Man synthesis. Incubation mixtures contained 0.13 mM-Ret-P and incubations were stopped after 2 min at 37°C. The lower-phase extracts were used for t.l.c. analysis in solvent A. Fluorographs for MnCl₂ (b), CoCl₂ (c) and MgCl₂ (d) were processed as described in the Materials and methods section. Film was exposed for 2 weeks for CoCl₂ (c) and MgCl₂ (d) and for 5 days in the case of MnCl₂ (b). Only selected concentrations are shown, for brevity. The positions of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

Ret-P-Man synthesis at 2.5 mM-MnCl₂ for vitamin A-deficient microsomes; however, the actual amount of Ret-P-Man synthesized is greatly decreased compared with microsomes from normal hamsters (Fig. 9a), and the ratio of Ret-P-Man to Dol-P-Man is greatly diminished, this being also due to a concomitant accumulation of Dol-P-Man in vitamin A-deficient microsomes, as shown in Table 2. This is

readily realized from the fluorographs shown in Fig. 9(c). Lower-phase extracts of incubation mixtures containing 2.5 mM-, 5 mM- and 10 mM-MnCl₂ are compared in microsomal systems from normal and vitamin A-deficient hamsters. A decrease in radioactivity associated with Ret-P-Man is observed. Here, as before, the doublet of bands opposite the Ret-P-Man marker was considered as Ret-P-Man

Fig. 9. Mn^{2+} -dependence of the synthesis of Ret-P-Man (○) and Dol-P-Man (●) from endogenous Ret-P and Dol-P by hamster liver microsomes

(a) Syrian-golden-hamster livers from normal animals were used for the preparation of microsomes. The incubation and processing of the lower-phase extract is as described for the assay of endogenous Ret-P and Dol-P of rat liver microsomes. (b) The details are the same as those indicated for (a), except that microsomes from vitamin A-deficient hamster livers were used. (c) Fluorography of $[^{14}C]$ mannolipids obtained at various concentrations of $MnCl_2$ with microsomes from normal and vitamin A-deficient hamsters. A mixture of standard Dol-P-Man and Ret-P-Man was applied in the column marked St. The film was exposed for 39 days. The lower-phase extract was used for t.l.c. in solvent A. (d) Fluorography of Mn^{2+} -dependence study of Ret-P-Man and Dol-P-Man synthesis from endogenous Ret-P and Dol-P of vitamin A-deficient hamster liver microsomes. The lower-phase extract was used for t.l.c. in solvent A. The film was exposed for 14 days. Concentrations of 0 mM-, 25 mM- and 50 mM- $MnCl_2$ are shown. The position of the named compounds indicate the chromatographic mobilities of samples of these when used as chromatographic standards.

because of the variability in the relative proportion of the two bands, possibly due to isomerization. It does seem, however, that a specific depletion of the lower band takes place in the vitamin A-deficient membrane. At the same time Dol-P-Man increases,

and multiple bands migrating in the area of standard undecaprenyl phosphate mannose are also seen (materials B and C). Material B is chromatographically similar to undecaprenyl phosphate mannose, and material C is slightly less hydrophobic, as

Table 2. *Effect of vitamin A deficiency on the Ret-P-Man/Dol-P-Man ratio from endogenous lipid acceptor at different concentrations of MnCl₂*

For explanation and full experimental details see the text.

[MnCl ₂] (mm)	Normal						Vitamin A-deficient					
	Ret-P-Man synthesis			Dol-P-Man synthesis			Ret-P-Man synthesis			Dol-P-Man synthesis		
	(c.p.m./ 15 min per mg of protein)		(pmol)	(c.p.m./ 15 min per mg of protein)		Ret-P-Man/ Dol-P-Man ratio	(c.p.m./ 15 min per mg of protein)		(pmol)	(c.p.m./ 15 min per mg of protein)		Ret-P-Man/ Dol-P-Man ratio
	(mm)											
0	0	0	420	1.26	0	0	0	0	1480	4.44	0	0
0.025	0	0	670	2.01	0	0	21	0.06	3240	9.72	0.006	0.006
0.125	32	0.09	920	2.76	0.03	0.03	42	0.12	4190	12.57	0.010	0.010
0.625	111	0.33	910	2.73	0.12	0.12	160	0.48	5140	15.42	0.031	0.031
2.5	369	1.10	1090	3.27	0.33	0.33	270	0.81	4560	13.68	0.059	0.059
5.0	364	1.09	1240	3.72	0.29	0.29	171	0.51	4730	14.19	0.036	0.036
10.0	329	0.98	780	2.34	0.42	0.42	130	0.39	2750	8.25	0.047	0.047
50.0	59	0.17	390	1.17	0.15	0.15	40	0.12	370	1.11	0.10	0.10

shown by the arrow. Moreover, an accumulation of a compound with chromatographic characteristics of Dol-P-P-GlcNAc₂-Man is also found (Fig. 9c). This is confirmed in Fig. 9(d), where a study of mannlipid synthesis in the 0–50 mm range of MnCl₂ concentrations is shown for the vitamin A-deficient microsomes. The results of an experiment designed to compare the synthesis of Ret-P-Man and Dol-P-Man and their ratios in normal and severely vitamin A-deficient hamster liver microsomes are shown in Table 2.

Discussion

The incubation in the presence of bovine serum albumin permits the detection of the following mannlipids from endogenous lipids: (1) Ret-P-Man; (2) a slightly more hydrophobic compound at times also found in incubations containing exogenous Ret-P and possibly representing an isomeric form of Ret-P-Man (Rosso *et al.*, 1975); (3) a mannlipid with the same chromatographic characteristics as undecaprenyl phosphate mannose; (4) Dol-P-Man. Radominska-Pyrek *et al.* (1979) have characterized a fully unsaturated decaprenol from bovine pituitary gland, and the putative undecaprenyl phosphate mannose may be related to that structure.

The amount of Ret-P available for mannosylation is about 1 ng/mg of microsomal rat liver protein for the rat (Shidoji & De Luca, 1981) and 0.71 ng/mg for the hamster, as assayed at 5 mM-MnCl₂ for 15 min at 37°C. These concentrations compare with Dol-P values of 20 ng/mg of microsomal rat protein and 6 ng/mg in the hamster. The finding that about 50–60% of Ret-P-Man partitions in the upper phase of the extraction procedure is not surprising, if one

considers the relatively short polyisoprenoid chain (C₂₀) of retinol. A more rapid assay for both Ret-P-Man and Dol-P-Man synthesis from endogenous lipid acceptors involves extraction with 15 vol. of chloroform/methanol (2:1, v/v) to yield a monophasic extract. However, determinations of Ret-P-Man are rendered difficult by large amounts of mannose present in monophasic extracts, and for this reason the procedure involving the use of the biphasic extract is preferable. Whatever the extraction procedure, it is crucial to dry down the extract, immediately redissolve it in chloroform/methanol (2:1, v/v) containing carrier Ret-P and perform the t.l.c. procedure within the same day, if one wants to avoid selective breakdown of Ret-P-Man within 24 h, even in the refrigerator at 4°C. Storage at –20°C does not protect Ret-P-Man from breakdown in the presence of salts. It should also be emphasized that membrane preparations used in this work were always utilized within 1–2 weeks. Prolonged storage of the membrane preparation in liquid N₂ (2–3 months) preferentially destroyed Ret-P-Man synthesis from endogenous Ret-P compared with Dol-P-Man synthesis from endogenous Dol-P.

By using these procedures, distinctive characteristics between Ret-P-Man and Dol-P-Man synthesis are found. Thus a bivalent cation (Mn²⁺, Co²⁺ or Mg²⁺) is essential for Ret-P-Man synthesis, but not for Dol-P-Man synthesis, in the assay in the presence of bovine serum albumin. The optimal concentration for Ret-P-Man synthesis from endogenous Ret-P is higher for Mg²⁺ (10 mM) than for Co²⁺ and Mn²⁺ (5–10 mM). Triton X-100 (0.5%) completely inhibits Ret-P-Man synthesis; it also causes partial inhibition of endogenous Dol-P-Man synthesis as assayed in the bovine serum albumin system. A similar inhibitory effect of Triton was

observed at 0.4% for Dol-*P*-Man synthesis in the rough endoplasmic reticulum but not the smooth endoplasmic reticulum of rat liver by Nilsson *et al.* (1978). Similarly to reports by Kerr & Hemming (1978), the metal-ion requirement becomes absolute in the presence of the detergent in Dol-*P*-Man synthesis from both endogenous and exogenous Dol-*P*. The optimal concentration for this synthesis shifts from 5 mM to 2.5 mM in the presence of the detergent. Similarly, optimal Ret-*P*-Man synthesis from exogenous Ret-*P* in rat liver microsomes has been consistently observed to require 2.5 mM-MnCl₂ instead of 5–10 mM, the observed maximum range for the endogenous system.

In the present paper we have shown for the first time that vitamin A deficiency specifically lowers the amount of Ret-*P* by 50% in the liver microsomes, and it increases the amount of Dol-*P*. The amount of enzyme activities responsible for Ret-*P*-Man and Dol-*P*-Man synthesis were the same as in normal liver microsomes (results not shown). It should be said that the hamsters displayed typical symptoms of vitamin A deficiency, including weight loss, closed eye lids and an un-co-ordinated walking pattern, and that their vitamin A liver stores had fallen to undetectable values. (Retinyl palmitate concentrations in matched normal hamsters were about 30 µg/g wet wt. of liver tissue.) Ret-*P*-Man behaved as either one or two chromatographic bands, without a predictable pattern. Therefore the total amount was always considered in the calculations of Ret-*P*-Man synthesis. The two bands may represent isomeric forms of Ret-*P*-Man, and their possible biological significance remains to be established.

The increase in the amount of Dol-*P* available for mannosylation appears to be at variance with our own previous findings obtained *in vivo*, in which a decrease in the incorporation of [¹⁴C]mannose into both Ret-*P*-Man and Dol-*P*-Man was observed in livers from severely vitamin A-deficient hamsters (De Luca *et al.*, 1975). Apparently this discrepancy cannot be resolved on the basis of differences in precursor pool sizes *in vivo*, inasmuch as the relatively small difference in mannose pools (699 nmol/g wet wt. of liver in normal and 560 nmol/g in deficient tissue) could not explain the large decrease (90%) in Dol-*P*-Man labelling *in vivo* (De Luca *et al.*, 1975). However, the relatively large accumulation of radioactive precursor (from 1.6×10^5 d.p.m./g in normal to 4.9×10^5 d.p.m./g of deficient liver after 30 min from intraperitoneal injection) suggests lack of utilization of the mannose as deficiency becomes more severe.

Taken together, the results obtained *in vitro* and *in vivo* suggest that the decrease in Ret-*P*-Man synthesis is the primary result of vitamin A deficiency, and that, in the highly organized structure of the intact cell, Ret-*P* depletion causes

accumulation of Dol-*P*, possibly because less Dol-*P*-Man is being utilized for protein mannosylation, consistent with a converging pathway for these lipid intermediates. Such accumulation is rendered evident in the microsomal system, where membrane continuity and control is at least partially broken down. These findings, though preliminary, are consistent with a regulatory role of Ret-*P* pools in glycoprotein synthesis, particularly if one considers recent findings that Dol-*P*-*P*-GlcNAc₂-Man₅ also appears to accumulate in vitamin A-deficient rat liver (Rosso *et al.*, 1981) and that our results suggest an increased pool of Dol-*P*-*P*-GlcNAc₂ available for mannosylation in the microsomes from vitamin A-deficient hamsters.

In addition to Dol-*P*, less hydrophobic mannosyl acceptors appear to accumulate in the vitamin A-deficient hamster liver. The function, if any, and the structure of these compounds (B and C) is unknown, but their accumulation becomes of relative interest if one considers that ubiquinone, a substituted benzoquinone with a C₄₅ polyisoprenoid side chain, also accumulates in vitamin A-deficient rat liver (Lowe *et al.*, 1953).

In conclusion, the present work has demonstrated unequivocally that mammalian liver contains Ret-*P* and that the condition of vitamin A deficiency specifically decreases the pool of Ret-*P*. It also has shown that a mannosyl lipid of chromatographic characteristics similar to undecaprenyl phosphate mannose is synthesized in the endogenous system. Therefore the conclusion that at least three mannosyl lipids are synthesized by rat liver microsomes seems warranted by these findings. The reason for the apparent accumulation of Dol-*P* and Dol-*P*-*P*-GlcNAc₂ in vitamin A deficiency is not clear, but it is consistent with a regulatory role of Ret-*P* in mannosylation of dolichyl pyrophosphate oligosaccharides.

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