MANNOSYL TRANSFER FROM MANNOSYLRETINYLPHOSPHATE TO GLYCOCONJUGATES OF RAT LIVER MEMBRANES

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Received 16 October 1979

1. Introduction

The metabolic mode of action of vitamin A in maintaining growth and normal phenotypic expression is still unknown, although its molecular function in the visual cycle has been elucidated [1,2].

Some insight into a possible metabolic function of the 'oldest' of the vitamins was gained, when it was observed that its deficiency caused a drastic reduction in the incorporation of labelled monosaccharides into glycoproteins of a variety of tissues [3–5]. The greatest effect (90%) was noted on the incorporation of [^{14}C]mannose into liver glycoproteins [6]. Conversely, excessive doses of this essential nutrient caused an increase in the amount of [^{14}C]mannose incorporated into rat liver glycoconjugates [7]up to 600% over controls.

On the basis of these and other data, it was proposed that retinol may be phosphorylated to retinylphosphate and that the phosphorylated vitamin may function as a carrier of glycosyl residues for the biosynthesis of some glycoproteins [8] in a manner akin to the mode of action of bacterial and mammalian polyprenols [9,10]. Phosphoryl [11–13] and glycosylphosphoryl [14–18] derivates of vitamin A have been characterized from several systems in vivo and in vitro. However, the function of MRP as donor of mannose to glycoproteins is not clear at present.

Here we demonstrate that mannosylretinylphosphate donates mannose to glycoconjugate acceptors of the rat liver, provide further characterization of the mannosyl-acceptors and compare them to those generated by dolichyl mannosylphosphate.

Abbrevations: MRP, mannosylretinylphosphate; RP, Retinylphosphate; DMP, dolichylmannosylphosphate; SDS, sodium dodecyl sulfate; GDP-mannose, guanosinediphosphomannose

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2. Materials and methods

2.1. *Preparation of rat liver membrane fraction*

Normal male rats (150–200 g) (Osborne-Mendel Stock) were killed by decapitation and livers were removed, weighed and washed with cold medium A (50 mM Tris–HCl (pH 7.6); 5 mM $MgCl_2$, 25 mM KCl; 0.25 M sucrose) [19]. All the following operations were conducted at 0–4°C. Livers were cut into small pieces and homogenized in 2 vol. medium A. The homogenate was centrifuged twice at 2500 × *g* for 20 min. The resulting supernatant was centrifuged at 105 000 × *g* for 1 h. The pellet was rinsed with medium A and suspended in medium A by gentle homogenization. The suspension was divided into 0.5 ml aliquots and stored in liquid nitrogen.

2.2. *Preparation of [^{14}C]mannosylretinylphosphate*

The incubation mixture was similar to that in [15], and all operations were conducted in red or yellow light. Retinylphosphate (42–45 μg) in methanol was dried under ultrapure nitrogen and dissolved in Triton X-100 (final conc. 0.5%); to this was added in the indicated final conc.: 30 mM Tris–HCl buffer (pH 8.0); 3 mM ATP; 10 mM $MnCl_2$; GDP-[^{14}C]mannose (New England Nuclear) 2 μCi of spec. radioact. 208.8 mCi/mmol and 100 μl rat liver membrane (~3 mg protein) containing 2.5 mM EDTA. The mixture, in 200 μl final vol., was incubated at

37°C for 30 min and the reaction stopped with methanol.

The methanolic extract was always adjusted to contain <1 mM salt. [^{14}C]Mannosylretinylphosphate was purified by chromatography on DEAE-cellulose acetate and eluted with 10 mM ammonium acetate in 99% methanol.

2.3. *Transfer of [^{14}C]mannose from [^{14}C]mannosylretinylphosphate to endogenous acceptors*

The incubation mixture contained: 60 mM Tris–HCl (pH 8); 5 mM $MnCl_2$; 0.5% Triton X-100; 35 400–39 200 cpm [^{14}C]MRP; 5 μl rat liver membrane preparation (409–525 μg protein) in 50 μl final vol. [^{14}C]Mannosylretinylphosphate was first added to the tube and the solvent was removed under a stream of nitrogen. After addition of the other constituents, the reaction was started by adding the liver membrane preparation and the mixture was incubated at 27°C for 30 min. After cooling in ice the mixture was applied directly on Whatman 3MM paper and the chromatogram was developed in 95% ethanol/1 M ammonium acetate (pH 7.5) 7/3 (solvent A) for 18 h [20]. The paper was dried and the origin was cut out and counted in 10 ml solution of 5 g 2,5-diphenyloxazone and 50 mg 1,4-bis-2-(5-phenoloxazolyl) benzene in 1 liter toluene. Counting efficiency was 80%. Alternatively, the incubation was stopped by adding 15 vol. chloroform/methanol (2/1). After centrifugation the denatured membranes were washed with chloroform/methanol (2/1), water and chloroform/methanol/water (1/1/0.3).

2.4. *Preparation of dolichyl[^{3}H]mannosylphosphate*

The incubation mixture contained the following constituents at the indicated final conc.: 60 mM Tris–HCl buffer (pH 7.0); 10 mM $MnCl_2$; 10 mM $MgCl_2$; 0.5% Triton X-100; 100 μCi GDP-[1-^{3}H]mannose of spec. radioact. 12.6 Ci/mmole (New England Nuclear) and rat liver membranes, 12.5 mg protein; 1 ml total vol. This mixture was incubated for 20 min at 37°C and the reaction was stopped by the addition of 5 vol. chloroform/methanol (2/1) to obtain two phases. Dolichyl[^{3}H]mannosylphosphate was purified from the lower phase by chromatography on a column of DEAE-cellulose acetate from which it was eluted at 10 mM ammonium acetate. Silica gel thin-layer chromatography in chloroform/methanol/water (60/25/4) yielded one radioactive spot at R_F 0.57, corresponding to synthetic dolichylmannosylphosphate, kindly provided by Dr Christopher Warren.

2.5. *Transfer of [^{3}H]mannose from dolichyl[^{3}-H]mannosylphosphate to endogenous acceptors*

Dolichyl[^{3}H]mannosylphosphate (4.2 μCi) dissolved in chloroform/methanol (2/1) was added first to the tube and the solvent was removed. The following constituents were added in the indicated final conc.: 0.1 M Tris–HCl buffer (pH 7.2); 10 mM $MnCl_2$; 10 mM $MgCl_2$; 0.5% Triton X-100 and rat liver membranes (25 mg protein) in 5 ml final vol. This mixture was incubated for 2 h at 37°C and the reaction was stopped by adding 5 vol. chloroform/methanol (2/1). The denatured interphase material was washed 3 times with each of the following solvents: chloroform/methanol (2/1); water; and chloroform/methanol/water (1/1/0.3), to yield a denatured pellet. This pellet was solubilized with 1% SDS containing 5 mM Tris–HCl buffer (pH 7.0) and used for chromatography as described.

2.6. *Other methods*

Retinylphosphate was prepared as in [21]. Mild acid hydrolysis was performed in tetrahydrofuran/0.5 N HCl (4/1) at 50°C for 1 h [22]. Pronase treatment was done in 0.1 M Tris–HCl buffer (pH 7.8) containing 10 mM $CaCl_2$ and 100 μg pronase/ml (activity 101 000 PUK/g; Calbiochem, San Diego, CA). A few drops of toluene was added and the mixture was incubated at 37°C for 72 h. Additional pronase in portions of 100 μg/ml was added at 24 and 48 h. Monosaccharides were determined by gas liquid chromatography of the hexitol boronates [6] essentially as in [23].

Ultraviolet light treatment of mannosylretinylphosphate was done with a shortwave (254 nm) lamp at ~2 cm at 875 W/cm^2. Electrophoresis of solubilized acceptors was done in SDS–polyacrylamide slab gels as in [24].

3. Results

Fig.1 shows the analysis by thin-layer chromatography on silica gel of the negatively charged [^{14}C]-labeled mannolipid fraction eluted from DEAE-cellulose column with 10 mM ammonium acetate. Fig.1a represents the incubation containing exogenous RP. The radioactive product at R_F 0.25 was

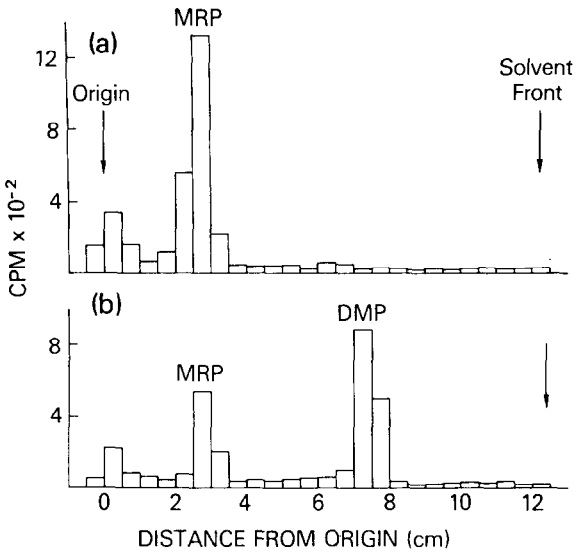

Fig.1. Silica gel thin-layer chromatography of mannolipids obtained from an incubation with (a) and without (b) exogenous retinylphosphate. The incubation mixture is in section 2. The labelled lipids were purified by chromatography on DEAE-cellulose as in [28]. Plates were developed in chloroform/methanol/water (60/25/4). Radioactivity was detected in the scraped gel as in [16].

identified [15] as [^{14}C]MRP. No [^{14}C]DMP (R_F 0.5–0.6) was detectable in this preparation, because of competition for GDP-mannose by excess RP. Fig.1b shows that, in the absence of exogenous RP, both [^{14}C]MRP and [^{14}C]DMP were present in the monophasic extract from rat liver membranes. The radioactivity at the origin of the thin-layer plates in both preparations was eluted from the plates with water with a recovery of 96%. Mild acid hydrolysis (see section 2) of this radioactive product yielded a compound which comigrated with [^{3}H]mannose upon paper chromatography in *n*-butanol/pyridine/water (6/4/3) (solvent B). Thus, the product at the origin was not a lipid-bound oligosaccharide and was probably formed by breakdown of [^{14}C]MRP on the silica gel. It was also formed upon storage of [^{14}C]-MRP in liquid nitrogen, since its proportion in comparison to [^{14}C]MRP increased during storage.

[^{14}C]Mannosylretinylphosphate (6.2 μCi) was obtained free of dolichyl[^{14}C]mannosylphosphate from an incubation containing 20 μCi of GDP-[^{14}C]mannose as in section 2.

Additional identification of [^{14}C]MRP was performed by alkaline and acid hydrolysis [25] as well as by UV absorption spectroscopy after removal of RP [11].

Hydrolyses in 0.1 M NaOH, 65°C, 20 min and 0.1 N HCl, 100°C 10 min yielded mannosylphosphate (R_F 0.54) and a mixture of mannosylphosphate and mannose (R_F 0.82), respectively, as determined by paper chromatography in solvent A. Intact [^{14}C]-MRP displayed a UV absorption spectrum (fig.2a) typical of retinol and retinylphosphate [26] with a maximum at 325 nm. After 1 min of treatment (fig.2b) with the UV probe, most [^{14}C]MRP was destroyed. After 3 min, no maximum at 325 nm was detected (fig.2c).

The ability of intact and UV-treated [^{14}C]MRP preparations to donate [^{14}C]mannose to endogenous acceptors of rat liver membranes is shown in fig.3. Intact [^{14}C]MRP functioned as a donor of [^{14}C]mannose to nonlipidic endogenous acceptors. The transfer reaction was linear for 30 min and proceeded at a lesser rate up to 2 h. About 16% of the original radioactivity was transferred at this time. In contrast, only ~3% of the original radioactivity was transferred, if UV-treated [^{14}C]MRP was used as the donor or if the incubation was conducted with boiled enzyme.

Some properties of the enzymatic system for the transfer reaction were studied. Mn^{2+} was slightly stimulatory, in contrast to Mg^{2+} and Ca^{2+}, which were not effective; EDTA displayed a slightly inhibitory effect. The pH optimum was 8–9. Triton X-100 stimulated the reaction 2-fold at 0.5% in an incubation containing 0.5 mg protein. Other detergents tested at 0.5% (Tween 20, sodium deoxycholate, Tween 80) were also effective, but to a lesser extent; SDS had a slightly inhibitory effect.

An attempt at characterizing the endogenous acceptors was made. Labelled membranes were prepared using [^{14}C]MRP in an incubation scaled up 70-fold over the standard incubation in section 2. The membranes were then extracted 3 times with each of the following solvents: chloroform/methanol (2/1); water; and chloroform/methanol/water (1/1/0.3). A small proportion of the radioactivity, 1% of the total, was found in the chloroform/methanol/water extract. This and the water extract were hydrolized separately by mild acid and the resulting material was chromatographed on paper to test for the release of [^{14}C]oligosaccharides from MRP. The radioactivity cochromatographed with standard mannose, thus excluding the formation of lipid-linked oligosaccharides. The chloroform/methanol extract

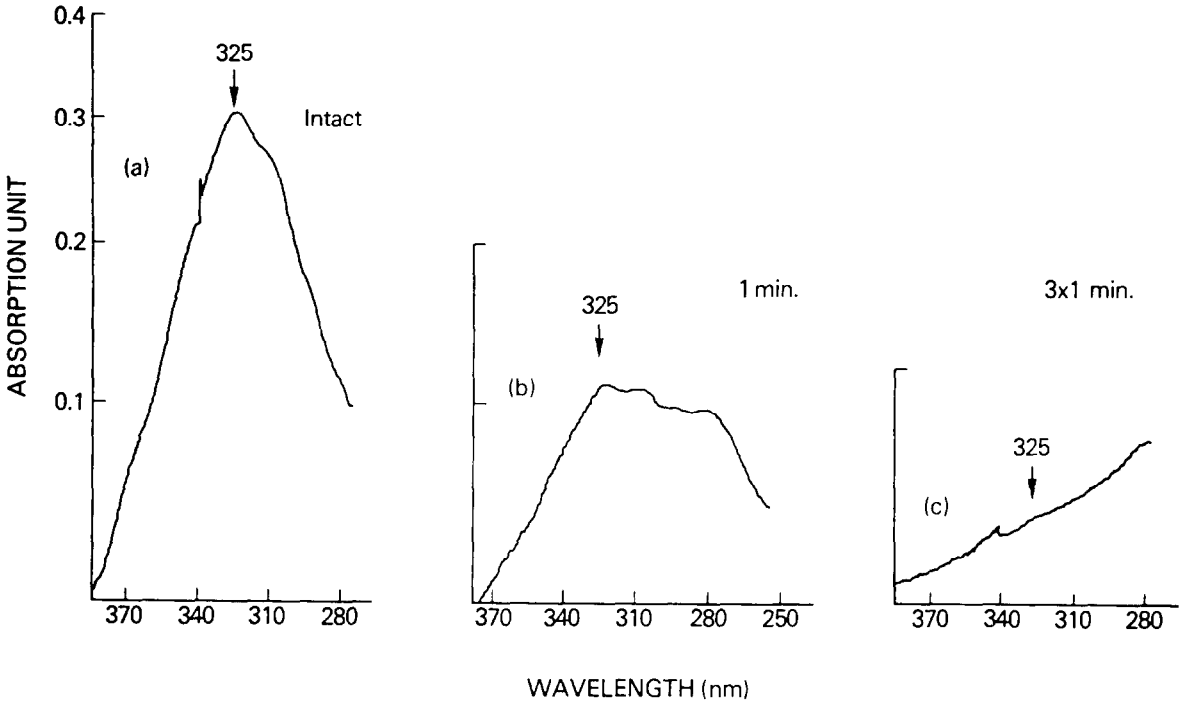

Fig.2. Ultraviolet absorption spectra of intact (a) and UV-treated (b,c) [^{14}C]mannosylretinylphosphate. About 3 μg [^{14}C]MRP was dissolved in 1 ml methanol and placed in a test tube with an internal diam. 1.3 cm. The test tube was kept in ice and the UV light source was placed 2 cm from the surface of the liquid, as specified in section 2.

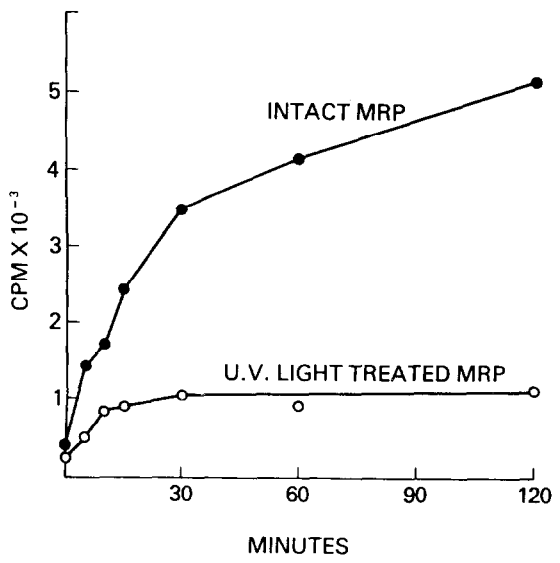

Fig.3. Time course of mannosyl transfer reaction from intact and UV-treated (3 min) [^{14}C]MRP into endogenous acceptors of rat liver membranes.

was subjected to hydrolysis in 0.1 N methanolic solution of LiOH at room temperature for 30 min to hydrolyze glycerolipids. The resulting material was chromatographed on DEAE-cellulose acetate and the 10 mM ammonium acetate eluate was chromatographed on thin layer of silica gel to test whether [^{14}C]DMP was formed from [^{14}C]MRP during the incubation: no radioactivity was detected in the area of DMP (R_F 0.5–0.6).

The denatured membranes were then extracted with 1% SDS, which solubilized from 65–80% of the radioactivity in 4 experiments. Another portion of the denatured membrane was treated with pronase, which solubilized 90–93% of the radioactivity. Fig.4a shows the elution profile from Sephadex G-150 of the [^{14}C]mannosyl-acceptors solubilized by treatment with SDS. Of the eluted radioactivity, 90% was found to be associated with macromolecules. Sephadex G-150 chromatography, after pronase digestion, showed that the macromolecular acceptors were susceptible to proteolysis (fig.4b).

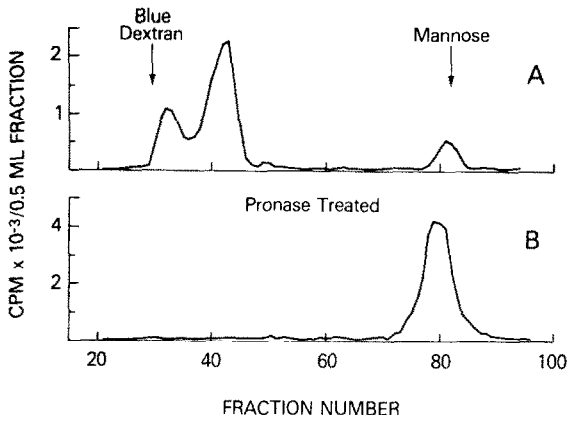

Fig.4. Sephadex G-150 chromatography of ^{14}C-labelled acceptors of rat liver membranes. (a) Labelled products extracted with 1% SDS containing 5 mM Tris–HCl (pH 7.0). (b) Labelled products after pronase treatment. Column (1.5 × 30 cm) was eluted with 5 mM Tris–HCl (pH 7.0) containing 10^{-4} M EDTA, 0.1% mercaptoethanol and 0.2% SDS [32]. Fractions (0.5 ml) were collected.

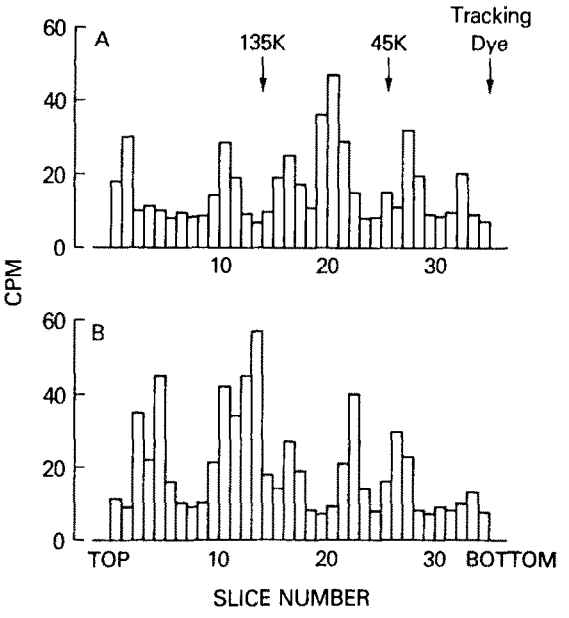

Fig.5. SDS–polyacrylamide gel electrophoresis of (A) [^3H]mannosyl- and (B) [^{14}C]mannosyl-acceptors of rat liver membranes obtained from dolichyl[^3H]mannosylphosphate and [^{14}C]mannosylretinylphosphate, respectively. The denatured pellet after three extractions with each of the following solvents: chloroform/methanol (2/1), water; and chloroform/methanol/water (1/1/0.3), was solubilized in 1% SDS containing 5 mM Tris–HCl (pH 7.0). This extract was brought to sample buffer, boiled for 2 min and applied to a gel for electrophoresis. The gel was cut into 2.5 mm slices combusted in a Packard Oxidizer and counted. Arrows indicate the position of standard proteins visualized by Coomassie blue staining: β-galactosidase (135 000 mol. wt) and ovalbumin (45 000 mol. wt).

Recently, mannosyl and galactosyl residues have been demonstrated to be covalently linked to specific mammalian ribonucleic acids [27]. However, ribonuclease treatment of the [^{14}C]mannosyl-macromolecular acceptors did not yield any ethanol-soluble radioactive products.

SDS–polyacrylamide gel electrophoresis of mannosyl-acceptors showed (fig.5) a population of distinct radioactive products, for the two lipid intermediates. This experiment was repeated with essentially the same results. Acid hydrolysis (3 N HCl, 100°C, 3 h) of the macromolecular acceptors and analysis of the hexitol boronates by gas–liquid chromatography showed mannose as the only radioactive monosaccharide.

4. Discussion

In vivo studies have shown that depletion of retinol (vitamin A) and its excess profoundly affect the biosynthesis of some glycoproteins in a variety of tissues [4,7,28]. Thus, the idea was proposed that retinylphosphate may function in membranes as a carrier for specific glycosyl residues [28].

The biosynthesis of retinylphosphate and its mannosyl derivative has been demonstrated in vivo and in cultured cells (reviewed [28]). Also, depletion or repletion of vitamin A specifically decreases or stimulates the biosynthesis of MRP in rat livers, in vivo, without affecting the biosynthesis of DMP [29]. However, excessive doses of retinol cause an increase also in the synthesis of DMP [7].

Contrary to dolichylphosphate which accepts a variety of sugar residues from their nucleotides, retinylphosphate appears to be a specific acceptor for mannosyl residue. Thus the sugar nucleotides: UDP-*N*-acetylglucosamine; UDP-glucose; UDP-galactose; CMP-*N*-acetyl-neuraminic acid, were ineffective as donors of glycosyl residues to retinyl phosphate in rat liver membranes [30]. However, galactosyl-retinylphosphate may be synthesized by other systems [13].

It has been shown that MRP functions as a donor

of mannose to endogenous acceptors of rat liver membranes [31]. However, negative controls were not included in these studies. Here, we have demonstrated the necessity for an intact retinol residue in MRP for the transfer activity, inasmuch as the UV-treated MRP lost its activity as a donor. Moreover, we have shown for the first time differences in the subunit molecular weight of the acceptors derived from the two lipid intermediates. This observation coupled with the characterization of MRP as a physiological compound [28] is suggestive of a role of this derivative of vitamin A in glycoprotein synthesis, distinct from the role of the dolichol derivatives.

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