EFFECTS OF EXOGENOUS RETINOL AND RETINOIC ACID ON THE BIOSYNTHESIS OF 14C-MANNOSE LABELLED GLYCOLIPIDS AND GLYCOPROTEINS IN RAT LIVER

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Summary Studies were conducted to investigate the in vivo and in vitro effects of retinol and retinoic acid on the synthesis of mannolipids and mannopeptides in rat liver. The incorporation of 14C-mannose into glycolipids and glycoproteins showed a decrease in vitamin A-depleted rats as compared with vitamin A-fed rats. By means of DEAE-cellulose, silicic acid and thin-layer chromatography, the mannose-containing lipids were separated into mannosyl retinyl phosphate (MRP, Rf 0.2) and dolichyl mannosyl phosphate (DMP, Rf 0.4), respectively. A rapid increase in the synthesis of labelled MRP was observed, exhibiting a peak between 25 and 60 min after intraperitoneal administration of retinol to vitamin A-depleted rats. Similarly, administration of retinoic acid brought about elevation of 14C-mannolipid (Rf 0.2) synthesis with a peak at 60 min after injection. On the other hand, the incorporation of 14C-mannose into DMP (Rf 0.4) remained unchanged by such treatment. In vitro addition of retinyl phosphate, but not retinoyl phosphate, markedly stimulated the synthesis of 14C-mannolipid (Rf 0.2), using crude membrane of rat liver and GDP-14C-mannose as the donor. These findings strongly suggest that not only retinol but also retinoic acid plays an important biological role in mannosyl transfer reaction in rat liver. However, the molecular participation

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Abbreviations: MRP, mannosyl retinyl phosphate; DMP, dolichyl mannosyl phosphate; RP, retinyl phosphate; Royl-P, retinoyl phosphate; C–M–W, chloroform–methanol–water; DMSO, dimethyl sulfoxide; BHT, butyrylhydroxytoluene; RBP, retinol-binding protein.
of a metabolite of retinoic acid in the formation of a mannolipid and the structure of such a metabolite remain to be established.

Recently, the molecular involvement of vitamin A (retinol) in glycoprotein synthesis has been well established: either biologically (1) or chemically (2) synthesized retinyl phosphate (RP) accepts mannose moiety from GDP-mannose to form mannosyl retinyl phosphate (MRP) on the biological membrane (3), subsequently functioning as a donor of mannose to endogenous acceptors (4, 5). DeLuca et al. (6) have further demonstrated that biosynthesis of the mannose-containing glycolipid and glycopeptide of hamster liver is regulated by the vitamin A status of the animal.

It is also well documented that retinoic acid has selective vitamin A biological activity. Thus, rats which are maintained on a vitamin A-deficient diet supplemented with retinoic acid become blind (7) and sterile (8), in parallel to a specific block of retinol-binding protein (RBP) secretion by the liver (9). Otherwise, the rats grow at a normal rate and in generally good health (i.e., mucus-secretion, appetite, taste acuity, etc. are maintained). Hence, it was interesting to assess whether or not retinoic acid is molecularly involved in glycoprotein synthesis, in analogy to retinol as mentioned above.

The present study was undertaken to examine the response of mannolipids and mannoproteins biosynthesis in rat liver as induced by exogenous retinol as well as by retinoic acid.

EXPERIMENTAL

Animals and diets

Weanling male, Wistar-strain rats (Japan Clea Inc., Tokyo), weighing 35 to 40 g, were housed in hanging wire bottom cages and maintained on a vitamin A-deficient diet which was prepared in this laboratory as described previously (10). Vitamin-free casein (Nutritional Biochemicals Co., Cleveland) proved to be good for ready use without previous extraction by HCl-ethanol (9). Diets were mixed bi-weekly and stored at 4°C. The rats were allowed to eat and drink ad libitum and were given fresh food every other day. Body weight was recorded 3 times per week. When needed, rats were fed a freshly prepared vitamin A-deficient diet supplemented with all-trans-retinoic acid (Hoffmann-LaRoche Co., Basle), containing 25 µg per 10 g of the diet.

Experimental design (in vivo)

Experiment A: Weanling rats were fed the vitamin A-deficient diet for 43 days.

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3 No contamination of retinol in this preparation was found by high performance liquid chromatography, using model 013-S (Mitsumi Scientific Industries Inc., Tokyo): a column (2.1×500 mm) of Hitachi Gel No. 3010, n-hexane–tetrahydrofuran (98:2) as eluting solvent, flow rate 1.5 ml/min, 700 psi, and wavelength at 350 nm.
days, and were switched to the retinoic acid-supplemented diet between day 44 and 53, followed again by the deficient diet up to day 63 (i.e., retinol depletion for 63 days and retinoic acid depletion for 10 days before sacrifice). Thirty-three rats were divided into 11 groups, consisting of one control, 5 retinol-treated and 5 retinoic acid-injected groups, respectively. Either all-trans-retinol (Sigma Chemical Co., St. Louis) or retinoic acid was dissolved in DMSO (Sigma) to give a final concentration of 8 mM. Each 0.25 ml solution (2 μmoles per rat) was injected intraperitoneally 25 min, 1, 3, 8 and 15 hr before sacrifice. [1-14C]-D-mannose (53.1 Ci/mol; New England Nuclear, Boston) was also injected intraperitoneally 20 min before sacrifice at a dose of 28 μCi per rat (dissolved in 0.28 ml of 0.9% NaCl). Immediately after decapitation, livers were removed, frozen in dry-ice, and lipid was extracted on the following day.

Experiment B: Nine rats were maintained on the vitamin A-deficient diet without any retinoic acid supplementation (i.e., simple retinol depletion for 53 days), and then divided into 3 groups: one control group (Group I), and Group II and III were given intraperitoneally 60 min before sacrifice retinoic acid and retinol, respectively. Group IV (3 rats) was supplemented orally with 280 μg of retinyl palmitate in oil (Wako Pure Chemical Industries, Ltd., Osaka) per rat 3 times weekly between day 23 and 53. The 14C-mannose (25 μCi per rat) solution was also injected intraperitoneally 20 min before sacrifice.

In addition, rats in Group I and IV were used for in vitro study (see below) without injection of the radioactive mannose.

Extraction and separation of 14C-mannolipids (small scale)
All operations were conducted in dim light at room temperature.

Lipid extraction (Method A): Crude lipid in the liver of Exp. A was extracted as described previously (6). Each liver (0.8 g) was homogenized (3 strokes) with equivolume of ice-cold distilled water by a glass-Teflon homogenizer, and then extracted with 8 ml of C–M 2:1 (v/v). When the phases were separated by standing for 1 hr without centrifugation, the lower phase was obtained by aspiration with a transfer pipette. The three individual lower phases in each group were combined and stored at −20°C for further separation of 14C-mannolipids.

Lipid extraction (Method B): In order to achieve better recovery of the labelled mannolipid (Rf 0.2), slight modifications of lipid extraction were performed as follows: each 3 g of frozen liver in Exp. B was also homogenized with 3 ml of ice-cold distilled water. After mixing and shaking well with 30 ml of C–M 2:1 and standing for 4 hr, the three phases were clearly separated by low speed centrifugation. The upper phase (about 6.4 ml) was washed once with 9.6 ml of a theoretical lower phase, a mixture of C–M–W 86:14:1 (II), and successively re-extracted twice with equivolume of C–M 2:1. In Exp. B, both the lower phase and the second re-extracted lower phase from the original upper phase (designated U-E2) were mostly used for analyses of acidic mannolipids.
The intermediate layer was stored for glycopeptide analysis after three successive washings with C–M 2: 1, C–M–W 10: 10: 3 and methanol.

**DEAE-cellulose and thin-layer chromatography:** DEAE-cellulose (Eastman Kodak Co., Rochester, New York) was prepared in acetate form according to the method of Dankert et al. (12). The radioactive lipid extract was directly applied to a column (0.9 × 4.5 cm), which was firstly washed with 100 ml of M–W 99: 1, and then eluted with 35 ml of 0.05 m ammonium acetate (Nakarai Chemicals Ltd., Kyoto) in M–W 99: 1 (recovery of authentic 14C-MRP was found to be greater than 95%).

Each 0.05 m ammonium acetate eluate from the DEAE-cellulose column was concentrated by a rotatory vacuum evaporator at less than 37°C to remove methanol, and then exhaustively lyophilized overnight to eliminate ammonium acetate. The lyophilized residue was redissolved in 0.1 ml of C–M 2: 1, and applied onto the thin-layer plate, precoated with silica gel 60 (E. Merck, A. G., Darmstadt) which was neutralized with 0.1 n ammonia solution and dried in advance. A developing solvent system, such as C–M–W 60: 25: 4 (13), was used in a tank pre-filled with nitrogen. The authentic 14C-MRP was usually co-chromatographed as a reference. After development, silica gel on the plate was scraped every 5 mm width into a counting vial to which 0.1 ml of methanol and 5 ml of 0.4% Omnifluor (New England Nuclear) in toluene (w/v) were added. Radioactivity was measured by a Packard Tri-Garb liquid scintillation spectrometer, model 3380 (counting efficiency: 95%). Average recovery of total radioactivity on TLC was found to be about 80%.

**Large scale extraction and further examination of 14C-mannolipid**

According to lipid extraction (Method B), U-E2 fraction was obtained from the combined livers (about 10–15 g) in each group of Exp. B. The U-E2 fraction was then applied to a DEAE-cellulose column (1.6 × 15 cm) and separated by a stepwise elution as indicated in Fig. 4 (upper panel). The 14C-mannolipid; Rf 0.2 (peak b), which was eluted with 10 mM ammonium acetate in M–W 99: 1, was concentrated, lyophilized, and stored at −20°C for both silicic acid column chromatography, and acid and alkaline hydrolysis.

**Silicic acid column chromatography:** Unisil (100–200 mesh, Clarkson Chemical Co., Inc., Pennsylvania) was prewashed with 3 volumes of 0.1 m ammonium hydroxide solution and then activated at 110°C for 2 hr before use. The sample was chromatographed by a stepwise elution of various C–M mixtures (see Fig. 4, lower panel).

**Acid and alkaline hydrolysis:** Lipids eluted from the DEAE-cellulose column were treated with acid, mild alkaline and mild acid hydrolysis as previously reported (6). A portion of the eluate was dried under nitrogen, suspended in 0.2 ml of 2 N HCl, and heated in a sealed ampoule with nitrogen at 100°C for 3 hr. The labelled authentic MRP was prepared in DeLuca's laboratory, according to the method of Rosso et al. (3).
hydrolyzed material was flash-evaporated to dryness below 40°C, redissolved in 1 ml of distilled water and re-evaporated to remove residual HCl. The dry residue was partitioned between each 1 ml of chloroform and water, and then the aqueous phase was chromatographed on Toyo filter paper No. 52 (The Toyo Roshi Kaisha, Ltd., Tokyo), using butanol–pyridine–water 9: 5: 4 as solvent, along with standard D-mannose, glucose and galactose (Kokusan Chemical Co., Tokyo).

For mild alkaline hydrolysis, the dry eluate was dissolved in 0.09 ml of methanol, to which 0.01 ml of 1 N NaOH was added, and incubated for 15 min at 68°C. After hydrolysis, the content was neutralized by adding 0.01 ml of 1 M acetic acid. Mild acid hydrolysis was also carried out as follows: the dry eluate was dissolved in 0.01 ml of methanol, to which 0.04 ml of 0.125 N HCl was added, heated for 10 min at 20°C, and then neutralized by adding 0.01 ml of 0.5 N NaOH. Both mild alkaline and acid hydrolysates were concentrated under nitrogen, and applied onto the TLC plates, being developed using C–M–W 60: 25: 4 as solvent as described earlier. Hydrolysis was expressed by % reduction in radioactivity of 14C-mannolipid (Rf 0.2).

### Digestion and separation of 14C-mannopeptides

Labelled mannose-containing glycopeptides were separated by a sequence of procedures which included: protein digestions, cold-ethanol precipitation, and successive gel filtration on Sephadex G-25 as previously reported (6, 14).

In Exp. A, each 2 g of the frozen liver was freshly homogenized with 2 ml of distilled water and used for this study. After lipid extraction by 5 volumes of C–M 2: 1, solid materials were collected on a filter paper with a funnel, further washed by 50 ml of methanol, and dried overnight (about 0.46 g of dried materials was obtained). In Exp. B, the washed intermediate layers as mentioned above was used for protein digestion. Each dried residue was suspended in a sterile solution of Protease VI (Sigma), containing 0.8 mg per ml of 1 mM sodium acetate buffer, pH 7.5 (20 mg of Protease per g of the dried residue). Digestion proceeded for 48 hr at 37°C in the presence of toluene. During the incubation, pH was adjusted to be 7.4 by adding 1 N NaOH solution. The second digestion was performed for 24 hr at 37°C with a sterile solution of Pronase (750,000 tyrosine unit/g; Kaken Chemical Co., Tokyo) dissolved in 1 mM sodium acetate buffer, pH 7.5 and 0.12 M CaCl₂ to give a final concentration of 12 mg/ml (30 mg of Pronase per g of the dry material).

After digestion, the hydrolysate was filtered and washed twice with each 5 ml of distilled water, and then 2 mg of heparin sodium salts (158 units/mg; Wako) per ml of the hydrolysate was added. After cooling, 4 volumes of ice-cold ethanol was added and the mixture was allowed to stand at −20°C overnight. The precipitated glycopeptides were obtained by centrifuging at 2,000 g for 30 min at 4°C (In Exp. A, about 0.121 g of the precipitate was obtained from the original dried residue). Each 20 mg of the glycopeptide was dissolved in 1 ml of 0.1 M am-
monium bicarbonate solution, 0.1 ml of which was mixed with 1.0 ml of distilled water and 10 ml of 0.4% Omnifluor in a mixture of Triton X-100 (Wako)-toluene (3:7), and then radioactivity was measured (counting efficiency: about 90%).

Gel filtration on a column (1 × 60 cm) of Sephadex G-25, fine (Pharmacia Fine Chemicals, Uppsala), equilibrated with 0.1 M ammonium bicarbonate, was also used for further separation of glycopeptide. The column was standardized using a blue dextran polymer with a molecular weight of 2 × 10⁶ (Pharmacia) for estimation of void volume and [1-¹⁴C]glucose (Daiichi Pure Chemicals Co., Tokyo) for bed volume, respectively. A portion of ¹⁴C-mannopeptide eluted at the void volume (peak 1) was also hydrolyzed in 1 N HCl at 100°C for 3 hr, followed by paper chromatography as mentioned above.

In vitro formation of ¹⁴C-mannolipids

Mannosyltransferase reaction from GDP-¹⁴C-mannose was assayed as previously described (15), using crude liver membranes obtained from a vitamin A-depleted (Group I) and -supplemented rat (Group IV) in Exp. B. The incubation mixture (total volume: 100 µl) contained: 3.7 mg of enzyme protein suspended in 40 µl of a buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 50 mM KCl and 0.25 M sucrose), each 10 µl of 0.025 M EDTA, 0.3 M Tris-HCl, pH 8, 0.1 M MnCl₂, 22 mg/ml ATP, and GDP-[U-¹⁴C]mannose (210 Ci/mol; New England Nuclear) water solution, containing 0.1 µCi and 1 nmole after dilution by cold GDP-mannose (Sigma). The equivalent of rat liver lipid extract obtained from 0.3 mg of enzyme membrane protein was dried and redissolved in 10 µl of DMSO with or without vitamin A compounds. The two incubations containing 0 and 20 µg of vitamin A and its derivatives were run at 37°C for 30 min, and the reaction was stopped with 15 volumes of C-M 2:1 and 4 volumes of 0.9% NaCl. The upper and lower phase were obtained and analyzed as previously reported (15).

The phosphorylated retinol and retinoic acid were prepared by a modification (2) of the method of Popjak et al. for farnesyl phosphate (16). Di-triethylamine phosphate was crystallized as follows: 10 g of ortho-phosphoric acid was dissolved in 25.6 ml of acetonitrile (Wako) to which 20.65 g (28.4 ml) of triethylamine (Wako) was added. The mixture was allowed to stand overnight at room temperature. The resulting crystals (about 3.6 g) were washed three times with 5 ml of ice-cold acetonitrile in a cold room. In a typical experiment, 5 mg of retinol or retinoic acid dissolved in 1.25 ml of acetonitrile was added to 0.125 ml of trichloroacetonitrile (Tokyo Chemical Industry Co., Tokyo), containing 1.25 mg of the di-triethylamine phosphate, and continuously stirred for 2 hr under nitrogen at room temperature in the dark. When pH was found to be 7, the whole content was applied onto a column of DEAE-cellulose as described above. RP was eluted by 0.1 M ammonium acetate in M-W 99:1 after eluting the column with M-W 99:1, and Royl-P eluted by 0.15 M ammonium acetate in M-W 99:1 after washing the column with 0.01 M ammonium acetate to remove residual bound
retinoic acid. To each eluate 300 μg of BHT (Tokyo Chemical Industry Co.) per 1 mg of the phosphorylated compounds was added before concentration and lyophilization. Each phosphorylated compound was dissolved in ethanol to give a final concentration of 4 mg/ml, and stored under nitrogen at -20°C. Yields were usually found to be about 16% for RP and more than 80% for Royl-P, respectively. Royl-P was easily distinguished from retinoic acid by its UV spectrum; whereas RP was distinguished from retinol by detection of anhydroretinol with absorption maxima at 388, 368 and 346 nm (1), immediately after adding one drop of 4N HCl to RP. In the presence of BHT, not only Royl-P, but also RP were fairly stable: The RP preparation was found to be intact for approximately 7 weeks without any production of anhydroretinol. When some degradation with absorption maximum around 230 nm was detected, the preparation was discarded.

Protein concentration was assayed by micro-biuret method (17), using bovine serum albumin as a standard.

RESULTS

Changes of body weight in vitamin A-depleted rats

Growth of rats in Exp. A was stunted around day 35 at about 200 g of body weight. After supplementation with retinoic acid, rats gained 50 g of body weight between day 44 and 53. The final body weight was found to be, on average, 245 g on day 63; 10 days after depletion of retinoic acid.

In Exp. B, growth was also stunted around day 35, and the maximum body weight was about 230 g on day 45. Due to no supplementation with retinoic acid during an entire observation period, the final body weight declined to about 216 g on day 53. On the other hand, rats supplemented with retinol palmitate (Group IV) for 30 days were still growing: final body weight was about 300 g on average.

Changes of 14C-mannolipids after in vivo administration of retinol and retinoic acid

As shown in Fig. 1, the incorporation of [1-14C]mannose into glycolipid (Rf 0.2 material), eluted off the DEAE-cellulose column as authentic MRP, was increased, exhibiting a peak between 25 and 60 min after intraperitoneal injection of retinol, and stayed at a higher level up to 15 hr thereafter (Exp. A). A similar pattern was also observed by in vivo administration of retinoic acid, showing a peak at 60 min. On the other hand, the incorporation into 14C-mannolipid (Rf 0.4) remained unchanged or was slightly decreased by administration of both retinol and retinoic acid.

In Exp. B, where 3 g of livers was used for lipid extraction, no significant amounts of 14C-mannolipid (Rf 0.2) were detected in the lower phase of lipid extracts, as shown in Fig. 2 (dotted line). Several attempts were made to obtain the Rf 0.2 material from the upper phase with reasonably good yields. The second extract of the upper phase by C–M 2: 1 (designated U-E2, see EXPERIMENTAL) was
Fig. 1. Incorporation of $^{14}$C-mannose into mannolipid (R$_f$ 0.2) at different times after in vivo administrations of retinol (○--○) and retinoic acid (●--●) to vitamin A-depleted rats (Exp. A). The labelled mannose was injected intraperitoneally 20 min before sacrifice. $^{14}$C-Mannose-containing glycolipid was separated by means of DEAE-cellulose and thin-layer chromatography after lipid extraction (see Experimental).

Fig. 2. Thin-layer chromatography of $^{14}$C-mannolipids obtained from the lower (dotted line) and upper phase (U-E2; solid line) in Exp. B. Both phases were applied to DEAE-cellulose column prior to TLC (see Experimental). Authentic $^{14}$C-MRP was co-chromatographed and its position is illustrated by the black spots at R$_f$ 0.2. Panel a and b refer to mannolipids obtained from retinoic acid-treated Group II and retinol-injected Group III, respectively.

finally found to contain the R$_f$ 0.2 mannolipid in large amounts. The R$_f$ 0.2 compound co-chromatographed with standard $^{14}$C-MRP and was well separated from other labelled materials on TLC (solid line, Fig. 2). In addition, the R$_f$ 0.2 material finally obtained from the retinoic acid-treated rat livers was also found to be indistinguishable from the authentic MRP on TLC (Fig. 2, a).

The incorporation of $^{14}$C-mannose into the R$_f$ 0.2 materials (i.e., MRP), expressed as dpm per total liver, were increased in both retinol-treated groups (Group III and IV), as compared with vitamin A-depleted group (Group I), although differences were not statistically significant (Fig. 3). Formation of the R$_f$ 0.2 material was also induced by retinoic acid injection (Group II), showing an intermediate value between Group I and Group III (or IV). In contrast, $^{14}$C-mannolipid (R$_f$ 0.4) with properties of DMP was not affected by any of the treatments (shaded column, Fig. 3).

Further characterization of $^{14}$C-mannolipid (R$_f$ 0.2 compound)

Properties of $^{14}$C-mannolipids extracted from rat livers of each group in Exp. B were further examined in some details; by stepwise elutions on both DEAE-
Fig. 3. Changes in the incorporation of $^{14}$C-mannose into both mannolipids $R_f$ 0.2 (white column) and $R_f$ 0.4 materials (shaded column), one hour after in vivo administrations of retinoic acid and retinol to vitamin A-depleted rats (Exp. B). The labelled mannose was injected 20 min before sacrifice. Group I, deficient rats without treatment; Group II, retinoic acid-treated; Group III, retinol-injected, and Group IV, vitamin A-supplemented for 30 days before sacrifice.

Fig. 4. DEAE-cellulose (upper panel) and silicic acid (lower panel) column chromatography of $^{14}$C-mannolipid extracted from the liver of Group IV (Exp. B). Lipid extract (U-E2, $1.1 \times 10^6$ dpm) was applied to a column (1.6 $\times$ 15 cm) of DEAE-cellulose and separated by a stepwise elution of 0, 10, 20 and 50 mM ammonium acetate in M-W 99:1, containing 2 mg/100 ml BHT (each 150 ml). Fractions of 10 ml were collected at a flow rate of 200 ml/hr. A portion of peak b (11,850 dpm) was further chromatographed on a column (0.7 $\times$ 5 cm) of the alkaline-treated Unisil (see EXPERIMENTAL), and separated by a stepwise elution of C–M 8:1, 4:1, 2:1, 1:1 and 1:2 (each 30 ml). Fractions of 3 ml were collected.

cellulose and silicic acid chromatography, and by both acid and alkaline hydrolysis.

The $R_f$ 0.2 compound, which was contained in the U-E2 extract of Group IV, was eluted with 10 mM ammonium acetate in M-W 99:1 (peak b) from the DEAE-cellulose column (Fig. 4, upper panel). On the other hand, peak c which was eluted with 20 mM ammonium acetate from the same column, was found to remain at the origin on TLC (see Fig. 2). When the peak b was subjected to Unisil column chromatography, most of the $R_f$ 0.2 material (about 64%) was eluted with C–M 4:1 (Fig. 4, lower panel). Similar elution patterns on both DEAE-cellulose and silicic acid column chromatography were observed in the $^{14}$C-mannolipids ($R_f$ 0.2) extracted from Group I, II and III. In contrast, the $R_f$ 0.4 compound (i.e.,
DMP), which was extracted into the lower phase and indistinguishable from the $R_f$ 0.2 on DEAE-cellulose column chromatography, was exclusively eluted with C–M 8:1 on Unisil column chromatography (data not shown).

After strong acid hydrolysis of the $R_f$ 0.2 material, most of the radioactivity was found to be mannose on paper chromatography, as shown in Fig. 5. Moreover, mild alkaline treatment (0.1 N NaOH, 68°C, 15 min) hydrolyzed about 70% of the $^{14}$C-mannolipid ($R_f$ 0.2). Mild acid treatment (0.1 N HCl, 20°C, 10 min) also hydrolyzed 76% of authentic MRP and 60% of the $R_f$ 0.2 material, without any significant hydrolysis of the $R_f$ 0.4 compound (i.e., DMP). No distinct differences of both acid and alkaline hydrolysis of the $R_f$ 0.2 compounds among the four groups of Exp. B were noticed.

**Changes of $^{14}$C-mannopeptides after in vivo administration of retinol and retinoic acid (Table 1)**

A slightly increasing trend of the incorporation of $^{14}$C-mannose into glycopeptides, digested with proteolytic enzymes and cold-ethanol precipitation, was observed in the rat livers obtained from 3 hr after *in vivo* administration of both retinoic acid and retinol, as compared with the control deficient rat livers (Table 1, Exp. A). Upon gel filtration on Sephadex G-25, about 70% of radioactive glycopeptides was eluted at the void volume. In Exp. B, formation of $^{14}$C-mannopeptides was increased in the rat livers supplemented with retinol for 30 days, as compared with the control. However, only a slight increase was seen 1 hr after administration of both retinoic acid and retinol (no statistical difference was seen).

![Fig. 5](image_url)  
**Fig. 5.** Paper chromatography of $^{14}$C-mannolipid ($R_f$ 0.2) after acid hydrolysis. A portion of the eluate from DEAE-cellulose (peak b in Fig. 4, upper panel), containing 3,160 dpm, was hydrolyzed in 2 N HCl at 100°C for 3 hr (see EXPERIMENTAL). Butanol–pyridine–water 9:5:4 was used as a developing solvent. Authentic mannose (Man), glucose (Glu), galactose (Gal) and mannose phosphate (Man-P), which was obtained after hydrolysis of GDP-mannose, were co-chromatographed, and visualized by either the silver nitrate or molybdate reagent. The paper strips, cut with each 1 cm width, were dipped in 0.5 ml of water for 1 hr, and then radioactivity was counted using 5 ml of 0.4% Omnifluor in a mixture of toluene–Triton X-100 (7:3).
Table 1. Incorporation of 14C-mannose into rat liver glycopeptides before and after administration of retinoic acid, retinol and retinyl palmitate.

| Experiment | Vitamin A-deficient | Vitamin A-supplemented |
|------------|---------------------|------------------------|
|            | Mean ± SEM          |                        |
| None       | dpm x 10^-8/liver   |                        |
| Retinoic acid (3 hr) | 192 ± 41           | 211 ± 11               |
| Retinol (3 hr) | 209 ± 17           |                        |
| Group I    | 122 ± 28            |                        |
| Group II   | 156 ± 28            |                        |
| Group III  | 142 ± 32            |                        |
| Group IV   | 232 ± 44            |                        |

After strong acid hydrolysis of the glycopeptide eluted at the void volume, most of the radioactivity was found to be mannose on paper chromatography (data not shown).

In vitro effects of vitamin A and its phosphorylated derivatives on mannosyltransferase activity (Fig. 6)

The incorporation of 14C-mannose from GDP-[U-14C]mannose into the mannolipid (Rf 0.2) was markedly increased by in vitro addition of chemically synthesized RP. No difference in this mannosyltransferase activity was observed between the retinol-supplemented and deficient rat liver (Fig. 6, a and b). On the other hand, no changes were observed in formation of the mannolipid (Rf 0.4; DMP) by in vitro addition of RP. Moreover, no changes in mannosylation were seen by in vitro additions of retinol, retinoic acid as well as Royl-P, using the crude liver membranes obtained from the vitamin A-fed rat (Fig. 6; a).

DISCUSSION

The present in vivo study has demonstrated that the incorporation of 14C-mannose into a labelled glycolipid chromatographically identical with mannosyl retinyl phosphate (MRP; Rf 0.2) is increased by the administration of retinol to vitamin A-depleted rats without any changes in the synthesis of 14C-dolichyl mannosyl phosphate (DMP; Rf 0.4). In addition, an increasing trend in the incorporation of 14C-mannose into glycopeptides is also observed when the vitamin A-depleted rat is orally or intraperitoneally supplemented with retinol. These results are generally consistent with the previous observation of DeLUCA et al. (6), although the changes obtained in rats are found to be less dramatic than those in
Fig. 6. Effects of in vitro addition of retinoic acid, retinol and their phosphorylated derivatives on mannosyltransferase activity from GDP-\(^{14}\)C-mannose to mannolipids of \(R_f\) 0.2 (white column) and \(R_f\) 0.4 (shaded column). Panel a and b represent the crude membrane enzyme sources from vitamin A-supplemented Group IV and vitamin A-depleted Group I in Exp. B, respectively. Royl-P (retinoyl phosphate) and RP (retinyl phosphate) were chemically synthesized (see EXPERIMENTAL). All vitamin A compounds were added to give a concentration of 20 \(\mu\)g per 100 \(\mu\)l of total incubation mixture, and incubation was done for 30 min at 37°C.

Hamsters. This study, however, has provided additional evidence that in vivo administration of retinoic acid also brings about a similar though smaller elevation of the synthesis of a \(^{14}\)C-mannolipid (\(R_f\) 0.2 material), which is indistinguishable from the authentic MRP with regard to polarity on phase separation of lipid extraction, DEAE-cellulose, silicic acid and thin-layer chromatography. Moreover, and most importantly, formation of the mannolipid is rapidly induced by both retinol and retinoic acid, already showing a peak within 60 min (Fig. 1). This finding strongly supports the concept that vitamin A is directly involved in biosynthesis of glycolipid and glycoprotein rather than functioning indirectly by improving general conditions (i.e., appetite, food intake, etc.).

It must be emphasized that most of \(^{14}\)C-mannolipid (\(R_f\) 0.2) was recovered in the upper phase of lipid extract when more than 3 g of liver was used for analysis. Better recovery of the \(R_f\) 0.2 material was achieved by the second re-extraction of the upper phase with C–M 2:1 (designated U-E2 of the lipid extraction; method B). The U-E2 extract was analyzed further in this study, since this fraction showed significant differences between the groups of Exp. B: The total radioactivities in both Group III (276 ± 24 \(\times\) 10\(^3\) dpm/liver) and Group IV (287 ± 28 \(\times\) 10\(^3\)) were found to be significantly increased, as compared with that in Group I (139 ± 17 \(\times\) 10\(^3\)) (\(p < 0.05\)). Moreover, the \(^{14}\)C-mannolipid (\(R_f\) 0.2) was well separated from the \(R_f\) 0.4 material as well as the origin material(s), when each U-E2 and the lower phase was individually applied on TLC after DEAE-cellulose chromatography (see Fig. 2). However, the difference in the total amount of radioactivity
incorporated into purified $^{14}$C-mannolipid ($R_f$ 0.2) was not statistically significant among the four groups, due mainly to possible individual variation during the process of isolation (see Fig. 3).

Consistent with previous in vivo study (6), a $^{14}$C-mannolipid ($R_f$ 0.2), stimulated by in vivo administration of retinol to the deficient rats, has been isolated with properties similar to authentic MRP and the material ($R_f$ 0.2) which was also produced by in vitro addition of RP to the liver membrane (Fig. 6): The $R_f$ 0.2 compound was eluted with 10 mM ammonium acetate in M–W 99:1 from the DEAE-cellulose, with C–M 4:1 from the Unisil (Fig. 4), and easily hydrolyzed by mild alkaline and acid treatment. After strong acid hydrolysis, the radioactivity of the $R_f$ 0.2 material was exclusively detected as mannose (Fig. 5). Moreover, Masushige and Wolf (5) have recently reported the isolation of doubly labelled MRP from rat livers after in vivo administrations of both $^{14}$C-mannose and [15-3H]retinol. Therefore, it is highly possible that the $R_f$ 0.2 material obtained in this experiment is MRP.

An unexpectedly high blank value of $^{14}$C-mannolipid ($R_f$ 0.2) in vitamin A-depleted rat liver was noticed (Figs. 1 and 3), contributing to less dramatic increase in mannolipid formation than that in the hamster liver (6). Species difference may be involved, since similarly high blank value of labelled mannolipids was also observed by in vitro addition of retinol to crude membranes obtained from vitamin A-deficient rat liver (18, 19). This problem, however, remains to be solved, although the $R_f$ 0.2 compound obtained from the deficient rat livers was indistinguishable from those found in the retinol-treated livers and authentic MRP, with regard to behaviors on chromatographical analyses, and mild acid and alkaline hydrolysis. At present, two possibilities can be suggested: 1) vitamin A deficiency status may not allow complete depletion of endogenous retinyl phosphate and its derivatives, and 2) the possibility that the endogenous oligoprenyl phosphate present in vitamin A-depleted membranes may differ from retinyl phosphate and may consist of other polyprenoids with similar polarity to retinol.

One of the major reasons why the Exp. B was designed in this study was to exclude the possibility that the depletion of retinoic acid for 10 days in Exp. A was insufficient to bring about deficiency of the vitamin. However, no difference in the high blank value was seen between Exp. A and B, thus in both experiments manifest deficiency had been obtained. It is also certain that retinol in the vitamin A-depleted rats of this study was sufficiently exhausted, since not only vitamin A levels in the liver, but also in plasma (vitamin A and RBP) were found to be well depleted. However, nothing is known as to the attainment of a membrane deficient in RP, since this compound and other phosphorylates may turn over at a very slow rate. Further study is obviously required to isolate and

5 Oral presentation at the 29th annual meeting of the Japan Society of Vitaminology, Kyoto, June 1977.
characterize the $^{14}$C-mannolipid ($R_f$ 0.2) which is present in the vitamin A-depleted rat livers, and is chromatographically identical with authentic MRP.

In conclusion, a remarkable and rapid response in mannolipid synthesis to *in vivo* administration of retinol was observed. The rapidity of this response correlates well with the early stimulation of secretion of immunoreactive RBP from the liver to the plasma in the same series of rats (unpublished observations). Thus, this relationship between vitamin A-dependent mannosylation and secretion of RBP by the liver warrants further exploration.

Of interest is the fact that mannolipid formation was also induced by *in vivo* administration of retinoic acid, but not by *in vitro* additions of retinoic acid and its phosphorylated compound, Royl-P (see Fig. 6). It can be excluded that some contamination of retinol and/or other biological active compounds existed in the preparation of retinoic acid used, since the preparation was proved to be highly pure by high performance liquid chromatography before use, and no remarkable increase of plasma RBP was observed after injection of this preparation (see Ref. 9). It has been also observed (20) that Royl-P is not molecularly involved in mannolipid formation as an acceptor, at variance with RP. Therefore, further work is required to characterize and determine the biologically active metabolite(s) of retinoic acid for biosynthesis of glycolipid and glycoprotein in the body.

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