Enzymatic synthesis of mannosyl retinyl phosphate from retinyl phosphate and guanosine diphosphate mannose

Gloria Chi Rosso, Luigi De Luca, Christopher D. Warren, and George Wolf

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; Lung Cancer Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014;[1] and Laboratory for Carbohydrate Research, Department of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114[2]

Abstract A study was conducted to determine whether retinyl phosphate would act as substrate for the enzymatic synthesis of mannosyl retinyl phosphate. Retinyl phosphate, prepared chemically, supported the growth of vitamin A-deficient rats at the same rate as retinol. It also stimulated the uptake of [^{14}C]mannose from GDP-[^{14}C]mannose into total chloroform–methanol-extractable lipid. This reaction occurred in the presence of ATP, Mn^{2+}, detergent (Zonyl A), and a membrane-rich enzyme preparation from the livers of vitamin A-deficient rats, provided that a lipid extract of the membrane preparation of α-L-lecithin was also added. Total chloroform–methanol-extractable, labeled mannolipid was separated into two principal labeled mannolipids by thin-layer or column chromatography or by differential solvent extraction. The properties of these mannolipids identified them as glycophospholipids: one was identical with authentic synthetic dolichyl mannosyl phosphate, and the other was concluded to be mannosyl retinyl phosphate because of its incorporation of radioactivity from [^{3}H]retinyl phosphate, its rapid hydrolysis by dilute acid, and the formation of a substance that cochromatographed with retinol upon its acid hydrolysis. The presence of ATP or GTP was essential for the stimulation of mannolipid synthesis, probably because of their protective action on the substrates against phosphatases present in the crude enzyme fraction. A pH of 6.0–6.2 favored the formation of dolichyl mannosyl phosphate; a higher pH (6.7–7.0) that of mannosyl retinyl phosphate.

Supplementary key words retinol · mannolipid · dolichyl mannosyl phosphate · mannose · membrane-rich enzyme fraction

Glycophospholipids, consisting of a retinyl residue linked by a phosphodiester bond to a sugar moiety, have been found to be synthesized by mammalian enzyme preparations (1–3) or in vivo (4, 5). We postulated that a phosphate ester of retinol would be an intermediate and therefore investigated the synthesis of mannosyl retinyl phosphate from retinyl phosphate.

This report, from which some preliminary data have been published (6), describes the chemical synthesis and purification of [^{1}H]- and [^{3}H]retinyl phosphates (RP), their effect on the uptake of labeled mannose into lipid, and the separation of the labeled lipid products and the identification of one as mannosyl retinyl phosphate (MRP).

MATERIALS AND METHODS

Materials

All-*trans*-15-[^{3}H]retinol (1.25 Ci/mmole) and GDP-[^{14}C]mannose (154 mCi/mole) were obtained from New England Nuclear Corp.; α-L-lecithin type IIe and type IIIe (from egg yolk) from Sigma Chemical Co.; Zonyl A from E. I. du Pont de Nemours & Co., Organic Chemicals Div.; all-*trans*-retinol, trichloroacetonitrile, acetonitrile, and DEAE-cellulose from Eastman Kodak Co.; and silicic acid (Bio-Sil HA, 200–325 mesh) from Bio-Rad Laboratories. Thin-layer plates were precoated plates of silica gel G (E. Merck A.G., Darmstadt, Germany).

Animals and diets

The preparation of the animals and their diets followed the procedures previously described (7). In vitro experiments were carried out with the livers of vitamin A-deficient rats when the animals had lost 5–10% of their weight, with the exception of one experiment (Fig. 6), in

Abbreviations: C–M, chloroform–methanol; DMP, dolichyl mannosyl phosphate; MRP, mannosyl retinyl phosphate; RP, retinyl phosphate; TLC, thin-layer chromatography.

[1] Dr. De Luca.

[2] Dr. Warren.

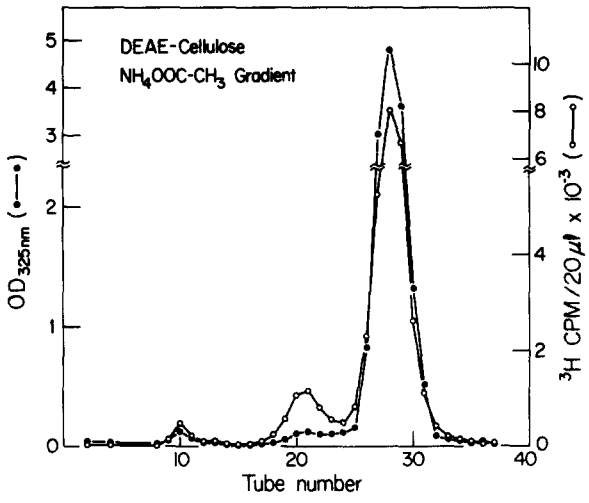

Fig. 1. Purification of synthetic RP. The sample was purified on a 1 × 30 cm column of DEAE-cellulose (in acetate form). It was eluted, after washing with 10 column vol of 99% methanol, with a 0–0.1 M linear gradient of ammonium acetate in 99% methanol. Fractions of 4 ml were collected. Aliquots of 20 μl were counted (counting efficiency, 36%), and their absorbance was measured at 325 nm. RP was eluted with 0.07 M ammonium acetate.

which a pair-fed normal rat was used. The experiments on the biological activity of RP and retinol were performed with vitamin A-deficient rats at the weight-plateau stage. RP (or control retinol) was dissolved in ethanol, and this solution was added to a mixture of Tween 80 and water to give a final concentration of either 0.6 or 2.5 μg of RP or retinol per 0.3 ml of a mixture of ethanol–Tween 80–water 10:16.2:78.8 (8). The rats were injected daily at 2 p.m., intravenously by the tail vein, with this solution.

Synthesis of retinyl phosphate

The synthesis was carried out by a modification of the method of Popjak et al. (9) for the preparation of farnesyl phosphate. Trichloroacetonitrile (0.9 ml) and retinol (30 mg) were added to 100 mg of bis(triethylammonium) phosphate in acetonitrile (10 ml). The mixture was stirred in a closed flask that had been previously flushed with nitrogen gas for 1 hr at room temperature in the dark. Ether (15 ml) was then added to the mixture, and the [^{1}H]RP was extracted into two 50-ml aliquots of ice-cold 0.1 M NH_4OH, in which the product was freely soluble. The combined aqueous extracts were washed once with 15 ml of ether and evaporated to dryness in a flash evaporator under N_2 and in dim light. The residue was taken up in a small volume of chloroform–methanol (C–M) 2:1. When [^{3}H]RP was prepared, all quantities were reduced to one-fifth of those described above, and 300 μCi of [^{3}H]retinol was added along with the nonradioactive retinol. Purification in either case was performed on a column of DEAE-cellulose (1 × 30 cm) in acetate form. The sample in C–M was then applied, and residual retinol was removed by washing with 10 column vol of 99% methanol. The RP was then eluted by a linear gradient (0–0.1 M) of ammonium acetate in 99% methanol (200 ml, total volume of the gradient).

An alternative procedure, preparative chromatography (TLC), was used to prepare [^{3}H]RP with higher specific activity and purity. After reacting trichloroacetonitrile, bis(triethylammonium) phosphate, and retinol, as described above, we stopped the reaction by cooling the flask in ice and added 1 M NH_4OH to pH 7.5. The resulting solution was concentrated to a small volume and applied as a band to a preparative thin-layer plate (50 mm thick) of silica gel G; this was then developed in chloroform–methanol–water 60:25:4. After the band of RP had dried, it was located by spraying a narrow guide strip with the molybdate spray for phosphate esters (10). The corresponding band was eluted with chloroform–methanol–water 1:1:0.3 in a closed jar flushed with nitrogen. Using [^{3}H]retinol of specific activity 2.4 Ci/mmole with the above procedure, the yield was 2% (specific activity, 37.3 μCi/μmole).

Incubation and separation

The incubation system used for the synthesis of mannolipid was closely modeled after that previously used in our laboratories (3). GDP-[^{14}C]mannose and a membrane fraction from vitamin A-deficient rat livers were incubated with either unlabeled or ^{3}H-labeled RP, with or without the addition of a lipid extract or α-L-lecithin. The membrane fraction contained 0.7–1.0 mg of protein in approximately 0.1 ml of 0.25 M sucrose in Tris–potassium–magnesium buffer prepared as previously described (3). To this was added, in a volume equal to that of the protein solution: 0.2 μmole of ATP, 1.4 × 10^{-3} μmoles of GDP-[^{14}C]mannose (0.1 μCi), 10–20 μl of Zonyl A, 3.12–4.20 × 10^{-2} μmoles (6–8 μg) of RP (0.3–0.4 μCi), α-L-lecithin or lipid extract in amounts described in the figure legends, Tris buffer (0.03 M, final concentration), $MnCl_2$ (0.01 M, final concentration), and EDTA (0.0025 M, final concentration). α-L-Lecithin or lipid extract was dissolved in C–M 2:1 and mixed with the RP; the solvent was removed with N_2 and replaced by the Zonyl A. The lipid extract was obtained by extracting the above membrane-enzyme fraction (10–12 mg protein) with 20 vol of C–M 2:1, washing with 0.2 vol of 0.9% saline, and then concentrating the lower (organic) phase to 3 ml. A determination of inorganic phosphate in this solution showed a total of 243 μg of P_i. All incubations contained α-L-lecithin unless otherwise indicated in the figure legends.

At the end of the incubation time (generally 20 min), the lipid was extracted by addition first of C–M 2:1 (15 times the volume of the incubation) then of 0.9% saline (0.2 times the volume of the C–M 2:1), mixing each time with a Vortex mixer. The resulting two phases separated upon centrifugation. The lower (organic) phase was

drawn off with a Pasteur pipette and washed with 0.9% saline (0.2 times the volume of the organic phase); the resulting lower (organic) phase is referred to as "1st extraction." The combined aqueous phases and washing were extracted again with C–M 2:1 (10 times the volume of the original incubation); this lower (organic) phase is referred to as "2nd extraction." When combined, the two extracts are referred to as "total C–M-extractable lipid."

When GDP-[^{14}C]mannose was the only radioactive compound, the organic phase was directly evaporated and replaced by a toluene–diphenyloxazole scintillation fluid, and its radioactivity was measured in a Beckman LS-250 liquid scintillation counter. When [^{3}H]RP was used, the mannolipid was purified on DEAE-cellulose and silicic acid columns as described below.

The solvent used for TLC with plates of silica gel G was chloroform–methanol–water 60:25:4, specifically developed for the separation of polyisoprenyl mannosyl phosphates by Tkacz et al. (11). Plates were dried and exposed to Kodak No-Screen medical X-ray film for either 1 or 2 wk, as indicated under the figures, and the film was then developed. The spots were located, scraped into liquid scintillation counting vials, and counted directly on the gel in 17% Bio-Solv BBS-3 (Beckman) and in 0.5% diphenyloxazole in toluene. Alternatively, 1-cm bands were scraped into vials and counted.

RESULTS

Properties of retinyl phosphate

The elution profile from DEAE-cellulose for RP shown in **Fig. 1** was obtained with [^{3}H]RP. Using $E^{1\%}_{1cm}$ = 1600 for the spectrophotometric assay of retinol and the method of Ames and Dubin (10) for phosphate determination, we found the retinol/phosphate ratio to be unity for the material from the column. The absorption spectrum of RP was identical with that of retinol between 290 and 350 nm. TLC of RP in two solvent systems gave the following R_f values: chloroform–methanol–15 M NH_4OH–water 80:30:0.5:3, 0.25; chloroform–methanol–water 60:35:6, 0.60. Either the anisaldehyde spray (12) or the phosphate-specific spray (13) was used to visualize the RP. When injected into vitamin A-deficient rats, RP was roughly similar to retinol in maintaining the weight of the animals (**Fig. 2**).

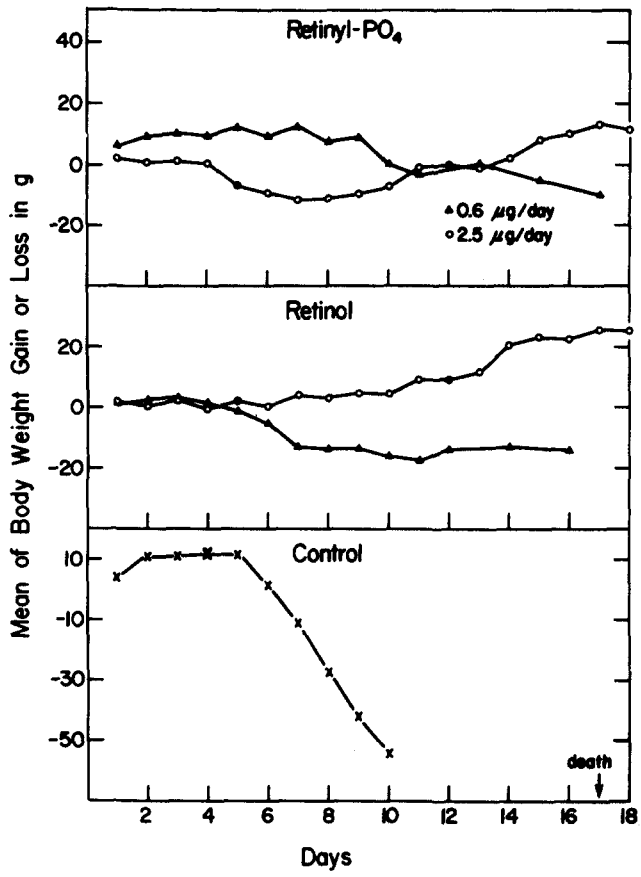

Fig. 2. Weight gains and losses of rats receiving RP. Animals at the weight-plateau stage in groups of four were injected intravenously daily with either retinol or RP (0.6 or 2.5 μg) in 0.3 ml of solvent described in the text, or with the solvent alone. The solid circles or triangles indicate days when injections were started and terminated.

Effect of retinyl phosphate on synthesis of total C–M-extractable lipid

The basic assay developed in our laboratories (3) consists of measuring the stimulation of uptake of [^{14}C]mannose from GDP-[^{14}C]mannose into lipid, which is caused by added retinol, and comparing it with a base-line uptake without retinol. The base-line value was shown to be due to a mannolipid formed with an endogenous acceptor compound(s) (3). Incubation of RP with GDP-[^{14}C]mannose and with the membrane-rich fraction from vitamin A-deficient rat liver did not significantly stimulate mannolipid formation. However, the presence of a lipid extract from the membrane-rich fraction (identical with that used as a source of the enzyme, prepared and incubated as described in the experimental section) did stimulate the synthesis of mannolipid (**Fig. 3**). This curve shows the effect of increasing amounts of RP on stimulation of mannose uptake in the presence and absence of lipid. The dependence of stimulating activity on the added lipid extract is illustrated in **Fig. 4. Fig. 5** shows that α-L-lecithin could also stimulate the reaction.

Separation of C–M-extractable lipid into constituents

Lipid labeled with GDP-[^{14}C]mannose. Using the TLC separation procedure of Tkacz et al. (11), it was possible to separate the two principal mannolipids formed on incubation of the membrane fraction from vitamin A-deficient rat liver with RP and GDP-[^{14}C]mannose (**Fig. 6**). The radioactive spot at the origin contained re-

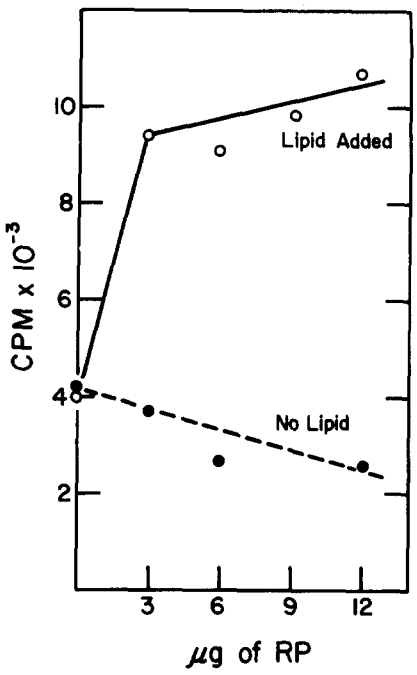

Fig. 3. Dependence on RP of stimulation of mannolipid formation in the presence and absence of lipid. The composition of the incubation mixture and the method for obtaining lipid extract is described in Materials and Methods. The final volume was 0.12 ml (0.61 mg of enzyme protein). The extract (0.3 ml, and determined to contain 24.5 μg of P_i) was evaporated to dryness under N_2 together with the RP and redissolved in Zonyl A (10 μl). The incubation mixture, including the enzyme, was added to this lipid extract. The counting efficiency was 87%.

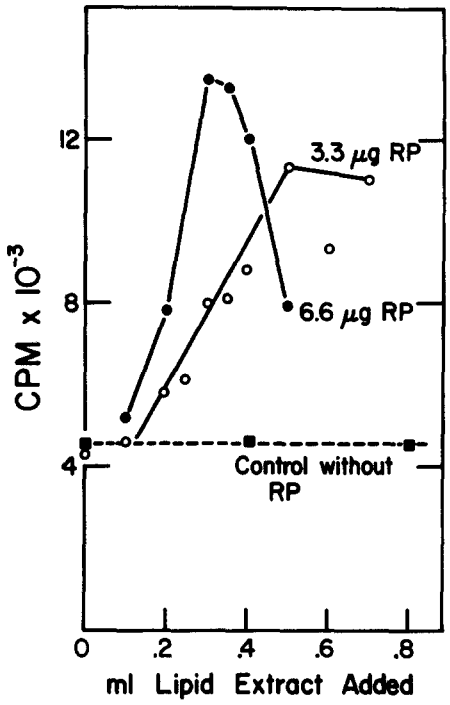

Fig. 4. Dependence of stimulation of mannolipid formation on lipid extract. Composition of the incubation mixture is described in Materials and Methods. Final volume was 0.14 ml (0.7 mg of protein). Lipid extract (found to contain 82 μg of P_i per ml) from the membrane fraction was prepared and added as described for Fig. 3, except that different amounts of extract were incubated either with 3.3 or 6.6 μg of RP, or without RP (control).

sidual GDP-[^{14}C]mannose or [^{14}C]mannosyl phosphate. The spot at R_f 0.15 contained [^{14}C]mannose, carried into the lipid extract. The spot with R_f 0.5 was identified as dolichyl mannosyl phosphate (DMP) by comparison with authentic DMP prepared by chemical synthesis (14). The double spot with R_f 0.25 disappeared in incubations without RP (Fig. 6, *A*), appeared when RP was added to the incubation (*B*), and greatly increased with the presence of α-L-lecithin (*D, E*) or when the concentration of RP was increased (*C, E*). Upon scraping the relevant spots into counting vials and determining their radioactivity, we observed the quantitative results shown in **Table 1,** corresponding to the radioautograph in Fig. 6.

TABLE 1. Uptake of radioactivity into DMP and compound with R_f 0.25 after incubation with GDP-[^{14}C]mannose

	Incubation with α-L-Lecithin			Incubation without α-L-Lecithin	
	Control (no RP) A	3 μg RP D	6 μg RP E	0.9 μg RP B	1.8 μg RP C
	cpm			*cpm*	
Experiment 1					
DMP	932	730	919	1237	1012
Compound R_f 0.25	43	538	702	222	338
Experiment 2					
DMP		1401	835	1832	1729
Compound R_f 0.25		731	1401	314	456
Experiment 3					
DMP	2748	2202	1144	2028	3450
Compound R_f 0.25	122	1005	1778	140	234

Incubations, extractions, and TLC were performed as described in Fig. 6. Respective spots were located by radioautography, scraped into vials, and counted. Letters correspond to those used in Fig. 6.

The data demonstrate that there is considerable uptake of mannose into an endogenous lipid acceptor, rendering a product now identified as DMP; this agrees with the results of other investigators (3, 4, 11, 15). Exogenous RP greatly stimulated uptake of labeled mannose into the double spot with R_f 0.25. The addition of lecithin enhanced this incorporation, but not that into DMP. On the other hand, when increasing amounts of RP were incubated, they gave increasing amounts of the substance with R_f 0.25 and led to decreased synthesis of DMP (Table 1). It should be noted that although the preparation from deficient rats incubated in the absence of RP showed little or no uptake of labeled mannose into the spot with R_f 0.25 (Fig. 6, *A'*), the preparation from normal, pair-fed rats without RP showed a single spot with R_f of about 0.25 (Fig. 6, *F*).

A second method of isolation consisted of fractionating the total mannolipid, as extracted into C–M, on silicic

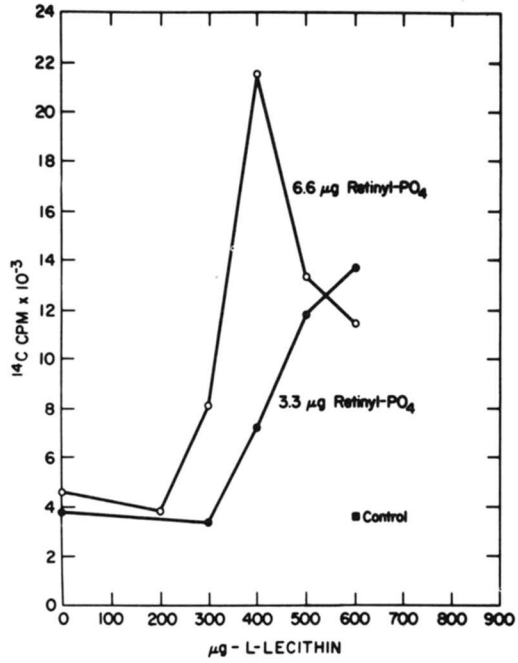

Fig. 5. Dependence of stimulation of mannolipid formation on α-L-lecithin. Conditions were identical with those described in Fig. 4 except for the addition of α-L-lecithin instead of lipid extract.

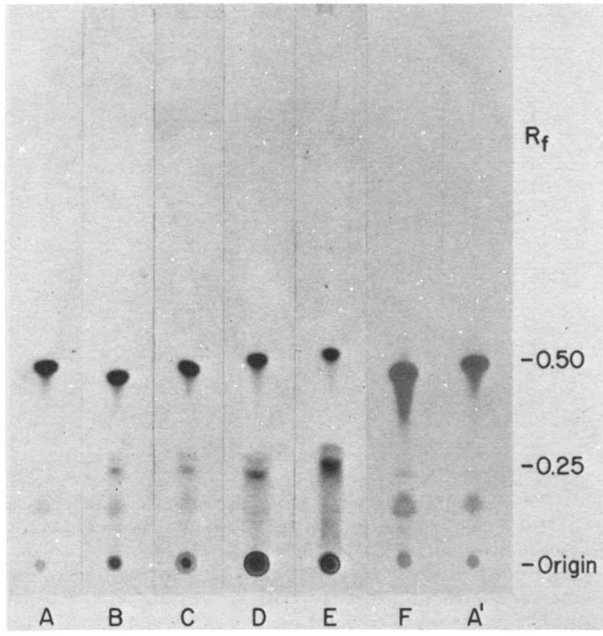

Fig. 6. Separation of total mannolipid into constituents by TLC. Incubations were as described under Materials and Methods, with 0.61 mg of membrane protein in 0.12 ml total incubation volume. *A* indicates the incubation without RP; *B* and *C*, incubations with 1 and 2 μg of RP, respectively, without lecithin; *D* and *E*, incubations with 3 and 6 μg of RP, respectively, each with 500 μg of lecithin; *F*, incubation with membrane fraction from normal pair-fed rat without RP. Each incubation was extracted with C–M 2:1. The extracts were evaporated to a small volume and applied to a TLC plate that was developed in chloroform–methanol–water 60:25:4 and exposed to X-ray film for 1 wk. TLC of *F* was exposed for 2 wk, together with *A'*, which is TLC of *A* exposed for 2 wk.

acid directly after the incubation. A column was packed with a slurry of silicic acid (200–300 mesh) in C–M 8:1 and adjusted to pH 7.0 by adding a few drops of 1 M NH_4OH in methanol to avoid hydrolysis of the substance with R_f 0.25. Elution was stepwise, starting with C–M 8:1, and each batch was again neutralized with the NH_4OH solution as it emerged. Only with such precautions could the substance with R_f 0.25 be recovered. Elution was followed by TLC and radioautography. The fraction eluted with C–M 2:1 was found to contain the substance with R_f 0.50; the fraction eluted with C–M 1:1 contained the substance with R_f 0.25. The yield of substance with R_f 0.25 was only 10–20% (based on direct TLC of the total lipid extract of the incubation as 100%).

A third method for separation of the constituents of total mannolipid by differential solvent extraction is described below (under Effect of pH).

Lipid labeled with [^{3}H]RP and GDP-[^{14}C]mannose. The method developed in this laboratory for purification of C–M-extractable lipid by gradient elution from DEAE-cellulose (3) removed unreacted RP from the lipid extract after incubation (**Fig. 7**). The ^{3}H peak was shown to be residual [^{3}H]RP by TLC and comparison with authentic, chemically synthesized RP. The ^{14}C–^{3}H peak was chromatographed again on a silicic acid column prepared as described above. Elution was stepwise, starting with C–M 8:1; each batch was again neutralized with the NH_4OH solution as it emerged. **Fig. 8** illustrates TLC of the C–M 1:1 eluate from this silicic acid column. The ^{14}C activity and ^{3}H activity coincided with R_f 0.25, and there was a small residual peak of ^{14}C-labeled DMP with R_f 0.5.

Identification of substance with R_f 0.25

Upon incubation of [^{3}H]RP with GDP-[^{14}C]mannose, purification of the total C–M-extractable mannolipid first by DEAE-cellulose (to remove residual [^{3}H]RP) (Fig. 7) and then by neutralized silicic acid, and further purification of the C–M 1:1 eluate by TLC, a ^{3}H–^{14}C peak occurred with R_f 0.25 (Fig. 8). This clearly demonstrated that the labeled mannose had combined in one compound with the ^{3}H-labeled RP.

If ^{3}H-labeled RP was incubated with unlabeled GDP-mannose, to exclude labeling of DMP, and if residual [^{3}H]RP was removed by DEAE-cellulose fractionation, the resulting TLC and radioautograph showed the double spot with R_f 0.25 labeled with ^{3}H (**Fig. 9,** *A*). This radioautograph also revealed a ^{3}H-labeled spot with R_f 0.35, which was not further investigated. For comparison, a radioautograph is shown of the residual [^{3}H]RP removed by DEAE-cellulose chromatography (Fig. 9, *B*). RP had the same R_f in this solvent system as the compound surmised to be MRP (0.25). This finding is not

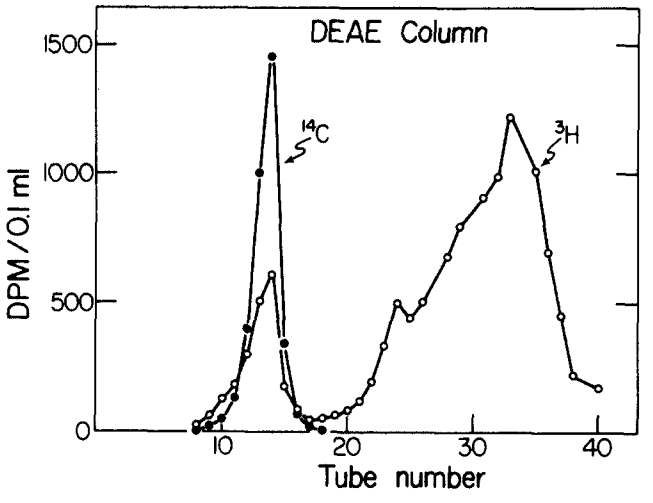

Fig. 7. Purification on DEAE-cellulose of total mannolipid prepared with [^{3}H]RP. Five separate incubations with double amounts of protein were made with GDP-[^{14}C]mannose. Each contained 11.8 μg of [^{3}H]RP (specific activity, 7.6 $\times$ 10^4 μCi/μg), which was added to the incubation dissolved in 10 μl of Zonyl A, together with 800 μg of α-L-lecithin. At the end of the incubation time, the lipid extract in C–M was purified on a column of DEAE-cellulose (1 $\times$ 30 cm) prepared in 99% methanol. The fraction volume was 2.5 ml. The first ^{3}H–^{14}C-labeled peak was eluted with 0.014 M ammonium acetate. The second ^{3}H-labeled peak (fractions 25–40), eluted by 0.07 M ammonium acetate, was shown by TLC to be recovered RP.

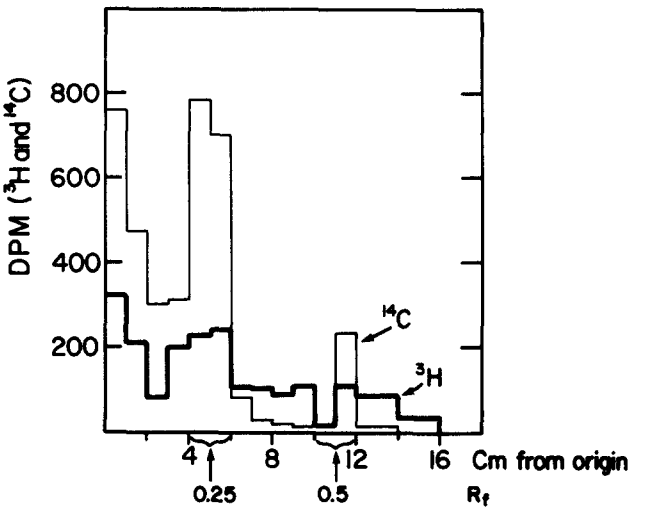

Fig. 8. Coincidence of ^{3}H and ^{14}C peaks in the spot with R_f 0.25 after TLC. The ^{3}H–^{14}C peak from the DEAE-cellulose column (Fig. 7) was applied to a silicic acid column neutralized with NH_4OH, as described under Results. The C–M 1:1 eluate was then analyzed by TLC. The plate was scraped and counted. There was a ^{3}H–^{14}C peak with R_f 0.25 and residual DMP with R_f 0.5.

surprising because we found dolichyl phosphate and DMP to have very similar R_f values in this system (0.55 and 0.50, respectively). This experiment, then, demonstrates that the peak eluted with 0.014 M ammonium acetate from DEAE-cellulose (as in Fig. 7) shows a ^{3}H-labeled spot on TLC and radioautography with R_f 0.25, after complete removal of residual [^{3}H]RP by elution with 0.07 M ammonium acetate.

In previous work from these laboratories (3), it was demonstrated that the hydrolysis rate curve in dilute acid (0.1 M HCl at 20°C) of total extractable mannose-labeled lipid was biphasic. It was surmised that the slowly hydrolyzing component was DMP and that the rapidly hydrolyzing one was MRP, because of the known hydrolysis properties of authentic DMP and because of MRP's allylic ester bond, which would probably render it less stable than DMP. By now separating DMP and the compound thought to be MRP, we were able to confirm our hypothesis. **Fig. 10** illustrates that the substance eluted from silicic acid with C–M 2:1 and with an R_f on TLC of 0.5, already identified as DMP, has indeed a slow rate of hydrolysis; the substance eluted with C–M 1:1 (R_f 0.25) is hydrolyzed rapidly. The total mannolipid (extracted with C–M 2:1) has the biphasic curve.

If the incubation was carried out with [^{3}H]RP and the C–M 1:1 eluate from the silicic acid column (R_f 0.25), hydrolyzed as described, the hydrolysis product had the same mobility (R_f 0.9) as retinol on TLC (**Fig. 11**). Thus, from the coincidence of the ^{14}C and ^{3}H labels on DEAE-cellulose fractionation and on TLC and from the hydrolysis rates and TLC of the hydrolysis product, we conclude that the substance with R_f 0.25 is MRP.

TABLE 2. Uptake of [^{14}C]mannose into total glycolipid from GDP-[^{14}C]mannose

Time	Incubations: +GDP[a] +ATP[a]	+GDP −ATP	−GDP +ATP	−GDP −ATP	−GDP +GTP[b]
min	*dpm in total extractable glycolipid*				
5	693	615	8105	4560	556
10	2307	387	8642	1706	491
15	2773	536	9865	567	
20	2147	204	8014	607	5243

Incubations were performed as described in Materials and Methods in 0.12 ml of buffer containing 6 μg of RP and 500 μg of α-L-lecithin.
[a] 50 μg.
[b] 150 μg.

Properties of the reaction

Effect of nucleotides. The formation of total mannolipids, both from [^{3}H]RP and GDP-[^{14}C]mannose, was clearly stimulated by ATP (**Table 2**), although we have as yet no information regarding a direct participation of ATP in the reaction. Instead, we regard ATP as a protective agent for the stabilization of GDP-mannose and/or RP against the attack of phosphatases present in the crude enzyme fraction. Because a large excess of RP was incubated (3 $\times$ 10^{-2} μmoles), support for this hypothesis could be obtained by the fact that, in the absence of ATP, 1.1 $\times$ 10^{-4} μmoles of RP was recoverable at the end of the incubations and, in its presence, 9.2 $\times$ 10^{-4} μmoles was recoverable. The stabilization of GDP-mannose by

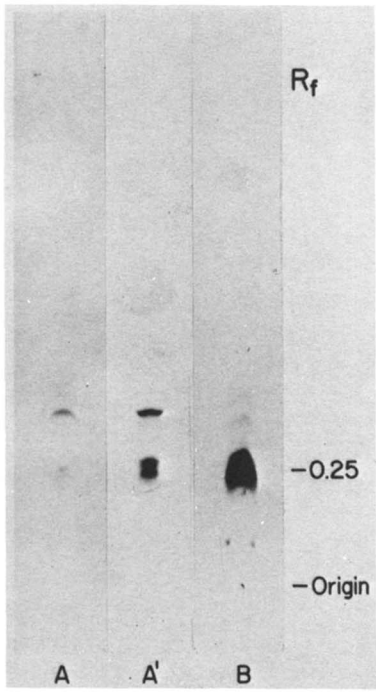

Fig. 9. Preparation of compound with R_f 0.25 labeled with ^3H from [^3H]RP. Incubation of [^3H]RP was identical with that in Fig. 7 except for the addition of unlabeled GDP-mannose. The labeled material was fractionated by chromatography on DEAE-cellulose. *A* indicates the TLC of the first eluted ^3H peak (0.014 M ammonium acetate), showing a double spot with R_f 0.25; *B*, the TLC of the second eluted ^3H peak (0.07 M ammonium acetate), identified as [^3H]RP with authentic RP. *B* was exposed for 2 wk to X-ray film; *A'* was identical with *A*, except that it was exposed for 1 mo.

ATP is supported by a much improved recovery of GDP-mannose from incubations in the presence of ATP and the fact that GTP (but not GDP) could substitute for ATP in stimulating uptake of mannose into lipid (Table 2).

Effect of pH. The formation of the two mannolipids differed in their dependence on pH (**Fig. 12**). The reaction at pH 6.0 favored DMP, and pH 7.0 favored MRP formation. In this experiment, it was found that a differential solvent extraction method could be used to separate DMP and MRP (**Fig. 13**). The incubation mixture was extracted exactly as described in the section "Incubation and separation" under Materials and Methods. The fraction referred to as "1st extraction" contained only the DMP; the "2nd extraction" contained mostly MRP. Both together, of course, accounted for total lipid-extractable radioactivity.

DISCUSSION

Lipid intermediates active in the transfer of carbohydrate from sugar nucleotides to polysaccharides have repeatedly been postulated to occur in mammalian organisms (4, 15–17). We have shown that retinol stimulates

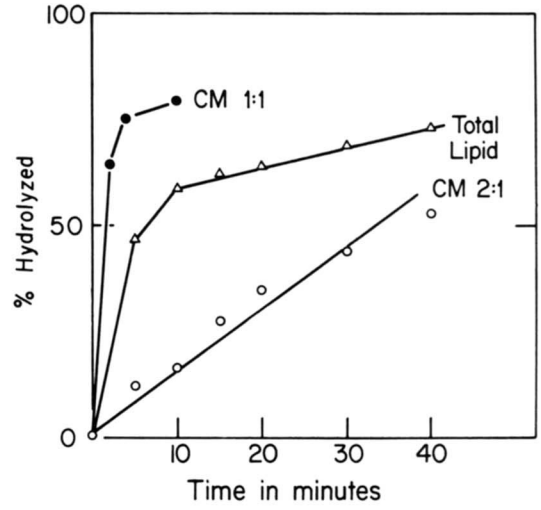

Fig. 10. Hydrolysis rates of [^{14}C]DMP and [^{14}C]MRP in 0.1 M HCl at 20°C. Incubations were made and separation was achieved on a neutralized silicic acid column as described under Fig. 7. C–M 2:1 refers to DMP and C–M 1:1 to MRP, eluted with the respective C–M proportions. For comparison, hydrolysis of a sample of the original total mannolipid extract is shown ("total lipid").

formation of glycophospholipids (3). The synthesis of MRP would probably require the formation of RP as an intermediate.

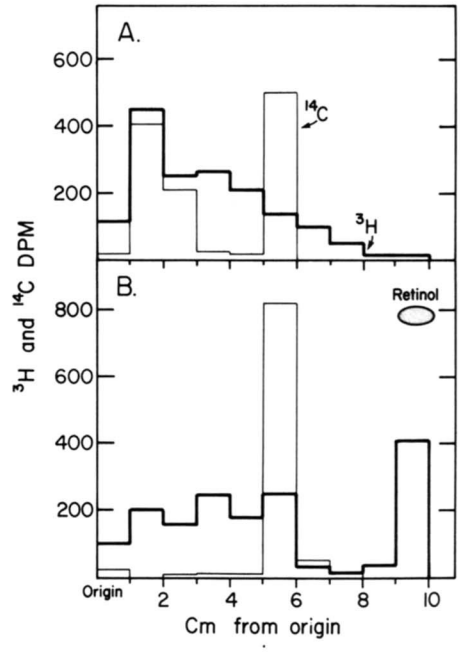

Fig. 11. Formation of a product with chromatographic mobility of retinol upon hydrolysis of MRP. [^3H]RP was incubated with GDP-[^{14}C]mannose as described in Fig. 7. Purification was by DEAE-cellulose. The first ^3H–^{14}C-labeled peak was collected and concentrated to a small volume, and a portion of it was chromatographed by TLC (*A*). Another portion was treated with 0.1 M HCl at 20°C for 5 min under N_2 in dim light, as described under Fig. 10. The hydrolysate was extracted into C–M 2:1, washed, and applied to TLC (*B*) in the solvent described in Fig. 6. A guide spot of authentic retinol was cochromatographed, R_f 0.9.

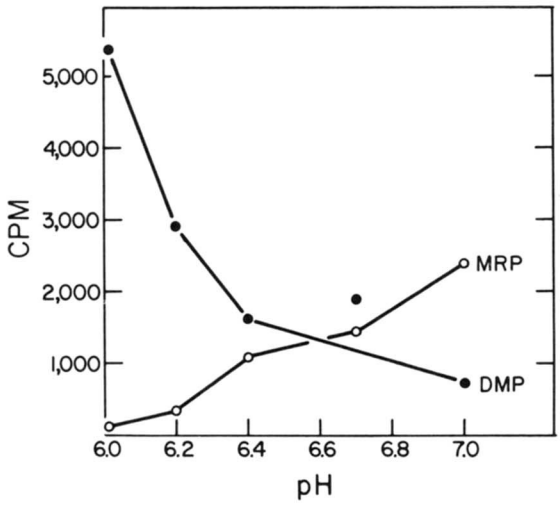

Fig. 12. Dependence on pH of DMP and MRP formation. Incubations were made with RP and GDP-[^{14}C]mannose, as described under Materials and Methods, except that the enzyme fraction was suspended again in Tris buffer and sucrose at the various pH values. The pH values were those measured in the final incubation mixture. Total extractable lipid after the incubation was applied to TLC. The spots were located by radioautography, scraped into vials, and counted.

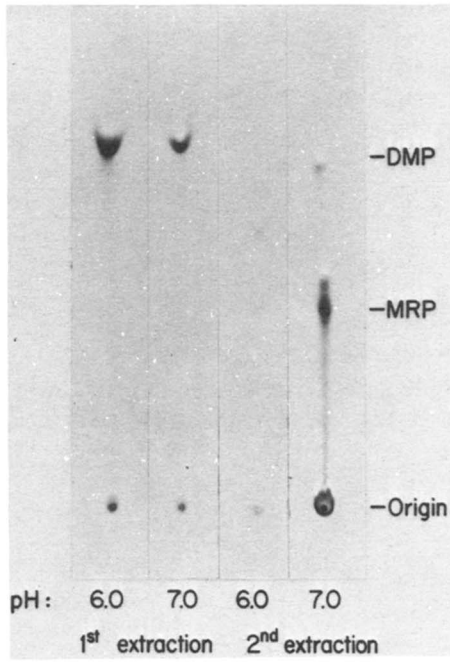

Fig. 13. Differential solvent extraction of DMP and MRP after incubation at two pH values. Incubations were identical with those described for Fig. 7 and had the indicated final pH values. At the end of the incubation, the mixture was extracted exactly as described in the section "Incubation and separation" under Materials and Methods, rendering one solution termed "1st extraction" and one termed "2nd extraction." Each extract was concentrated to a small volume and applied to TLC, giving the indicated radioautograph.

RP was synthesized chemically. Its biological activity in maintaining the weight of vitamin A-deficient rats was roughly similar to that of retinol.

When incubated, RP could serve as substrate for the membrane-rich enzyme fraction previously shown to catalyze the incorporation of retinol into mannolipid. Although the addition of detergent (Zonyl A) was sufficient to stimulate the reaction with retinol (3), the reaction with RP required either membrane-lipid extract or lecithin for the stimulation of MRP synthesis. This was due, possibly, to the polar nature of the RP molecule compared with retinol.

The enzyme that incorporates mannose into lipid is much more active with RP as substrate than with retinol, since the concentration of RP needed for maximal stimulation was $\frac{1}{15}$ to $\frac{1}{20}$ of that required for retinol. This result supports the hypothesis that RP is the immediate mannose acceptor and refutes the possibility that RP is first hydrolyzed to retinol.

Thus far, the formation of a retinyl glycophospholipid has been measured only by the stimulation of [^{14}C]mannose uptake beyond that found as a result of the reaction of an endogenous acceptor(s) and by the incorporation of labeled precursors into total mannolipid (2–5). We have now developed three different separation methods that enable us to explore the separate reactions for formation of DMP and MRP. Tkacz et al. (11) first described the TLC method.

Table 1 shows that, despite considerable variability when increasing amounts of RP were added to the incubations, the uptake of mannose into DMP decreased, suggesting a competing reaction for the substrate, GDP-mannose, between endogenous dolichyl phosphate and added RP. The variability in uptake of mannose from preparation to preparation may be due to uncontrollable variables in the preparation of the membrane fractions or, possibly, to the variable stages of vitamin A deficiency from animal to animal.

From a comparison of the incubations with and without RP (Fig. 6), it appears that the spot with R_f 0.25 derives from RP. Its behavior on DEAE-cellulose and silicic acid columns leads to the conclusion that its charge is negative. When RP was ^{3}H-labeled, the spot became ^{3}H-labeled (Figs. 8 and 9). Preparations from normal animals incubated without RP showed this spot (Fig. 6, *F*). The cochromatography of the hydrolysis product with retinol and the substance's rapid hydrolysis rate made possible its identification as MRP.

The fact that this substance, when derived from RP, even if labeled with ^{3}H, had the appearance of a double spot on TLC (Figs. 6 and 9) has remained unexplained. Other researchers have also obtained a double spot on TLC for a mannolipid formed from GDP-[^{14}C]mannose using RP as the acceptor lipid.[3] RP itself, when prepared

[3] Herscovics, A., J. S. Tkacz, C. D. Warren, and R. W. Jeanloz. Unpublished results.

by chemical synthesis, appeared as a single spot on TLC; however, we surmise that a partial isomerization of the double bond system may occur in the course of incubation. It is well known (*a*) that in derivatives of retinol and retinal the configuration of the C-11 double bond is readily isomerized from *trans* to *cis* and (*b*) that isomeric compounds often show an incompletely resolved double spot on TLC. An example of this behavior is shown by synthetic citronellyl mannopyranosyl phosphate (14), citronellol being a mixture of 3,6-dimethyl-6-octen-1-ol and 3,6-dimethyl-7-octen-1-ol.

Hydrolysis of MRP labeled with ^{3}H resulted in a compound that cochromatographed with retinol (Fig. 11). Because MRP is an allylic phosphate diester, hydrolysis will take place via an elimination reaction, accompanied by rearrangement of the retinol moiety. The product may therefore contain retinol itself, as well as a tertiary alcohol isomeric with it and having a closely similar R_f.

The protective and stabilizing effects of ATP and GTP (Table 2) on the substrates were not unexpected.

The possibility had to be considered that the formation of MRP from RP is due to the low specificity of the enzyme that converts dolichyl phosphate to DMP in vivo. In fact, Richards and Hemming (18) and Tkacz et al. (11) have shown that this enzyme reacts with a variety of long-chain isoprenyl phosphates. However, our study suggests that there is a specific enzyme for the conversion of RP to MRP, because of the differences in the pH dependence and time courses of the two reactions of endogenous dolichyl phosphate to DMP and exogenous RP to MRP.

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