Identification and characterization of mannosyl retinyl phosphate occurring in rat liver and intestine in vivo

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Abstract A study was conducted to determine whether mannosyl retinyl phosphate occurred in rat liver and intestine in vivo, and, if so, to partially purify it and investigate its properties. After injection of [^{3}H]retinol and [^{14}C]mannose, a chloroform–methanol 2:1 extract of rat liver and small intestinal mucosa yielded two ^{3}H/^{14}C-labeled peaks on DEAE–cellulose column chromatography: peak I eluted with 10 mM and peak II eluted with 29 mM ammonium acetate. Peak II, subjected to silicic acid column chromatography, gave principally two ^{3}H/^{14}C-labeled fractions, one eluted with chloroform–methanol 2:1 and the other with chloroform–methanol 1:1. The latter showed, on thin-layer chromatography in a chloroform–methanol–water 60:25:4 system, an R_f of 0.25 (with coincidence of the ^{3}H and ^{14}C radioactivity), which is identical to the R_f of authentic mannosyl retinyl phosphate. The chloroform–methanol 1:1 peak, on mild acid hydrolysis, yielded [^{3}H]retinol (identified by two thin-layer chromatography systems), [^{14}C]mannose, and [^{14}C]-mannose phosphate (identified by paper chromatography). On mild alkali hydrolysis, the peak yielded [^{3}H]retinol and [^{14}C]mannose phosphate. The substance eluted in the chloroform–methanol 1:1 peak from silicic acid was therefore concluded to be mannosyl retinyl phosphate. When chromatographed on silicic acid, peak I from the DEAE–cellulose column primarily showed a fraction eluted with chloroform–methanol 2:1. When chromatographed on thin-layer plates in the above solvent, this fraction showed an R_f of 0.3, with coincidence of ^{3}H and ^{14}C radioactivity; it was resistant to mild acid hydrolysis, mild and strong alkali hydrolysis, and glucuronidase action. Mannosyl retinyl phosphate occurs, therefore, in vivo in liver and intestinal mucosa, and it is accompanied by a closely similar, though slightly less polar, compound that remains unidentified.

Supplementary key words retinol · mannose · vitamin A deficiency · DEAE–cellulose chromatography · silicic acid chromatography · thin-layer chromatography

Mannosyl retinyl phosphate (MRP) has been made from retinol (1) and from retinyl phosphate (2), with liver microsomes catalyzing its synthesis. Barr and De Luca (3) reported the isolation of MRP from hamster liver in vivo, but did not completely characterize it.

We were prompted to reexamine the problem of the in vivo occurrence of MRP largely because a substance that had many of the properties of MRP was found in both rat liver and cell cultures of chick embryo fibroblasts; it differed, however, from MRP by being highly resistant to acid hydrolysis. MRP is, of course, very unstable towards acid. Until we learned how to separate this unidentified substance from MRP, we had mistaken it for MRP, and others may also be so mistaken. In learning to separate it, we characterized the MRP, partially purified from rat liver and intestine, by acid and alkali hydrolysis and an analysis of the hydrolysis products.

MATERIALS AND METHODS

Materials

All-*trans*-[15-^{3}H]retinol (2.66 Ci/mmol) and D-[1-^{14}C]-mannose (53.1 mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA. Tween 80 was from Fisher Scientific Co., Pittsburgh, PA; mannose 1-phosphate, β-glucuronidase, and phenolphthalein-β-monoglucuronide were from Sigma Corp., St. Louis, MO; DEAE–cellulose was from Eastman Kodak Co., Rochester, NY; and silicic acid (Bio-Sil HA, 200–325 mesh) was from Bio-Rad Laboratories, Richmond CA. Precoated plates of silica gel G for thin-layer chromatography (TLC) were from E. Merck A.G., Darmstadt, Germany. Purity of [^{3}H]retinol was determined by TLC (4) and found to vary between 92 and 98% for the several batches used; purity of the [^{14}C]mannose was reported by the supplier to be greater than 99%.

Abbreviations: BHT, butylated hydroxytoluene; DMP, dolichyl mannosyl phosphate; MRP, mannosyl retinyl phosphate; TLC, thin-layer chromatography.

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Animals and diets

The preparation of the animals and their diets followed the procedures previously described (5). Two vitamin-A deficient rats were injected intraperitoneally at the plateau stage of deficiency (253 and 255 g) with: *a*) 100 μCi of [^{3}H]retinol dissolved in 0.5 ml of a mixture of ethanol–Tween 80–0.85% saline 10:16:74 at zero time; *b*) 25 μCi of [^{3}H]retinol after 22 hr; and *c*) 83.3 μCi of [^{14}C]mannose in 0.5 ml of saline after 25 hr. A normal rat (270 g) received 100 μCi of [^{3}H]retinol at zero time, 100 μCi more after 22 hr, 50 μCi more after 46 hr, and 83.3 μCi of [^{14}C]mannose after 49 hr. All animals were killed by decapitation 20 min after the mannose injection and bled; their livers and intestines were removed. All further work was performed under dim incandescent light.

Extractions

The livers of the two vitamin A-deficient rats were combined (total weight, 14.4 g) and homogenized in 60 ml of water containing 0.5% ascorbic acid as an antioxidant (4). The homogenate was lyophilized. The residue was then extracted twice with 130 ml of methanol, once with 93 ml of chloroform–methanol 2:1, and seven more times with 70 ml of the same solvent, until no more radioactivity was extractable (the organic solvents all contained 50 μg of butylated hydroxytoluene [BHT] per ml). The extracts were combined and flash-evaporated to 2 ml under nitrogen. During this process, a small amount of white precipitate appeared; it was removed by centrifugation and washed several times with chloroform–methanol 2:1. The washings were added to the extract.

The normal liver (7.9 g) was homogenized and the homogenate was lyophilized in the same way, except that the first two methanol extractions were with 90 ml and the next two chloroform–methanol 2:1 extractions were with 40 ml, followed by six more extractions with 30 ml.

The intestinal mucosa from all three rats were obtained by scraping the mucosa of the small intestine as previously described (6). The scrapings of the two vitamin A-deficient rats were combined and homogenized with 25 ml of water containing 0.5% ascorbic acid, and the homogenate was lyophilized. The residue was extracted with 48 ml of methanol, then with 22.5 ml of methanol, then with 23 ml of chloroform–methanol 2:1, and finally seven more times with the same solvent (all solvents contained 50 μg BHT per ml). The mucosa of the normal rat was treated in the same way, and the two extracts were combined and flash-evaporated under nitrogen, as were the liver extracts.

Radioactivity determinations

Because all of the compounds counted were soluble in organic solvents, we evaporated aliquots of their solutions to dryness under nitrogen and dissolved them in 10 ml of toluene containing 0.5% diphenyloxazole in order to prepare them for liquid scintillation counting. We determined the radioactivity of compounds on TLC plates and on paper chromatograms as described (2, 7). Sugars and mannose phosphate were located by sprays as previously described (7).

RESULTS

Previous work from our laboratory (8) has shown that the rise in the weight curve of vitamin A-deficient rats begins 24 hr after they receive as little as 8 μg of retinyl acetate. In the present work, two mildly deficient rats each received 13.5 μg of labeled retinol intraperitoneally and were killed 25 hr 20 min later. A normal rat, which had been kept at the same weight as the deficient ones by pair-feeding, received 27 μg of labeled retinol and was killed 47 hr 20 min later. One experiment was done with the extract of the combined livers of the deficient rats, one with the extract of the liver of the normal rat, and one with the combined intestinal mucosal extracts of all three rats.

Isolation and identification of MRP-like compound from liver and intestine

We chromatographed flash-evaporated chloroform–methanol extracts of livers on DEAE–cellulose columns in 99% methanol by the method of Dankert et al. (9) as used previously in our laboratory for MRP (1, 2). Because the total radioactivity for the separate extracts of intestinal mucosa was small, the extracts of the two deficient livers were combined with the normal extract for fractionation.

One large peak, having ^{14}C radioactivity derived from mannose and ^{3}H radioactivity derived from retinol, was eluted with 10 mM ammonium acetate (**Fig. 1**, peak I); a second smaller peak was obtained with 29 mM ammonium acetate (Fig. 1, peak II). The pattern was similar for extracts of vitamin A-deficient liver (Fig. 1*A*), normal liver (Fig. 1*B*), and intestinal mucosa (Fig. 1*C*).

To standardize the DEAE–cellulose columns and the elution procedure, we repeated the experiment exactly as illustrated in Fig. 1*A*, except that no [^{14}C]-mannose was injected. To the ^{3}H-labeled chloroform–methanol extract of the livers was added [^{14}C]MRP (129,500 dpm) as internal standard, made from retinyl

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phosphate and GDP-[^{14}C]mannose (2). The ^{3}H-labeled peak II was eluted at 22 mM ammonium acetate, as in Fig. 1*A*. The [^{14}C]MRP marker gave a ^{14}C-labeled peak at exactly the same molarity. In the same experiment, unlabeled retinyl phosphate was eluted at 34 mM ammonium acetate, separate from peak II, thus excluding the possibility that peak II is a mixture of retinyl phosphate and an unknown compound.

From past experience (1–3), we expected a mixture of dolichyl mannosyl phosphate (DMP) and MRP to be eluted from the DEAE–cellulose column in the region of peak I or II (Fig. 1). We first concentrated our attention on peak II and, armed with the knowledge that silicic acid chromatography can separate DMP and MRP (1–3), we fractionated this peak on a silicic acid column (**Fig. 2**), collecting fractions eluted with chloroform–methanol 2:1 and 1:1. We knew from previous work (2, 3) that the chloroform–meth-

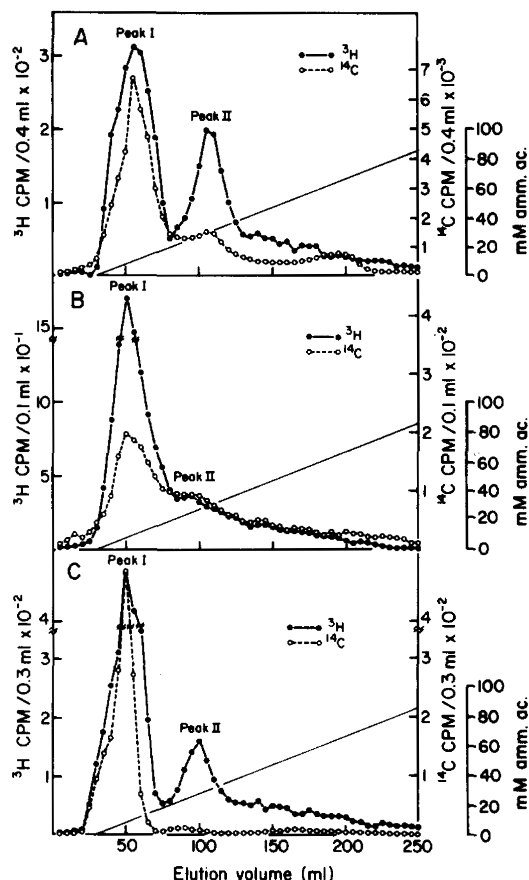

Fig. 1. Fractionation of liver and intestinal extracts on DEAE–cellulose. (*A*) Extract of two deficient livers (2 ml), prepared as described in Materials and Methods, was placed on a 1 × 33-cm column of DEAE–cellulose, by the method of Dankert et al. (9) as previously described (1, 2), washed with 300 ml of 99% methanol, and then eluted with a linear gradient, 0–0.1 M ammonium acetate (amm. ac.). Material placed on the column: 8.40 × 10^{6} dpm ^{3}H radioactivity (1.53% of injected), 5.57 × 10^{6} dpm ^{14}C radioactivity (3.04% of injected). (*B*) Extract of normal liver fractionated exactly like that of deficient livers. Material placed on column: 43.47 × 10^{6} dpm ^{3}H radioactivity (7.90% of injected), 4.40 × 10^{6} dpm ^{14}C radioactivity (2.40% of injected). (*C*) Extract of combined intestinal mucosa of two deficient and one normal rat fractionated exactly like the extract of deficient livers. Material placed on column: 2.78 × 10^{6} dpm ^{3}H radioactivity (0.25% of injected), 1.78 × 10^{6} dpm ^{14}C radioactivity (0.16% of injected). Total recoveries from the columns: (*A*) ^{3}H, 108%; ^{14}C, 84%; (*B*) ^{3}H, 101% ^{14}C, 88.2%; (*C*) ^{3}H, 96%; ^{14}C, 80%.

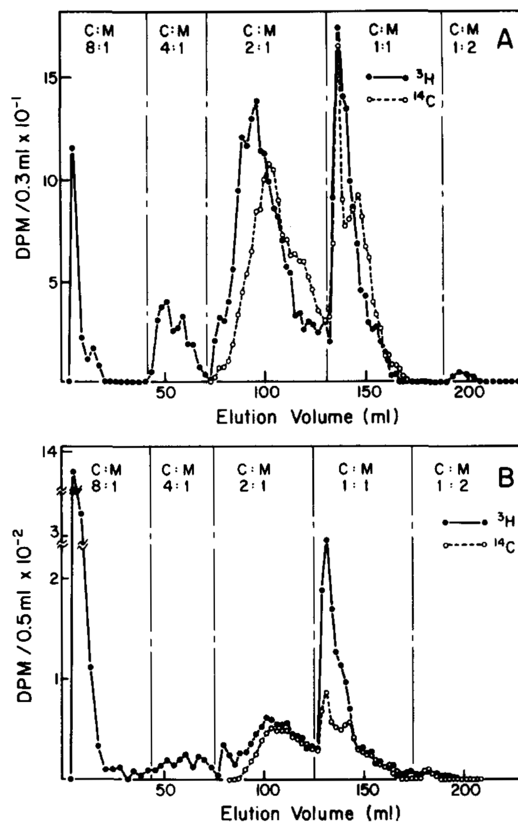

Fig. 2. Fractionation of peak II from DEAE–cellulose on silicic acid. Fractions under peak II (Fig. 1) were pooled, neutralized by passing through NH_3 gas, and flash-evaporated under N_2 to 2 ml. Chloroform (4 ml) and water (1.2 ml) were added, and the amm. ac. was extracted into the water phase. The latter was then reextracted with 5 ml of chloroform, followed by 2.5 ml of chloroform–methanol (C–M) 2:1, 3 ml of C–M 2:1, and 3 ml of chloroform. The organic phases were combined, flash evaporated to dryness, taken up in 2 ml of C–M 8:1, and placed on the silicic acid column. The silicic acid had previously been heated to 105°C for 2 hr for activation, and a 1 × 6-cm column was made with chloroform. For the deficient livers (*A*), peak II ^{3}H radioactivity placed on the column, as obtained from DEAE–cellulose, after washing, was 3.128 × 10^{4} dpm; ^{14}C radioactivity was 2.554 × 10^{4} dpm. For the normal livers (*B*), peak II ^{3}H radioactivity placed on the column was 2.654 × 10^{4} dpm; ^{14}C radioactivity was 9.559 × 10^{3} dpm. Elution was batchwise, starting with 40 ml of C–M 8:1, followed by 30 ml of C–M 4:1, 50 ml of C–M 2:1, 50 ml of C–M 1:1, and 40 ml of C–M 1:2. The pooled C–M 1:1 peak from deficient livers (*A*) had 5.71 × 10^{3} dpm ^{3}H radioactivity and 6.04 × 10^{3} dpm ^{14}C radioactivity. The pooled C–M 1:1 peak from the normal liver (*B*) had 4.60 × 10^{3} dpm ^{3}H radioactivity and 2.44 × 10^{3} dpm ^{14}C radioactivity. Total recoveries from the columns: (*A*) ^{3}H, 74.0%; ^{14}C, 67.6%; (*B*) ^{3}H, 89.8%; ^{14}C, 62.3%.

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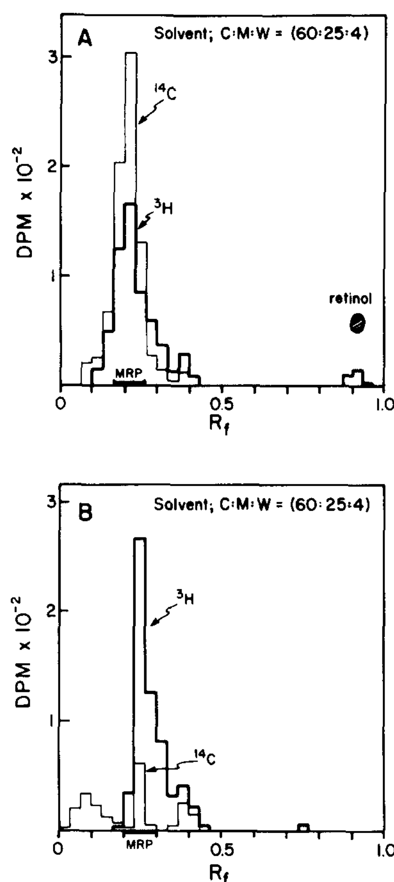

Fig. 3. TLC of the C–M 1:1 peak from liver and peak II from intestine with authentic MRP. (*A*) A portion of the C–M 1:1 peak from the silicic acid column illustrated in Fig. 2*B*, derived from extract of normal liver, was used for TLC with a marker spot of synthetic MRP (2), cochromatographed with retinol in chloroform–methanol–water (C–M–W) 60:25:4. (*B*) A portion of pooled peak II from the DEAE–cellulose column illustrated in Fig. 1*C* of combined extracts of intestinal mucosa was submitted to TLC with authentic MRP as marker in the same solvent system.

anol 1:1 peak would be likely to contain the MRP. This peak, both from deficient (Fig. 2*A*) and normal (Fig. 2*B*) liver, was therefore subjected to further analysis.

The pooled chloroform–methanol 1:1 fractions from the silicic acid columns were subjected to TLC. **Fig. 3*A*** shows the result of TLC of the fraction from normal liver. ^{14}C-Labeled MRP served as a marker; it was prepared with retinyl phosphate, GDP-[^{14}C]-mannose, and a rat liver microsome fraction (2). The TLC of the corresponding fraction from deficient liver gave the same result (not shown). The results of TLC of peak II (Fig. 1*C*) from combined intestinal mucosal extracts directly (without prior purification on silicic acid) are shown in Fig. 3*B*.

These results show that a substance, which closely resembles MRP in behavior on DEAE–cellulose, silicic acid, and thin-layer chromatography, and which shows coincidence of the ^{3}H- and ^{14}C-labels after administration of [^{3}H]retinol and [^{14}C]mannose, could be isolated from liver and intestine.

Hydrolysis experiments with MRP-like compound

Identification of retinol. Because we have found that mild acid hydrolysis (0.1 M HCl at 23°C for 20 min) could split MRP to yield retinol (2), we hydrolyzed the chloroform–methanol 1:1 peak from the silicic acid column of the extract of deficient livers (Fig. 2*A*) and the extract of combined intestinal mucosa (Fig. 1*C*; the fraction that chromatographed like MRP on TLC). With both tissues and in two solvent systems, a substantial portion of the ^{3}H radioactivity in the organic phase after hydrolysis migrated with retinol upon cochromatography (**Fig. 4**). It should be emphasized that acid hydrolysis, even though performed under very mild conditions, is not the best way of obtaining retinol from MRP. Hence, mild alkali hydrolysis was carried out (0.1 M NaOH at 65°C for 20 min), again using the pooled chloroform–methanol

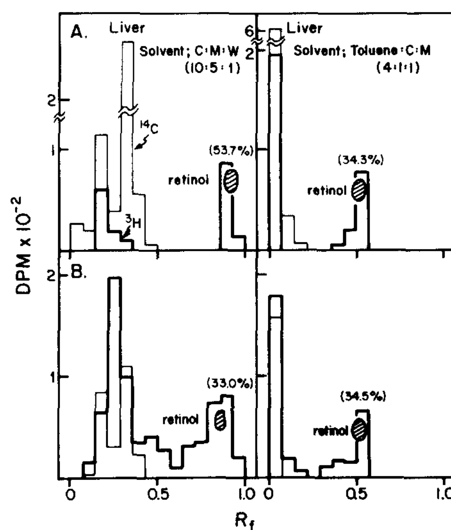

Fig. 4. TLC of the organic phase after mild acid hydrolysis of C–M 1:1 fraction of extracts of livers and peak II fraction of intestines. (*A*) The pooled C–M 1:1 peak from silicic acid chromatography of extracts of deficient livers (illustrated in Fig. 2*A*) was taken to dryness under nitrogen and taken up in 0.6 ml of methanol. A portion (0.2 ml) was treated with 0.2 ml of 0.2 M HCl for 20 min at 23°C, placed on ice, and neutralized with 0.4 ml of 0.1 M NH_4OH, 0.25 ml of methanol, and 0.95 ml of water; the lipid material was extracted into 5 ml of C–M 2:1. The organic layer was then washed once with 1 ml of C–M–W 3:48:47 and subjected to TLC in two different systems, C–M–W 10:5:1 and toluene–C–M 4:1:1, with retinol cochromatographed. (*B*) Intestinal extracts were treated identically, except that the hydrolysis was performed on peak II from the DEAE–cellulose column (shown in Fig. 1*C*), omitting fractionation on silicic acid. Recoveries on hydrolysis: (*A*) in the organic phase: ^{3}H, 42.8%; ^{14}C, 57.5%; in the aqueous phase: ^{3}H, 57.2%; ^{14}C, 42.5%; (*B*) in the organic phase: ^{3}H, 37.9%; ^{14}C, 29.4%; in the aqueous phase: ^{3}H, 62.1%; ^{14}C, 70.6%.

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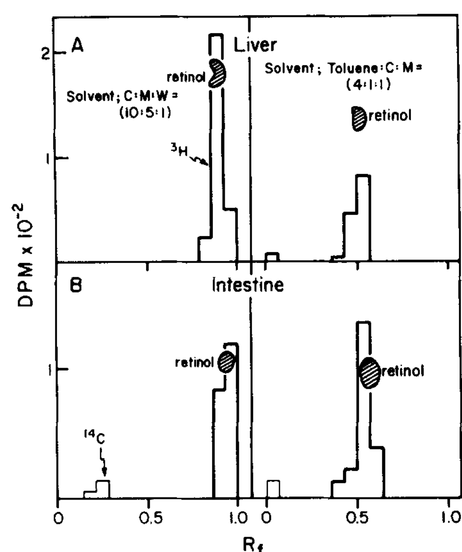

Fig. 5. TLC of the organic phase after mild alkali hydrolysis of the C–M 1:1 fraction of extracts of liver and peak II of extract of intestines. (*A*) A portion (1.2 ml; see Fig. 8*A*) of the pooled C–M 1:1 fraction of extract of deficient livers was taken to dryness and dissolved in 0.45 ml 1-propanol. Sodium hydroxide (1 M, 0.05 ml) was added; the solution was heated to 65°C for 20 min, cooled in ice, and neutralized with 0.5 ml of 0.1 M acetic acid. It was then extracted with C–M 2:1 exactly as for mild acid hydrolysis (see legend for Fig. 4). The organic phase was then subjected to TLC with cochromatography of retinol in the same solvents as for acid hydrolysis. (*B*) An identical procedure was followed for combined intestinal extracts, except that hydrolysis was performed on peak II from the DEAE-cellulose column (Fig. 1*C*), omitting fractionation on the silicic acid column. Recoveries on hydrolysis: (*A*) in the organic phase: ^{3}H, 34.5%; ^{14}C, 0.0%; in the aqueous phase: ^{3}H, 65.5%; ^{14}C, 100%; (*B*) in the organic phase: ^{3}H, 37.5%; ^{14}C, 27.3%; in the aqueous phase: ^{3}H, 62.5%; ^{14}C, 72.7%.

1:1 fraction from the silicic acid fractionation of extracts of deficient liver and combined intestine (Figs. 2*A* and 1*C*). With both tissues and in two solvent systems, the ^{3}H radioactivity in the organic phase after hydrolysis cochromatographed almost exclusively with retinol (**Fig. 5**).

Identification of mannose and mannose phosphate. The aqueous phases after mild acid and alkali hydrolysis from these two experiments (Figs. 4 and 5) were subjected to paper chromatography; after mild acid hydrolysis, these phases yielded mannose and mannose phosphate, respectively, exactly as we reported for authentic MRP (7). **Fig. 6** shows the result for the extract of deficient liver using the pooled chloroform–methanol 1:1 fraction from silicic acid. Intestinal extract gave a similar result (not shown). Mild alkali hydrolysis produced only mannose phosphate (7), as shown for deficient liver (**Fig. 7*A***) and intestine (Fig. 7*B*).

We concluded from the hydrolysis experiments that the compound from liver and intestine found in peak II (Fig. 1) is indeed MRP.

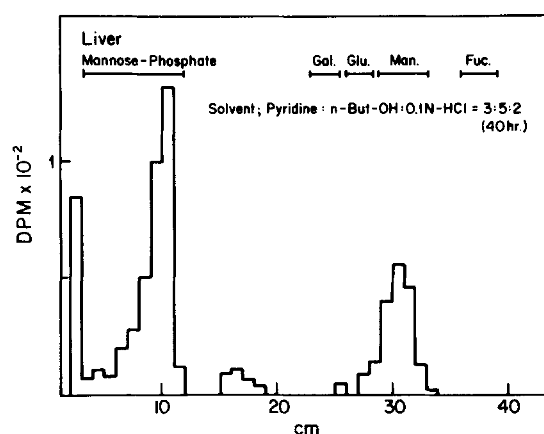

Fig. 6. Paper chromatography of the aqueous phase after acid hydrolysis (0.1 N HCl for 30 min to 95°C) of the C–M 1:1 peak from extracts of deficient livers. The aqueous phase remaining after extraction of the lipid into the organic phase following acid hydrolysis was applied to Whatman No. 1 filter paper and chromatographed descendingly in pyridine–1-butanol–0.1 M HCl 3:5:2 for 40 hr with marker spots as shown. After drying, the paper was cut into 1-cm strips; radioactivity was determined and the marker spots were located by sprays as described (7).

Fractionation and properties of the compound accompanying MRP

Peak I, eluted with 10 mM ammonium acetate from DEAE–cellulose (Fig. 1) and by far the largest peak obtained from this fractionation, was treated exactly as peak II. It was subjected to chromatography on a silicic acid column. **Fig. 8*A*** shows the elution pattern

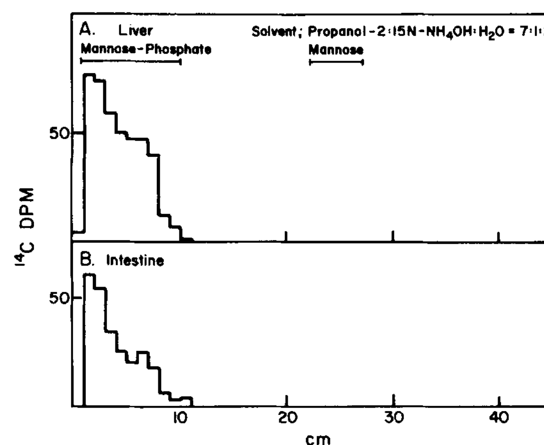

Fig. 7. Paper chromatography of the aqueous phase after alkali hydrolysis of the C–M 1:1 peak from extracts of deficient livers and peak II from extracts of intestines. The aqueous phase remaining after extraction of the lipid into the organic phase following mild alkali hydrolysis (Fig. 5) was applied to Whatman No. 1 filter paper and chromatographed descendingly in isopropanol–15 M NH_4OH–water 7:1:2 with markers as shown, for the C–M 1:1 peak from extract of deficient livers (*A*) and the peak II of extract from intestines (*B*). Radioactivity was determined and the marker spots were located by sprays as described (7).

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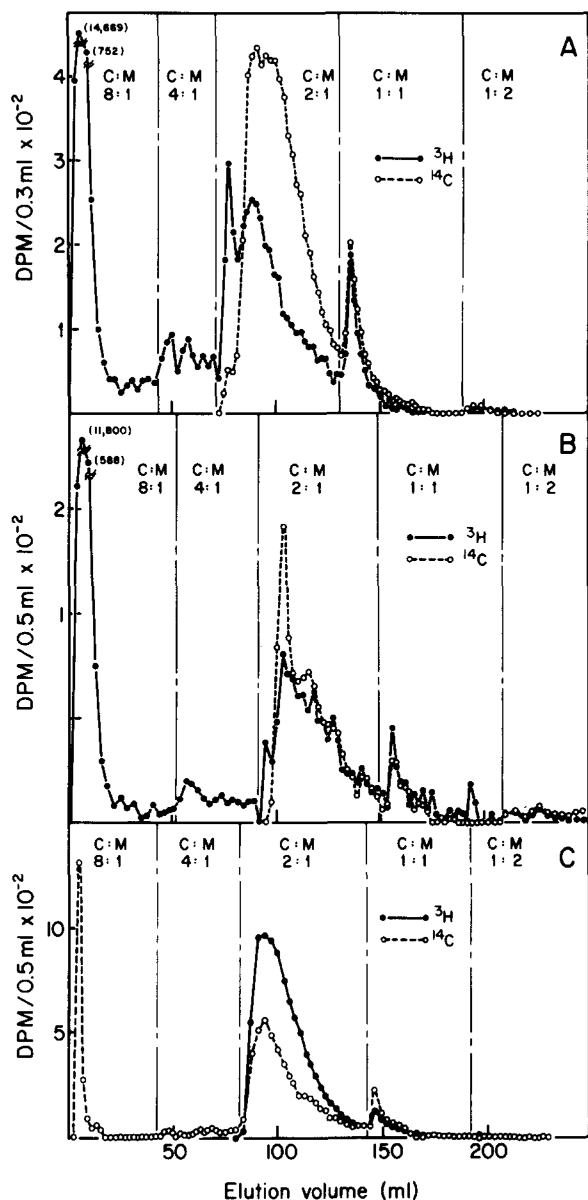

Fig. 8. Fractionation of peak I from DEAE-cellulose on silicic acid. (*A*) Pooled peak I from extract of deficient livers (as shown in Fig. 1*A*) was washed free of amm. ac. and applied to a 1 × 6-cm silicic acid column (18.8 × 10^4 dpm ^{3}H radioactivity and 7.55 × 10^4 dpm ^{14}C radioactivity) and chromatographed exactly as described for peak II (see legend for Fig. 2). Eluted in the C–M 2:1 peak: 2.85 × 10^4 dpm ^{3}H radioactivity and 4.64 × 10^4 dpm ^{14}C radioactivity. (*B*) The pooled peak I from extract of normal liver (as shown in Fig. 1B) (7.77 × 10^4 dpm ^{3}H radioactivity and 2.01 × 10^4 dpm ^{14}C radioactivity) was chromatographed on a silicic acid column exactly as the extract of normal liver, above. The peaks obtained from the batchwise elution with C–M 2:1 from both deficient (*A*) and normal (*B*) liver were pooled (4.73 × 10^4 dpm ^{3}H radioactivity and 6.33 × 10^4 dpm ^{14}C radioactivity) and rechromatographed on another silicic acid column (1 × 4.5 cm) in the same way, yielding the elution pattern shown (*C*). The pooled C–M 2:1 peak fraction contained 2.33 × 10^4 dpm ^{3}H radioactivity and 4.26 × 10^4 dpm ^{14}C radioactivity. Recoveries from the silicic acid columns: (*A*) ^{3}H, 112%; ^{14}C, 71.7%; (*B*) ^{3}H, 102%; ^{14}C, 66.2%; (*C*) ^{3}H, 87.8%; ^{14}C, 86.7%.

of peak I from the extract of deficient livers, with ^{3}H radioactivity and a very high level of ^{14}C radioactivity in the chloroform–methanol 2:1 peak. The small peak eluted with chloroform–methanol 1:1 was readily identifiable as MRP by TLC and was clearly derived from a small amount of admixture of peak II material in peak I, because separation of these two peaks on DEAE–cellulose is not perfect (see Fig. 1*A*). A similar pattern was obtained by silicic acid chromatography of peak I from the extract of normal liver (Fig. 8*B*). In order to obtain enough material for further characterization and purification, we combined the chloroform–methanol 2:1 peaks from Figs. 8*A* and *B* and rechromatographed them on silicic acid (Fig. 8*C*). Peak I of the extract of intestinal mucosa was not further investigated.

TLC of the chloroform–methanol 2:1 eluate from the extract of deficient liver showed an R_f of 0.3, slightly higher than that obtained with MRP (0.25) (**Fig. 9**). An identical pattern was obtained on TLC of the chloroform–methanol 2:1 fraction from the extract of normal liver. Indeed, the substance with R_f 0.3 was also detected when subjecting the chloroform–methanol 2:1 fractions of the silicic acid separation of peak II (Fig. 2) to TLC, since, just as peak I carries peak II with it, so peak II carried over some peak I.

Attempts to hydrolyze the chloroform–methanol 2:1 peak with mild acid, mild alkali, strong alkali (1 M

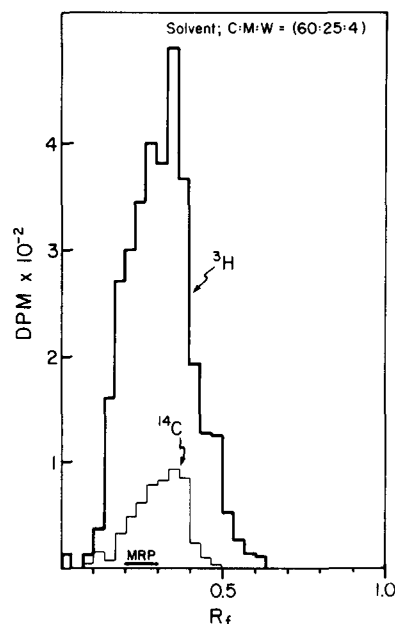

Fig. 9. TLC of the C–M 2:1 peak. A portion of the peak derived from peak I of the extract of deficient and normal livers (Fig. 8*C*) was submitted to TLC in C–M–W 60:25:4 with a marker of authentic synthetic MRP.

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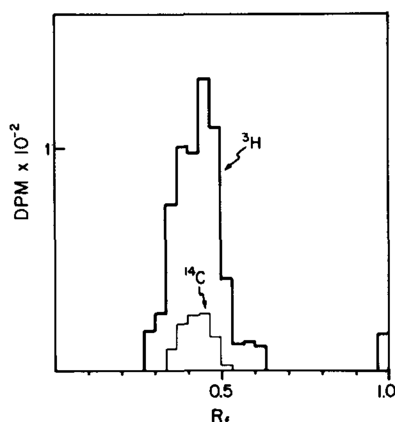

Fig. 10. TLC of the C–M 2:1 peak after mild acid hydrolysis. Another portion of the peak eluted with C–M 2:1 from peak I derived from extract of deficient and normal livers (Fig. 8*C*) was subjected to mild acid treatment as described in the legend to Fig. 4. The organic phase was then chromatographed on thin-layer as for Fig. 9.

sodium hydroxide at 70°C for 1 hr), and β-glucuronidase were unsuccessful in that the substance was recovered unchanged (**Fig. 10**). (The activity of the glucuronidase was, of course, checked by the known glucuronidase substrate phenolphthalein-β-monoglucuronide.) In a parallel experiment in which standard [^{14}C]MRP, made from retinyl phosphate and GDP-[^{14}C]mannose, was hydrolyzed with mild acid (see Fig. 4), insignificant radioactivity was recoverable in the chloroform–methanol extract, whereas the aqueous radioactivity behaved like mannose in the TLC system (Fig. 10).

Experiments were carried out with chick embryo fibroblasts grown in culture in the presence of [^{3}H]-retinol and [^{14}C]mannose.[2] The cells were homogenized and extracted exactly as described for liver, above, and a ^{3}H/^{14}C-labeled peak corresponding to peak I (as in Fig. 1) was obtained, but only a trace of peak II occurred (**Fig. 11**). The peak I from the fibroblast extract was chromatographed on silicic acid and yielded a chloroform–methanol 2:1 peak, which gave the R_f 0.3 upon TLC (**Fig. 12**). Again, this substance was totally resistant to mild or strong alkali treatment, and remained unchanged even after it was heated in 0.1 M HCl at 50°C for 1 hr. Although the compound behaved in a manner very similar to MRP and was labeled with ^{3}H and ^{14}C from [^{3}H]retinol and [^{14}C]mannose, we have no clue as to its nature and did not pursue its identification further.

[2] The authors are grateful to Drs. Sharon S. Krag and P. W. Robbins (Department of Biology, Massachusetts Institute of Technology) for providing chick embryo fibroblasts cultured with [^{3}H]-retinol and [^{14}C]mannose.

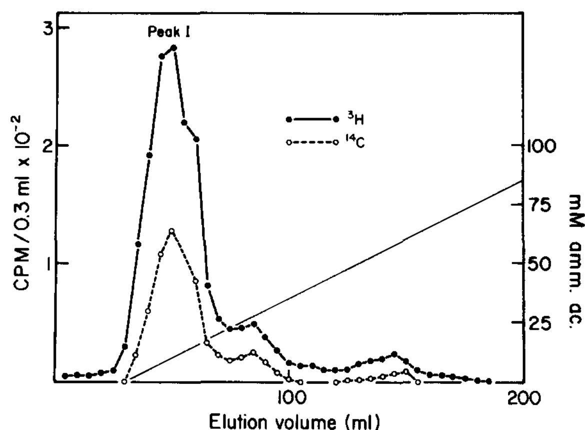

Fig. 11. Fractionation of extract from chick embyro fibroblasts on DEAE–cellulose. Cells (5 × 10^7/plate) were incubated for 3 hr at 39°C with 10 μCi of [^{3}H]retinol and 24 μCi of [^{14}C]mannose. Cells from four separate incubations were washed with 0.5% ascorbic acid and extracted as described in Fig. 1. Extract was placed on a 1 × 30-cm column of DEAE–cellulose, washed with 99% methanol, and then eluted with a linear gradient, 200 ml of 0–0.1 M amm. ac. Material placed on the column: 13.2 × 10^6 dpm ^{3}H radioactivity (15.1% of added); 1.64 × 10^5 dpm ^{14}C radioactivity (0.078% of added). Total recoveries from the column: ^{3}H, 97%; ^{14}C, 81%.

For purposes of comparison, and to avoid confusion between MRP and its companion substance when isolating the former, we have summarized the results in **Table 1**.

DISCUSSION

The conclusion from the experiments described here is that [^{3}H–^{14}C]MRP can be isolated from liver and

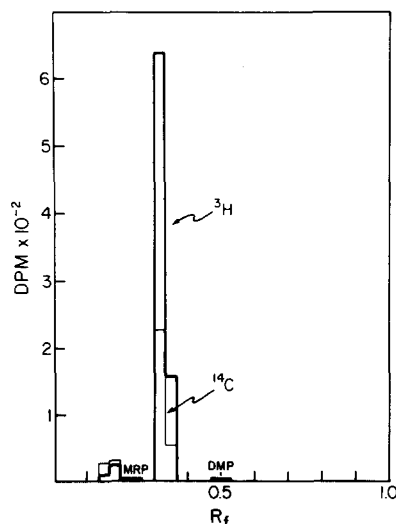

Fig. 12. TLC of the C–M 2:1 peak from chick embryo fibroblasts with authentic MRP and DMP. A portion of the C–M 2:1 peak from the silicic acid column was used for TLC, cochromatographed with synthetic MRP and DMP as markers in C–M–W 6:25:4 system.

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TABLE 1. Properties of MRP and related companion compound

MRP	Peak I Compound
Extractable into C–M[a] 2:1	Extractable into C–M 2:1
$^3H/^{14}C$-labeled from [^3H]retinol and [^{14}C]mannose	$^3H/^{14}C$-labeled from [^3H]retinol and [^{14}C]mannose
Eluted with 22–29 mM amm. ac.[b] from DEAE–cellulose	Eluted with 7–10 mM amm. ac. from DEAE–cellulose
Eluted with C–M 1:1 from silicic acid	Eluted with C–M 2:1 from silicic acid
R_f of 0.25 on TLC (C–M–W[c] 60:25:4)	R_f of 0.30 on TLC (C–M–W 60:25:4)
Hydrolysis in alkali yields mannose phosphate and retinol	Hydrolysis in alkali leaves compound unchanged
Hydrolysis in acid yields mannose, mannose phosphate, and retinol	Hydrolysis in acid leaves compound unchanged

[a] Chloroform–methanol.
[b] Ammonium acetate.
[c] Chloroform–methanol–water.

intestinal mucosa of rats after injection of [^3H]retinol and [^{14}C]mannose. The compound was identifiable *a*) by its behavior on DEAE–cellulose and silicic acid columns and on TLC, in all cases showing coincidence of ^3H- and ^{14}C-peaks; and *b*) by identification of retinol, mannose, and mannose phosphate as the products of mild acid and alkaline hydrolysis. MRP was accompanied on the DEAE–cellulose column by a substance eluted slightly in advance of it, which also had a very similar behavior on silicic acid and TLC, suggesting that it was slightly less polar than MRP. It could not be hydrolyzed by acid or alkali.

Because we had previously found preparations of synthetic MRP to be eluted from DEAE–cellulose with 14 mM ammonium acetate (2), we mistakenly assumed that peak I (eluted with 7–10 mM ammonium acetate) was MRP, until we identified peak II (eluted with 22–29 mM ammonium acetate) as this compound. In fact, peak II, identified as MRP, has been isolated in several additional experiments, not reported here, after injection of retinol labeled with either ^3H or ^{14}C.

The reason for the small shift in the elution position of MRP from 14 mM to 22–29 mM ammonium acetate is obscure and may depend on the batch of ion exchange resin. It is significant that the elution position of the retinol glycophospholipid labeled with [^{14}C]-retinol found by Barr and De Luca (3) is 33 mM ammonium acetate, which corresponds to our peak II. DMP in their work is eluted at 22 mM ammonium acetate, like our peak I. It is likely that in our extracts DMP is contained in peak I.

We should note that, even though an ostensible comparison is here made between MRP formation in "vitamin A-deficient" rats and that in a normal rat, the deficient rats were in fact no longer deficient; they had received the labeled retinol for a period just long enough for the weight curve to begin to rise (8). The comparison, therefore, is between depleted rats (without liver reserves of vitamin A) and a normal rat (with liver reserves).

From the partial purification of MRP from deficient rats injected with labeled retinol, one can make an approximate estimate, at least to an order of magnitude, of the amount of MRP present in rat liver. This estimate requires two assumptions: *a*) the amount of retinol found in rat liver at the plateau stage of deficiency is, very approximately, between 0.2 and 1.5 μg/g liver (wet weight)[3] (10); and *b*) the injected labeled retinol completely mixes with the existing pool of retinol in the liver. Were one to accept these assumptions, the total ^3H radioactivity in the chloroform–methanol 1:1 peak (Fig. 2*A*) should give a measure of the amount of MRP present. We estimated this quantity to be 1.8×10^{-13} mol/g liver (wet weight). (A correction was made for a 21% loss of MRP, which we determined to be in the water wash, occurring when the ammonium acetate was removed before applying the substance to silicic acid; see legend to Fig. 2.) It is of interest to compare this figure to the 9.6×10^{-11} mol/g intestine (wet weight) for retinyl phosphate, as determined by Frot-Coutaz et al. (4).

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