THE IN VIVO INCORPORATION OF MANNOSE, RETINOL AND MEVALONIC ACID INTO PHOSPHOLIPIDS OF HAMSTER LIVER

Robert M. Barr and Luigi M. De Luca

Differentiation Control Section
Lung Cancer Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

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SUMMARY

Hamsters were injected intraperitoneally with [^{14}C]mannose, [^{14}C]retinol and [^{3}H]mevalonic acid. The livers were removed, extracted with chloroform-methanol and the lipids chromatographed on DEAE-cellulose and silicic acid. The hamster liver lipid contained a component which could be labelled with mannose and mevalonic acid. The properties of this compound were in accord with it being dolichyl-mannosyl-phosphate, a possible lipid intermediate required for the biosynthesis of some glycoproteins. [^{14}C]Retinol and [^{14}C] mannose were incorporated into another phospholipid which was labile to mild alkali conditions commonly used for the preparation of dolichyl-mannosyl-phosphate. The retinol labelled compound had similar properties to *in vitro* prepared mannosyl-retinyl-phosphate.

INTRODUCTION

Biosynthesis of some polysaccharides in mammals requires lipid intermediates for transfer of glycose units from the sugar-nucleotide to polysaccharides or their precursors (1-3). Mannolipids (3-7), glucolipid (8), xylolipids (5, 9), galactolipid (10) can be made *in vitro*. Exogenously (6) supplied lipid increases the incorporation of monosaccharide into the lipid soluble material. In many instances, the lipid component of the glycolipid intermediate has not been positively identified although it has been shown to be a phospholipid. Because of its chromotagraphic properties, and in comparison

Abbreviations: MVA, Mevalonic Acid; DMP, dolichyl-mannosyl-phosphate; MRP, mannosyl-retinyl-phosphate; C:M 8:1, chloroform: methanol solution vol: vol ratio.

with bacterial systems involving lipid intermediates, the mammalian lipid acceptor was presumed to be dolichyl phosphate, an isoprenoid compound. The evidence for the in vivo occurrence of the glycophospholipids which can be made in vitro using cell-free preparations from the same tissues has been scant; dolichyl-mannosyl-phosphate has been isolated and fully characterized in liver (3, 11). It has been reported that there may be more than one phospholipid acceptor compound involved in the transfer of glycose residues. Retinol and ATP (5) or retinyl-phosphate (12) when incubated with GDP-[^{14}C]man and a particulate enzyme preparation from rat liver forms a retinyl-phosphoglycolipid. Brief reports on the in vivo labelling of a phospholipid by retinol in regenerating rat liver have appeared (7). The in vitro synthesis of retinyl-pyrophosphate by thyroid has also been reported (13). We report here the in vivo occurrence of retinol phosphoglycolipid and its distinction from dolichyl-mannosyl-phosphate.

MATERIALS AND METHODS

Syrian golden hamsters were used. Thirty day old vitamin A deficient hamsters were prepared by feeding a vitamin A deficient diet to the mother from birth of the experimental animals and weaning the animals onto deficient diet at 21 days. These animals were not completely deficient, they were still gaining weight, but vitamin A stores were nearly exhausted. [1-^{14}C]Mannose, sp. act 52.7 mCi/mmole, was obtained from New England Nuclear, Boston. [3R, 4S-4^{3}H + 3S, 4R-4^{3}H]mevalonic acid, sp. act 250 mCi/mmole and [carbinol-^{14}C] vitamin A sp. act. 13 mCi/mmole were purchased from Amersham-Searle, Chicago. The radioactive precursors were injected intraperitoneally into the animals: [^{14}C]mannose in saline (0.85% NaCl),[^{3}H]MVA in saline 0.01M phosphate buffer pH=7 and [^{14}C]vitamin A in dimethyl sulfoxide. Mannose was given 20-25 min before sacrifice, vitamin A 3.5 hrs prior to sacrifice and MVA in two equal doses at 21.5 and 12.5 hrs. The livers were removed, cooled in ice and weighed. The livers were homogenized in two volumes of medium A (14). The homogenate (1 vol) was extracted with chloroform: methanol 2:1 (5 vol). The chloroform

extract was washed with saline and taken to dryness for chromatography. Biosil A, 200-325 mesh, activated 1 hr at 100°C was used for silicic acid chromatography. Lipids were applied to the columns in C:M 8:1 solutions. DEAE-cellulose columns were used as previously described (5). TLC was on Merck Silica gel 60 F-254 plates; solvent $CHCl_3$:CH_3OH: H_2O, 60:25:4. Radioactivity was located by scraping off bands of silica gel using an Analabs Zonyl scraper and assayed in PPO-POPOP-Tuluene scintillant. All experiments were carried out in dim light.

RESULTS AND DISCUSSION

We have investigated the occurrence of mannolipids in hamster liver with particular reference being made to the identification of mannosyl-retinyl-phosphate and dolichyl-mannosyl-phosphate. Animals given [^{14}C]mannose were starved for 24 hrs prior to the experiment to reduce their hexose pool size. The incorporation of ^{14}C-mannose into total lipid was greatest between 15-30 minutes after intraperitoneal injection of the label. [^{14}C]Retinol was injected into a hamster with depleted vitamin reserves, but still growing. MVA, the precursor of dolichyl derivatives, was obtained specifically labelled so that the radioactivity was primarily incorporated into cis-isoprenoid compounds, the dolichols, rather than the all-trans isoprenoids which compose the bulk of the isoprenoid compounds. [^{14}C]Mannose labelled lipid was chromatographed on silicic acid (Fig. 1A) with C:M 8:1 and then a gradient. 75% of the radioactivity was eluted by C:M 8:1. The radioactivity eluted by the gradient was in one broad peak centering on C:M 3:1. Aliquots taken at different stages in the elution of this peak and chromatographed on plates showed that it was composed of several lipids. The [^{14}C]retinol labelled lipid, when chromatographed under the same conditions as the mannose labelled lipid, gave a major radioactive peak early in the gradient which trailed into the mannolipid area of the gradient (Fig. 1B). Thus, further purification was required to decide whether retinol was incorporated in the mannolipid. This was achieved by DEAE-cellulose chromatography. The majority of the retinol derived activity

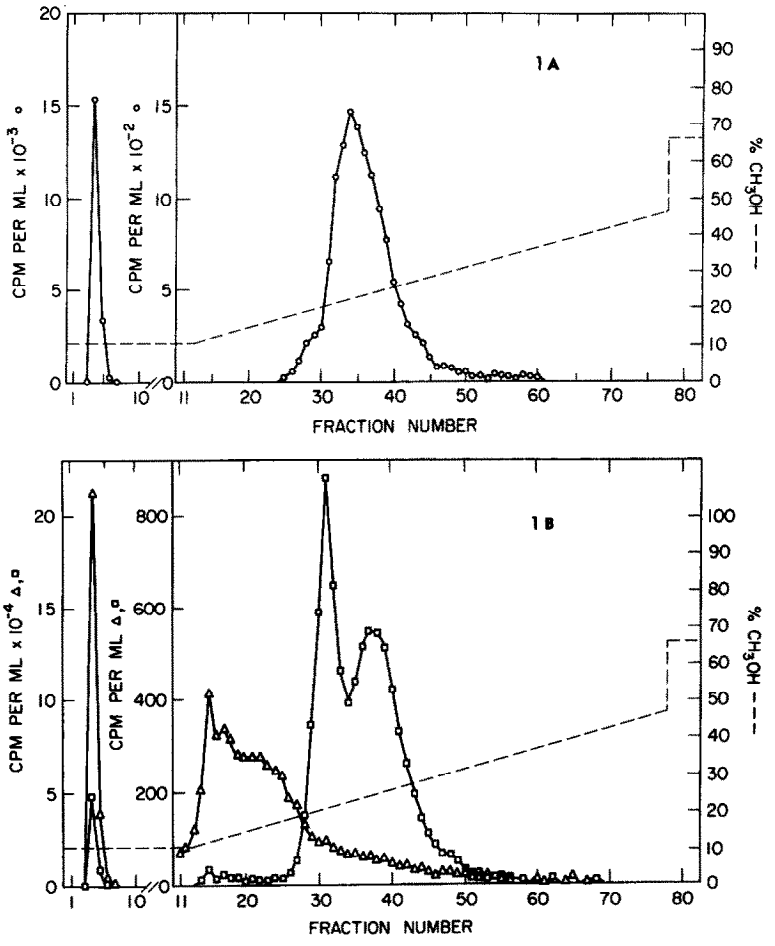

Fig. 1. Silicic acid chromatography of the lipid extracts.
The lipids extracted from the livers of two hamsters previously injected with the radioactive precursors were individually chromatographed on two columns of silicic acid. The columns, 12.5 x 250 mm, were eluted exactly in parallel using a single gradient maker and two pumps (LKB Instruments). Flow rate 65 ml per hour per column, 5.5-6.0 ml fractions collected. Fractions 1-15 eluted by C:M 8:1 fractions 16 - 78 by a gradient of C:M 8:1 - 1:1.
Fig. 1A - Chromatography of [1-^{14}C]mannose labelled lipid from a normal hamster liver. The animal was given 500 µCi [^{14}C]mannose 20 min prior to sacrifice. Radioactivity in the crude lipid extract was 5.65 x 10^5 DPM. Radioactivity eluted from the column by C:M 8:1 was 3.25 x 10^5 DPM, and during the gradient elution (C:M 4.9:1 - 1.8:1) 1.21 x 10^5 DPM total recovery of radioactivity of the column was 80%.
Fig. 1B - Chromatography of [^{14}C]retinol —Δ—, [^{3}H] MVA —□— labelled lipid from a vitamin A deficient hamster liver. The animal was given 50 µCi [^{14}C]retinol and 400 µCi [^{3}H] MVA. Radioactivity in the crude lipid was ^{14}C, 3.05 x 10^6 DPM; ^{3}H, 5.66 x 10^5 DPM. C:M 8:1 solvent eluted 3.19 x 10^6 DPM of ^{14}C and 2.18 x 10^5 DPM of ^{3}H activity. The gradient eluted 4.95 x 10^4 DPM ^{14}C and 1.36 x 10^5 DPM H. The total recovery of radioactivity of this column was ^{14}C 106%; ^{3}H 63%. Fractions 10-60 for each column were bulked and taken to dryness by flash evaporation. The residual lipid for each column was dissolved in 1 ml of C:M 2:1.

was eluted by C:M 8.1 as retinol. The ^{3}H activity, (derived from MVA and in the same lipid extract as the [^{14}C]retinol activity) was mainly eluted by C:M 8:1. Free and fatty acid esterified dolichol, are eluted in this fraction. Two peaks of ^{3}H activity were eluted by the gradient spanning the same region as the eluted mannose lipid. Chromatography of the lipids on equivalent columns of DEAE-cellulose removed a lot of activity (see Fig. 2 legend) in the 99% CH_3OH in each case. The phospholipids were eluted by the ammonium acetate gradient. The tritium activity from MVA was eluted ahead of the ^{14}C activity of retinol. The peak of the lipid labelled from the [^{14}C]mannose was between 0.03 - 0.045 M ammonium acetate, similar to the elution of *in vitro* prepared mannolipids and overlapped with both labelled retinol and MVA activities. The DEAE-cellulose fractions were bulked as described in the figure legend. Recovery of the MVA labelled lipid was 100% in the organic phase after extraction of the ammonium acetate but there was a 30% loss of [^{14}C]mannose derived activity and a 43% loss of [^{14}C]retinol derived activity, possibly indicating H_2O solubility of the retinol compound in the presence of ammonium acetate. Aliquots of the DEAE-cellulose purified phospholipids were chromatographed on TLC plates together with an authentic DMP marker, obtained from Dr. C. D. Warren of Massachusetts General Hospital. The marker was located after the plates had run by spraying with the annisaldehyde reagent (Fig. 3). The [^{14}C]retinol lipid separated into three peaks with the major part (about 65%) of the radioactivity running at Rf 0.20 similar to mannosyl-retinyl-phosphate prepared *in vitro*. The tritium activity from MVA ran with the marker DMP at Rf 0.3 (a lower Rf than the usual 0.35-0.45). The mannose derived activity gave a broad band centering at Rf 0.25 with a shoulder at Rf 0.20, as for [^{14}C]retinol. The leading edge of this band overlapped the DMP and the tailing edge the retinol compound.

DMP is stable to mild base treatment, whereas a mannosyl-retinyl-phosphate compound would be more labile, as shown by the complete breakdown of synthetic retinyl-phosphate under the same conditions. The DEAE-cellulose purified

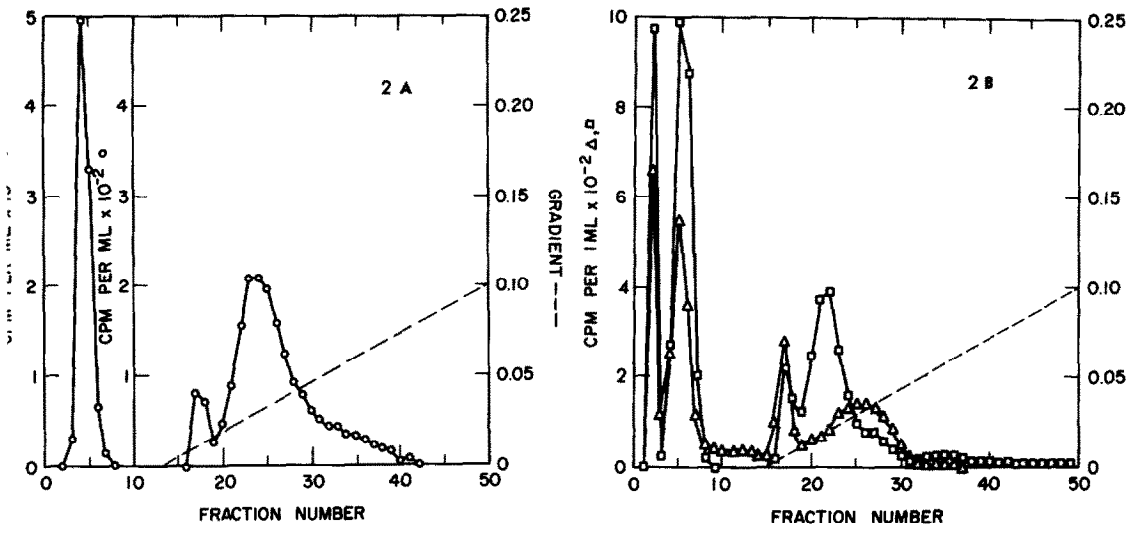

Fig. 2. Chromatography on DEAE-Cellulose

The [^{14}C]mannose labelled lipid and the [^{14}C]retinol [^{3}H] MVA labelled lipid eluted in fractions 10 - 60 from the respective silicic acid columns were chromatographed on two DEAE-cellulose columns in the acetate form. The two columns, 12.5 mm x 200 mm, were run in parallel using one gradient maker. The lipids were applied to the columns in C:M 2:1. The columns were then eluted with 99% CH_3OH to give fractions 1-13. A gradient of 0 - 0.1M ammonium acetate in 99% CH OH was then used to elute the phospholipids. Volume of each fraction 5.5 - 6.0 ml. Flow rate about 70 ml per hour per column.

Fig. 2A - [^{14}C]mannose labelled lipid. 1.13 x 10^5 DPM of lipid was applied to the column and 6.50 x 10^4 DPM were eluted by the 99% CH_3OH wash. 2.26 x 10^4 DPM were eluted by the gradient. Recovery 78%.

Fig. 2B - [^{14}C]retinol —Δ— [^{3}H] MVA —□— labelled lipid. The activity applied to column was ^{14}C, 3.63 x 10^4 DPM; ^{3}H 1.06 x 10^4 DPM. Activity eluted by the 99% CH_3OH wash was ^{14}C 1.40 x 10^4 DPM; ^{3}H 3.63 x 10^4 DPM. The ammonium acetate gradient eluted ^{14}C 2.69 x 10^4 DPM; ^{3}H 6.75 x 10^4. Recovery ^{14}C 114%, ^{3}H 98%.

Fractions 18-30 inclusive for each column were bulked and the ammonium acetate removed by extraction as follows: Bulked fractions (1 vol) were mixed with $CHCl_3$ (2 vol) and transferred to a separating funnel. Water (0.6 vol) was added and the contents mixed well. The phases were allowed to separate and the lower $CHCl_3$ phase taken. The solvent was removed by flash evaporation. Aliquots of both phases were assayed for radioactivity. Recovery of radioactivity in the organic phases: From [^{14}C]mannose lipid 70%; from [^{14}C]retinol lipid 57%; from [^{3}H] MVA lipid 100%.

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lipids were mildly saponified in 0.1N NaOH. The lipid was dissolved in 250 µl C:M 1:4 and 25 µl IN NaOH added. The mixture was incubated at 37°C for 10 min, cooled and neutralized with 1N acetic acid. The lipids were extracted by making the solution up to C:M 2:1 by addition of chloroform and methanol and 1/5 vol H_2O, mixing and centrifuging to separate the phases. Both the

organic and aqueous phases were assayed for radioactivity and the lipid re-run on TLC plates. The MVA labelled lipid remained in the organic phase and 80% of it still chromatographed with DMP, after saponification. Of the retinol lipid 40% became water soluble and that remaining in the organic phase mainly ran at the solvent front on TLC, as expected for free retinol. About 30% of the mannose labelling became water soluble thus indicating a linkage with phospholipids other than DMP. The mannose labelled lipid remaining in the organic phase gave two peaks of about equal activity on TLC, one at the front, the second at exactly the same place as DMP. Thus, only 30% of the [^{14}C]mannose activity was found as DMP, the remaining activity was labile to the alkaline conditions. If the saponified lipids were not extracted between H_2O and C:M but were chromatographed on small silicic acid columns and stepwise eluted (C:M 8:1 then C:M 1:1), the ^{3}H lipid from MVA was eluted in the 1:1 eluate and the ^{14}C mannolipids eluted partly in the C:M 8:1 eluate (the lipid which ran at the front on TLC) and in the 1:1 eluate. This was the lipid running with DMP on TLC. Mild acid hydrolysis of this latter lipid (0.01 N HCl in CH_3OH = H_2O 1:1 100°C 15 min) showed that it was acid labile. The activity became water soluble and when chromatographed on Whatman 3M paper using Butanol: Pyridine: H_2O 9:5:4 the radioactivity ran as mannose and methyl-mannoside. From these results it was concluded that the hamster liver contains dolichyl-mannosyl-phosphate. It had chromatographic properties on DEAE-cellulose, silicic acid and TLC comparable to synthetic DMP and a mannolipid which can be prepared *in vitro* from GDP-mannose. Its resistance to mild base but lability to mild acid reenforces this conclusion. [^{14}C]Retinol was incorporated *in vivo* into a compound with similar column and thin layer chromatographic properties to *in vitro* made mannosyl-retinyl-phosphate (5, 12). Interestingly this compound, which also contained [^{14}C]mannose derived activity, was labile to mild alkali as frequently used to prepare DMP. Thus we have established that a retinol (or metabolite of it) containing phospholipid is made by hamster liver *in vivo*. [^{14}C]Mannose was incorporated into a

were resolved by chromatography on carboxymethyl cellulose (18). Molecular weights of the α chains and cyanogen bromide cleavage products were estimated on a calibrated agarose molecular sieve column (18), and amino acid analyses were performed on an automatic amino acid analyzer employing a single-column procedure (18).

Analyses of the amount and nature of the carbohydrate prosthetic groups associated with the purified sturgeon α chains were performed as described previously (19).

RESULTS AND DISCUSSION. A carboxymethyl cellulose chromatogram illustrating the elution pattern of denatured sturgeon notochord or cartilage collagen is given in Figure 1. The collagen from both tissues was readily soluble in the

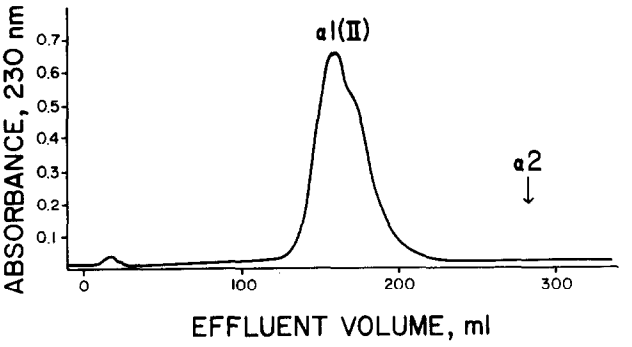

Figure 1: The carboxymethyl cellulose elution pattern of 75 mg of denatured sturgeon notochord or cartilage collagen. Chromatography was performed at 42° C on a 1.8 x 10 cm column. Elution was achieved at a flow rate of 100 ml/hr. in 0.04 M (Na^+) sodium acetate, pH 4.8, containing 1.0 M urea by employing a linear gradient from 0.0 to 0.12 M NaCl over a total volume of 400 ml.

starting buffer for chromatography, and essentially quantitative recovery of the material applied to the column was achieved. The chromatogram is characterized by a single somewhat asymmetric peak in the α1 chain position and the absence of material chromatographing in the region where α2 chains are eluted. These features are characteristic of a cartilage-type collagen from avian (2,3) and mammalian (4) sources. The protein eluted from carboxymethyl cellulose was

REFERENCES

1. Parodi, A.J., Behrens, N.H., Leloir, L.F., and Carminatti, H., (1972) Proc. Nat. Acad. Sci. 69, 3268-3272.
2. Maestri, N. and DeLuca, L., (1973) Biochem. Biophys. Res. Comm. 53, 1344-1349.
3. Baynes, J.W., Hsu, A.F., and Heath, E.C., (1973) J. Biol. Chem. 248, 5693-5704.
4. DeLuca, L., Rosso, G., and Wolf, G., (1970) Biochem. Biophys. Res. Comm. 41, 615-620.
5. DeLuca, L., Maestri, N., Rosso, G. and Wolf, G. (1973) J. Biol. Chem. 248, 641-648.
6. Richards, J.B. and Hemming, F.W., (1972) Biochem. J. 130 77-93.
7. Martin, H.G. and Thorne, K.J.I., (1974) Biochem. J. 138, 281-289.
8. Behrens, N.H. and Leloir, L.F., (1970) Proc. Nat. Acad. Sci. 66, 153-159.
9. Waechter, C.J., Lucas, J.J., and Lennarz, W.J., (1974) Biochem. Biophys. Res. Comm. 56, 343-350.
10. Helting, T. and Peterson, P.A., (1972) Biochem. Biophys. Res. Comm. 46, 133-138.
11. Evans, P.J. and Hemming, F.W., (1973)FEBS letters 31, 335-338.
12. Private Communication from Dr. G. Wolf. M. I. T., Massachusetts, USA.
13. Gaede, K. and Rodriguez, P., (1973) Biochem. Biophys. Res. Comm. 54, 76-81.
14. Littlefield, J.W. and Keller, E.B., (1957) J. Biol. Chem. 224, 13-30.