Phosphonolipid Metabolism in Control and Vitamin A Deficient Rats[1] (36499)

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We have reported that vitamin A deficiency results in an increased turnover of liver phospholipids (1, 2). This effect appears to be the result of an increased exchange of phospholipid moieties rather than *de novo* synthesis. We have suggested that this increased turnover might be the result of increased phospholipase activity produced by damaged lysosomes.

Since phospholipases attack only ester linkages in the phospholipid molecule, we thought it worthwhile to study the metabolism of an analogue of phospholipids, namely a phosphonolipid (3), which has carbon–phosphorus bond in place of a phosphate ester. This lipid might be expected to resist degradation by phospholipases. The phosphonolipid we have selected to study contains 2-aminoethylphosphonic acid (AEP), the naturally occurring analogue of phosphorylethanolamine (4). Organisms which do not synthesize AEP readily incorporate it into their tissue lipids (5–7).

This study deals with the incorporation of AEP into liver subcellular lipids of control and vitamin A-deficient rats and the decay rates of the phosphonolipids derived from subcellular fractions.

Materials and Methods. Animals. Disease-free, weanling, male albino rats of the Wistar strain were made vitamin A deficient. Pair-fed controls were also used (2). All animals were fasted 12 hr prior to sacrifice.

Preparation of labeled AEP. 1,2-(^{14}C)-aminoethylphosphonic acid was synthesized from 1,2-(^{14}C)-ethanolamine–HCl[2] (117 μCi/μmole) by the method of Kosolapoff (8) as described by Smith and Law (9). The material was purified by column chromatography (Dowex 50W - X8) and by thin-layer chromatography as employed by Smith and Law (9). Finally the AEP was recrystallized from ethanol to a constant radiospecific activity. The final product had a specific activity of 2 μCi/μmole. The radioactive AEP was dissolved in 0.9% saline, the pH was adjusted to 7.0 with 0.1 *N* NaOH and 0.5 ml (8 μCi/4 μmole) was injected intraperitoneally/rat.

Unlabeled AEP. Synthetic 2-aminoethylphosphonic acid obtained from Sigma Chem. Company, St. Louis, MO 63178.

Preparation of subcellular fractions. After the desired postinjection times, control and vitamin A deficient rats (300–400 g) were killed by a blow on the head followed by rapid decapitation. The subcellular fractions were prepared and characterized as previously described (2).

Lipid extraction. The extraction of lipid from tissues was carried out using a 2:1 (v/v) mixture of chloroform–methanol by the method of Folch, Lees and Sloane Stanley (10).

Lipid analysis. Phosphorus was measured by the method of Chen, Toribara and Warner (11). Thin-layer chromatography was carried out using silica gel G plates and the solvent system described by Thompson (12) for the separation of the phospholipids from

[1] The support of this work by U.S. Public Health Service Grant AM-11597 is gratefully acknowledged.

[2] Received from International Chemical and Nuclear Corporation, 2727 Campus Drive, Irvine, CA 92664.

TABLE I. Incorporation of Radioactivity into the Lipids of Control and Vitamin A-Deficient Rat Liver Subcellular Fractions After ^{14}C-AEP Intraperitoneal Administration (8 μCi/4 μmole/rat).

Time after injection (hr)	Sp act of lipids from subcellular fractions[a] (cpm/μg lipid-P)					
	Microsomes		Mitochondria		Soluble fraction	
	Control	Deficient	Control	Deficient	Control	Deficient
1	14	35	10	24	10	28
3	42	63	42	61	38	70
6	20	37	25	37	24	33

[a] Each value represents the pooled livers of three animals.

their structural analogues the phosphonolipids (*i.e.*, 2-aminoethylphosphonate phospholipid).

Lipid hydrolysis. A portion (5 mg) of the chloroform-soluble lipid extract was hydrolyzed with 15 ml of 6 *N* HCl at 110° for 24 hr. The hydrolysate was filtered and fractionated on a Dowex[3] 50W - X8, 50–100 mesh, hydrogen form, column 7.5 mm × 20 cm (20 g) and washed with 50 ml of distilled water. The column was eluted consecutively with 50 ml of 20% NH_4OH and 100 ml of 100% NH_4OH. Fractions (10 ml) were collected and the radioactivity was monitored by removing aliquots for scintillation counting and/or phosphorus determinations. By placing known quantities of labeled AEP and 1 mg of unlabeled AEP on the column it was found that more than 95% of the radioactivity added to the column could be recovered by combining the third, fourth, and fifth 10 ml fractions of the 20% NH_4OH eluent. These were the three fractions combined to determine the radioactivity of the AEP in subcellular lipid extracts obtained from animals injected with ^{14}C-AEP.

The identity of AEP in the above three fractions derived from the hydrolysate of liver subcellular lipids from rats given labeled AEP was established by paper chromatography (7), and by adding carrier AEP to radioactive samples and recrystallizing from ethanol to a constant radioactive specificity.

Preparation of samples for counting. Small aliquots of various types of samples were transferred to counting vials and counted in a scintillation counter as previously described (2). All samples had radioactivity above 100 cpm (corrected for quenching and background) and a counting error of less than 10%.

Results. The specific radioactivities of total lipid from liver subcellular fractions of control and deficient rats after the injection of ^{14}C-AEP are summarized in Table I. Note that the specific activity was higher in all subcellular fractions from deficient animals. The peak of activity was between 3 and 6 hr after injection. In general, the specific activities of the lipids from various subcellular fractions were approximately the same for each interval of time after injection.

Thin-layer chromatography (7) of the total lipid reported in Table I revealed that greater than 95% of the total lipid count of each fraction was recovered in an area just distal to phosphatidylethanolamine in an ascending chromatogram. According to Kennedy and Thompson (13) this is the approximate area where aminoethylphosphonate lipid (AEPL) is found. It was not possible to identify this area with iodine vapor because of the low concentration of AEPL.

Additional evidence that our radioactivity resided in the AEPL was by means of acid hydrolysis and Dowex chromatography. Our technique is described under the Methods section. In using these procedures we found better than 95% of the total lipid count in the combined third, fourth and fifth fractions (30 ml) of the 20% NH_4OH eluent. We have previously presented evidence that

[3] Bio-Rad Laboratories, 32nd and Griffin, Richmond, CA 94804.

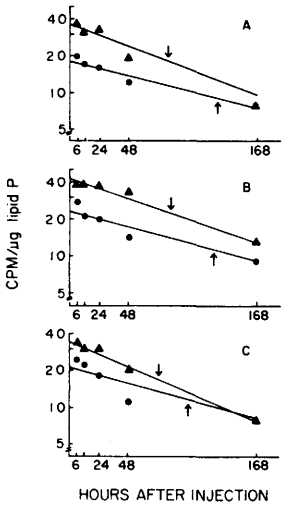

FIG. 1. Decay of specific radioactivity of total lipid obtained from isolated (A) microsomes, (B) mitochondria and (C) soluble fraction after a ^{14}C-aminoethylphosphonate injection. Each point represents the pooled livers of three animals. (Arrows) half-life times determined graphically. (●) subcellular fraction from control animals; (▲) subcellular fraction from deficient animals.

AEP is contained in these three fractions (see Methods).

Figure 1 illustrates the decay in specific radioactivity of lipid derived from subcellular fractions of control and vitamin A-deficient livers with time. The regression lines were drawn by the least squares method of the determined specific activity points. The first time point (6 hr after injection) is past the peak specific radioactivity determined for total lipid (Table I). Since all points measured are either on or very near the regression line we feel that these lines are suitable for calculating half-lives of the various labeled subcellular lipids.

The graphically determined half-lives for microsomal, mitochondrial and soluble fraction of AEP-labeled lipids from normal rats were 140, 136 and 100 respectivelv and 82, 80 and 80 hr, respectively, for deficient animals (Fig. 1). A statistical analysis of the six regression lines in Fig. 1 revealed that they are not significantly different ($p > .05$) (14). Therefore the half-lives for microsomal, mitochondrial and soluble fraction of AEP-labeled lipid were similar for control and vitamin A-deficient livers.

Discussion. Our findings support those of other investigators (5, 6) that organisms which do not synthesize AEP do incorporate this compound into their tissue lipids. It has been proposed that this synthesis of phosphonolipids occurs by the phosphonobases utilizing the same enzymes which transfer phosphobases from the cytidine phosphobase into phospholipid (*i.e.*, Kennedy pathway) (3). Microsomes appear to be the primary site for phosphonolipid synthesis (15). However, since the specific activity of the labeled phosphonolipids in this study was approximately the same for the three subcellular fractions at any specific time interval after injection it would appear that there is an exchange of phosphonolipids between the various subcellular fractions. The peak of radioactive incorporation for both control and deficient rats was prior to 6 hr after AEP administration and was higher in vitamin A-deficient rats. This later observation is similar to our previous finding that there is an accelerated incorporation of phospholipid precursors in the livers of vitamin A-deficient rats (2).

We have suggested that the increased turnover of liver phospholipids might be the result of increased phospholipase activity. Since phospholipase attacks only ester linkages we have indirectly studied its *in vivo* activity by measuring the half-lives of phospholipid from control and deficient livers labeled through ester linkages with various precursors (unpublished data) and by determining the half-life of phospholipids from control and deficient rats labeled with AEP which have carbon–phosphorus bonds in place of phosphate esters. As may be noted in Fig. 1 the half-lives of phosphonolipids from control and deficient animals were not statistically different. Similar results were found for phospholipids labeled with presursors through ester linkages. We feel that the similarity of half-lives for phospholipids and phosphonolipids from control and deficient rats do not support the thesis that vitamin A deficiency results in an increased phospholipase activity.

Since the life of the hepatocyte has been estimated to be between 160 and 400 days (16, 17) it would appear from our results that there is a significant turnover of AEP-lipid in the various subcellular fractions during the life of the cell and that the turnover of phosphonolipid in the different subcellular fractions are essentially similar. It is of interest that the half-life of AEP-lipid reported here is approximately one half as long as that reported by Bailey, Taylor, and Bartley (18) for one of the phospholipid components (^{32}P-labeled) of rat liver mitochondria. Also pertinent to our results are the findings of Omura, Siekevitz and Palade (19) who administered ^{14}C-acetate to rats, and reported two different half-lives for microsomal total phospholipid, one was 78 hr and the other 56 hr. In our study the AEP-lipid turnover corresponds best with the slower microsomal phospholipid component of Omura, Siekevitz, and Palade (19).

The active incorporation of AEP into liver lipids and their relatively long half-lives should be of particular nutritional significance in those instances where the dietary intake is high in marine or dairy products (possible sources of AEP) (3).

Summary. The rate of incorporation of labeled 2-aminoethylphosphonate (AEP) into liver lipids was similar for microsomes, mitochondria and soluble fraction. In vitamin A deficiency this rate was accelerated in all subcellular fractions. The peak of incorporation was between 3 and 6 hr.

The half-lives of lipid labeled with AEP from microsomes, mitochondria and soluble fractions were statistically similar (approx 4 days) for control and deficient rats.

The authors thank Dr. John M. Krall, Assistant Professor of Medicine, Public Health and Preventive Medicine, West Virginia University for his assistance in the statistical treatment of our data.

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Received Jan. 31, 1972. P.S.E.B.M., 1972, Vol. 140.