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Biosynthesis of 2–Aminoethylphosphonolipids in Fowl Liver Homogenates

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Synopsis

The incorporation of $^{14}$C-2-aminoethylphosphonic acid (2-AEP) into phospholipids of liver subcellular fractions were maximum in the unclei lipids, and similar for mitochondria and microsome.

Thin–layer chromatography was employed to demonstrate that $^{14}$C-2-AEP was incorporated in vitro into phospholipids of the fowl liver. $^{14}$C-2-AEP was incorporated into at least three phospholipids of the fowl liver. The formation of nucleotide–bound–2-AEP by liver homogenate was demonstrated. The rate of phosphonolipids formation from free–$^{14}$C–2–AEP was low compared to that from nucleotide–bound–2–AEP. The results indicate that nucleotide–bound–2–AEP is an intermediate in the synthesis of 2–aminoethylphosphonolipids.

Introduction

The phosphonic acid analog of phosphorethanolamine, taurine and $\beta$–alanine, 2–AEP (2-aminoethylphosphonic acid) occurs free and as major constituent of the phospholipid of protozoa$^{1–3}$, marine$^4$ and fresh water invertebrates$^5,6$ and mammalian tissues$^7–9$. Since the isolation of 2–AEP, many aminophosphonic acids structurally related to 2–AEP have been discovered in biological materials$^{10–12}$.

Studies on the incorporaton of 2–AEP and related compounds into animal tissues

Abbreviations:
2–AEP: 2–Aminoethylphosphonic acid

Key words:
2–aminoethylphosphonic acid;
2–aminoethylphosphonolipid;
cytidine–monophosphate–2–aminoethylphosphonate
were reported in several laboratories\textsuperscript{13-19}.

Kandatsu and coworkers\textsuperscript{13,14} found that \( ^{32}\text{P}-2\text{AEP} \) administered intraperitoneally was incorporated into rat liver lipids and insoluble residues to the extent of 3.3\% and 9.6\%, respectively.

Krause et al.\textsuperscript{17} reported that ciliatine was incorporated into lipids of rat liver, that the peak of incorporation was between 3 and 6 hr, and that the half-lives of labeled lipids were approximately 4 days. Curley and Henderson\textsuperscript{15} found that \(^{14}\text{C}-2\text{AEP} \) administered intravenously was incorporated into rat liver lipids (16\% of the total injected \( 2\text{AEP} \)).

Rosenthal and Pousoda\textsuperscript{20} reported that eight synthetic phosphonates containing analogs of lecithin, cephalin, and phosphatidic acid inhibited the hydrolysis by phospholipase C, the licithin analog were the most active. Dana and Douste–Blazy\textsuperscript{21} reported that \( 2\text{AEP} \) inhibites the utilisation of \(^{32}\text{P} \) for the synthesis of phosphatidic acids, phosphatidylethanolamines and phosphatidylcholines, and also observed that \( 2\text{AEP} \) inhibited decarboxylation of phosphatidylserine in the mitochondrial suspension of rat liver\textsuperscript{22}. Bjervel\textsuperscript{16} demonstrated a low incorporation of trimethylaminoethylphosphonic acid into phosphonolipids in a rat liver homogenate.

Liang and Rosenberg\textsuperscript{23} demonstrated the ability of a \textit{Tetrahymena} homogenate to form the complete lipid from diglyceride and cytidine monophosphate (CMP)–aminoethyphosphonate. We reported\textsuperscript{24,25} that phosphonolipids were biosynthetised from \( 2\text{AEP} \) in rat liver.

The present work aimed to confirm the incorporation of \(^{14}\text{C}-2\text{AEP} \) into fowl liver lipids and to investigate the possibility of its incorporation through a nucleotide–bound–intermediate (CMP– \( 2\text{AEP} \)).

Materials and Methods

\textit{Synthesis of \([ 1, 2-^{14}\text{C}] - 2\text{-aminomethylphosphonic acid}\)}

The procedure for the synthesis and purification of \([ 1, 2-^{14}\text{C}] - 2\text{AEP} \) was carried out by the method described by previous report\textsuperscript{25}.

\textit{Preparation of fowl liver homogenate}

A fowl weighing approx. 1 kg was killed by decapitation and liver immersed in cold 0.25M sucrose (pH 7.4) after perfusion by 0.9\% NaCl solution. 10\% homogenate was performed in Potter Elvenhjem homogenizer. Nuclei and cell debris were removed by centrifugation for 1,000 g x 10 min. The incubation medium contained ATP 5 x 10\(^{-3}\) M, CTP 5 x 10\(^{-4}\) M, MgCl\textsubscript{2} 5 x 10\(^{-3}\) M, tris–HCl buffer (pH 7.4) 5 x 10\(^{-3}\) M and homogenate corresponding to 0.5 g of liver in a total volume of 5.0 ml. In same experiments D–\(\alpha\)–diglyceride (5 x 10\(^{-3}\) M) was added in 10 mg of Tween 20. Incubations were performed at 37\(^\circ\)C in a shaking water bath. The reaction was stopped by the addition of 2.0 ml of 5\% trichloroacetic acid.
Proteins were removed by centrifugations and the supernatant was treated with 5.0 ml of a suspension of Charcoal (20 mg/ml)26).

After centrifugation the supernatant was sucked off, and the charcoal pellet washed 6 times with 15 ml of 0.9% NaCl.

Nucleotide-bound 2-2-AEP was eluted from the charcoal by the addition of 10 ml of formic acid. After centrifugation the supernatant was filtered and the charcoal washed 2 times with 10 ml of formic acid. The formic acid was evaporated to dryness and 1 ml of methanol was added. 0.2 ml of the nucleotides solution was transferred to a vial for counting. Subcellular fractionation of fowl liver homogenate was performed according to Vignais and Nachbaur27).

*Lipid extraction*

The extraction of lipids from proteins pellet was carried out using chloroform–methanol (2:1, v/v) and washed with 0.017% MgCl₂ as described by Folch et al28).

*Thin-layer chromatography*

For the separation of phospholipids and phosphonolipids on Silicagel plate (Merck, 20 x20cm, 2mm thickness) was performed using solvent systems:

1 : Chloroform : Methanol : Acetic acid : Water (75 : 45 : 12 : 6)29)

2 : Chloroform : Acetic acid : Methanol : Water (75 : 25 : 5 : 1.8)30)

3 : Chloroform : Methanol : Water (60 : 35 : 8)31)

4 : Chloroform : Methanol : Acetone : Acetic acid : Water (5 : 1 : 2 : 1 : 0.5)32)

Radioactive lipids were detected on developed plates with autoradiography. 2-AEP was detected on developed plates with Rosenberg's reagent33) and ninhydrine.

*Determination of phosphorus*

Determination of phosphorus was performed by the method of Chen et al34) after mineralization with HCIO₄ and H₂SO₄ mixture for 12 hours.

*Reagents*

All reagents used were either of analytical grade or of the highest purity. 2-AEP was prepared by the method described by Kosolapoff35).

**Results and Discussion**

*Incorporation in vitro of ¹⁴C-2-AEP into phospholipids of subcellular fractions of the fowl liver.*

Results of the incorporation of radioactivity into phospholipids of liver subcellular fractions of fowl after the incubation of ¹⁴C-2-AEP are summarized in Table I. These
results indicated that the specific radioactivity of phospholipids phosphorus was maximum in the nucleic lipid, and this activity was approximately 2 times greater than that of other fractions lipids except that there is no activity in supernatant. In the same experiment, radioactivity in fractions occurred at a higher percentage radioactivity in the nuclei and represented about 75% of the total phospholipids of liver. The radioactivity in mitochondria and microsome indicated the similar incorporation. Microsomes appear to be the primary site for phosphonolipid synthesis36).

Table 1. Incorporation of 14C-2-AEP (38690 dpm/µg p) into Phospholipids of Subcellular Fractions of the Fowl liver Fourty-eight Hours After an Incubation with Homogenate.

Subcellular fractionation of fowl liver homogenate was performed according to Vignais method27). The extraction of lipid from fractions was carried out using a chloroform: methanol by the method of Folch et al28).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total radioactivity (dpm)</th>
<th>total (%)</th>
<th>Specific activity (dpm/µg p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>358140</td>
<td>75.7</td>
<td>63</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>55890</td>
<td>11.8</td>
<td>30</td>
</tr>
<tr>
<td>Microsome</td>
<td>57780</td>
<td>12.3</td>
<td>36</td>
</tr>
<tr>
<td>Supernatant</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

However, since the specific activity of the labelled phosphonolipids in this study was approximately the same for the two subcellular fractions after incubation it would appear that there is an exchange of phosphonolipids between the various subcellular fractions. Our findings support those of other investigators7, 13) that organisms which do not synthesize 2-AEP do incorporate this compound into their tissues lipids. It has been proposed that this synthesis of phosphonolipids occurs by the phosphono base utilizing the same enzymes which transfer phosphobases from the cytidine phosphobase into phospholipid.

Figure 1 shows the distribution of radioactivity on the superimposed thin layer.

Fig. 1. Autoradiograms of 14C-2-AEP Incorporated into Phosphonolipids by Fowl Liver Homogenate.
Solvent systems: No. 3 (First) and No. 4 (Second)
chromatogram. A thin layer plate two-dimensionally developed with the two solvents revealed three $^{14}$C-2-AEP-containing substances. These results indicate that 2-AEP is incorporated into at least three lipids of the fowl liver.

Tamari et al.\textsuperscript{14} reported that the rat does not have the ability to decompose the C-P bond to phosphoric acid. Therefore, these observations indicate that the fowl can incorporate 2-AEP into liver phosphonolipids. But, it was not possible to identify this area with iodine vapor because of the low concentration of phosphonolipids.

**Incorporation of free $^{14}$C-2-AEP into lipids by fowl liver homogenate.**

Table II shows that fowl liver homogenates can incorporate $^{14}$C-2-AEP into phospholipids in vitro. The incorporation was very small, the maximal incorporation was $3 \times 10^{-3}$% at 60 min. of reaction. Bjerve\textsuperscript{16} reported that 3-trimethylaminopropylphosphonic acid was incorporated into phospholipids by rat liver homogenate in vitro, and the maximal incorporation was $2 \times 10^{-6}$% at 30 min. of reaction.

Table II. Incorporation of $^{14}$C-2-AEP into Phospholipids by Fowl Liver Homogenate.

A 10% fowl liver homogenate was prepared in 0.25 M sucrose. Nuclei and cell debris were removed by centrifugation for 1000g x 10 min. The incubation medium contained ATP: 5, $10^{-3}$ M; CTP: 5, $10^{-4}$ M; MgCl\textsubscript{2}: 5, $10^{-3}$ M; α, β-diglyceride: 5, $3 \times 10^{-3}$ M; Tween 20: 10 mg; Tris-HCl buffer (pH, 7.4): 5, $10^{-3}$ M; and homogenate corresponding to 0.5g of liver in a total volume of 5.0 ml. $^{14}$C-2-AEP (38690 dpm/µg P) was added as indicated.

Incubations were performed at 37°C in a shaking water bath. The phospholipids was extracted as described in methods.

<table>
<thead>
<tr>
<th>Incubation time(min)</th>
<th>Addition $^{14}$C-2-AEP (µg P/tube)</th>
<th>Total radioactivity (dpm)</th>
<th>Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>46</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>230</td>
<td>38</td>
<td>$4 \times 10^{-4}$</td>
</tr>
<tr>
<td>60</td>
<td>46</td>
<td>68</td>
<td>$3 \times 10^{-3}$</td>
</tr>
<tr>
<td>60</td>
<td>230</td>
<td>26</td>
<td>$4 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**Conversion of $^{14}$C-2-AEP to a charcoal adsorbable compound by fowl liver homogenate.**

The formation of nucleotide bound 2-AEP by liver homogenate was then investigated. Homogenates was prepared as described in the methods. Reaction mixtures were incubated for 15 min. and 60 min. at 37°C and precipitated by the addition of 2.0 ml of 5.0% trichloroacetic acid. The protein-free extracts were treated with charcoal as
Masato TAMARI

described above and the formation of charcoal-held radioactivity was assayed. The conversion of \( ^{14}\text{C}-2-\text{AEP} \) to a nucleotide derivative by fowl liver homogenate is shown in Table III.

When the \( ^{14}\text{C}-2-\text{AEP} \) was used as substrate, the formation rate was maximum for 15 min., 0.7% of nucleotide bound 2 -AEP were formed. These results indicate that nucleotide-bound phosphonate analogs, CMP-\( ^{14}\text{C}-2-\text{AEP} \) had been formed, although these compounds were not isolated, and these results indicate also that CMP-2 -AEP is an intermediate in the phosphonolipid synthesis.

Table III. Conversion of \( ^{14}\text{C}-2-\text{AEP} \) to a Charcoal-adsorbable Compound by Fowl Liver Homogenate.

The preparation of reaction medium and the incubations were performed as described in the Table II. The CMP-derivative was separated as described in the methods.

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Addition ( ^{14}\text{C}-2-\text{AEP} ) (( \mu\text{gP/tube} ))</th>
<th>Total radioactivity (dpm)</th>
<th>Specific activity (dpm/( \mu\text{g p} ))</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>46</td>
<td>6648</td>
<td>174</td>
<td>0.37</td>
</tr>
<tr>
<td>15</td>
<td>230</td>
<td>62675</td>
<td>1843</td>
<td>0.70</td>
</tr>
<tr>
<td>60</td>
<td>46</td>
<td>7296</td>
<td>38</td>
<td>0.40</td>
</tr>
<tr>
<td>60</td>
<td>230</td>
<td>26016</td>
<td>195</td>
<td>0.29</td>
</tr>
</tbody>
</table>

**Detection of nucleotide-bound 2 -AEP from the reaction mixture of fowl liver homogenate and \( ^{14}\text{C}-2-\text{AEP} \).**

The filtrate from the charcoal was suspended in 30ml of 6 N HCl, the suspension was refluxed at 120°C for 24 hrs and the HCl was removed under reduced pressure to dryness. The residue was dissolved in water and acid hydrolysate was chromatographed on Kieselgel G plate with solvent system of n-butanol:acetic acid:water (4 : 1 : 2, v/v). Thin-layer radioautogram showed that the spot contained a radioactive compound which was ninhydrine-and Rosenberg's reagent positive and migrated as 2 -AEP (Fig. 2).

Since exploratory experiments showed that free 2 -AEP was not absorbed on charcoal under the condition as described in methods, these results suggested the possibility of a nucleotide-bound 2 -AEP in the original extract.

**Incorporation of radioactivity from CMP-derivative into phospholipids by fowl liver homogenate.**

Table IV shows the incorporation of CMP-derivative into phospholipids by fowl liver homogenate. The rate of incorporation was small, the results was 0.32% and
0.14% at 2 hrs and 4 hrs of incubation, respectively. Our results indicate that the rate of phosphonolipids formation from CMP-derivative is high compared to that from $^{14}$C-2-AEP (see Table II).

From these results, there is possibility to synthesize phosphonocephalin from 2-AEP via the intermediate formation of CMP-2-AEP in animal liver.

These studies are of importance, in that 2-AEP is found in milligram amounts in common human foodstuffs such as edible shellfish and in smaller amounts in tissues of ruminants such as cattle and goats. Further studies on the in vivo and in vitro metabolism of this and related compounds are progress in our laboratory.

Fig. 2. Thin-layer Chromatogram of Water-soluble Phosphate Obtained from a Charcoal-adsorbable Compounds by Hydrolysis with 6 N hydrochloric acid for 24 hrs at 120°C.

Solvent system: n-butanol : acetic acid : water (4 : 1 : 2)

Sample 1: Authentic 2-AEP
2: Water-soluble phosphates obtained by hydrolysis
3: Standard phosphoric acid

Spot ○: Ninhydrine and Rosenberg's reagent positive
■: Radioautogram, ninhydrine and Rosenberg's reagent positive
○: Rosenberg's reagent positive
Table IV. Incorporation of Radioactivity from CMP-derivative into Phospholipids by Fowl Liver Homogenate.

The preparation of reaction medium and the incubations were performed as described in the Table II. CMP-derivative (71, 864 dpm) was added to incubation mixture.

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>Total radioactivity (dpm)</th>
<th>Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>234</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>98</td>
<td>0.14</td>
</tr>
</tbody>
</table>

References