In vitro Incorporation of Ciliatine (2-aminoethylphosphonic acid) into Lipids of Fowl Liver.

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ABSTRACT

1. The incorporation of ^{14}C-ciliatine into phospholipids of liver subcellular fractions were maximum in the nuclei lipids, and similar for mitochondria and microsome.

2. Thin-layer chromatography was employed to demonstrate that ^{14}C-ciliatine was incorporated in vitro into phospholipids of the fowl liver. ^{14}C-ciliatine was incorporated into at least three lipids of the fowl liver.

3. The formation of nucleotide-bound-ciliatine by liver homogenate was demonstrated.

4. The rate of phosphonolipids formation from free-^{14}C-ciliatine was low compared to that from nucleotide-bound-ciliatine.

5. The results indicate that nucleotide-bound-ciliatine is an intermediate in the synthesis of phosphonocephalin.

INTRODUCTION

The phosphonic acid analog of phosphorylethanolamine, taurine and β-alanine, ciliatine (2-aminoethylphosphonic acid) occurs free and as a 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 ciliatine, many aminophosphonic acids structurrally related to ciliatine have been discovered in biological materials[10-12].

Studies on the incorporation of ciliatine and related compounds into animal tissues were reported in several laboratories[13-19].

Kandatsu and coworkers[13, 14] found that ^{32}P-ciliatine administered intraperitoneally was incorporated into rat liver lipids and insoluble residues to the extent of 3.3% and 9.6%, respectively.

Krause et al.[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 et al.[15] found that ^{14}C-ciliatine administered intravenously was incorporated into rat liver lipids (16% of the total injected ciliatine).

Rosenthal et al.[20] reported that eight synthetic phosphonates containing analogs of lecithin, cephalin, and phosphatidic acid inhibited the hydrolysis by phospholipase C, the lecithin analog were the most active. Dana and Douste-Blazy[21] reported that ciliatine inhibites the utilisation of ^{32}P for the synthesis of phosphatidic acids, phosphatidylethanolamines and phosphatidylcholines, and also observed that ciliatine inhibited decarboxylation of phosphatidylserine in the mitochondrial suspension of rat liver[22]. Bjerve[16] demonstrated a low incorporation of trimethylaminoethylphosphonic acid into phosphonolipids in a rat liver homogenate. Liang and Rosenberg[23] demonstrated the ability of a *Tetrahymena* homogenate to form the complete lipid from diglyceride and cytidinemonophosphate (CMP)-aminoethylphosphonate.   We reported[24, 25] that phosphonolipids were biosynthetised from ciliatine via the intermediate formation of CMP-ciliatine in rat liver.

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

MATERIALS AND METHODS

(1) Synthesis of [1, 2-^{14}C]-Ciliatine

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

(2) Preparation of Fowl Liver Homogenate.

A fowl weighing approx. 1 kg was killed by decapitation and liver immersed in cold 0.25 M sucrose (pH 7.4) after perfusion by 0.9 % NaCl solution. 10 % homogenate was performed in Potter-Elvenhjem homogenizer. Nuclei andd cell debris were removed by centrifugation for 1,000 g×10 min. The incubation medium contained ATP 5×10^{-3}M, CTP 5×10^{-4}M, MgCl$_2$ 5×10^{-3}M, tris-HCl buffer (pH 7.4) 5×10^{-3}M and homogenate corresponding to 0.5 g of liver in a total volume of 5.0 ml. In same experiments D-α-diglyceride (5×10^{-3}M) was added in 10 mg of Tween 20. Incubations were performed at 37°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-ciliatine 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 method[27].

(3) Lipid Extraction.

The extraction of lipids from proteins pellet was carried out using chloroform-methanol (2:1, v/v) and washed with 0.017 % $MgCl_2$ as described by Folch et al[28].

(4) Thin-layer chromatography.

For the separation of phospholipids and phosphonolipids on Silicagel plate (Merck, 20×20 cm, 2 mm thickness) was performed using following 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. Ciliatine was detected on developed plates with Rosenberg's reagent[33] and ninhydrine.

(5) Determination of Phosphorus.

Determination of phosphorus was performed by the method of Chen et al[34] after mineralization with $HClO_4$ and H_2SO_4 mixture for 12 hours.

(6) Reagents.

All reagents used were eithers of analytical grade or of the highest purity. Ciliatine was prepared by the method described by Kosolapoff[35].

RESULTS AND DISCUSSION

(1) Incorporation in vitro of ^{14}C-Ciliatine 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 ^{14}C-ciliatine are summarized in Table 1. 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 radioactiv-

Table 1.   Incorporation of ^{14}C-Ciliatine (38690 dpm/μ_gP) 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 method. The extraction of lipid from fractions was carried out using a chloroform: methanol by the method of Folch et al.

Fraction	Total radioactivity (dpm)	total (%)	Specificactivity (dpm/μ_gp)
Nuclei	358140	75.7	63
Mitochondria	55890	11.8	30
Microsome	57780	12.3	36
Supernatant	—	—	—

ity 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 synthesis[36].

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 investigators[7, 13] that organisms which do not synthesize ciliatine do incorporate this compound into their tissues lipids. It has been proposed that this synthesis of phosphonolipids occurs by the phosphonobase utilizing the same enzymes which tranfer phosphobases from the cytidine phosphobase into phospholipid.

Figure 1 shows the distribution of radioactivity on the superimposed thin layer chromatogram. A thin layer plate two-dimensionally developed with the two solvents revealed three ^{14}C- ciliatine -containing substances. These results indicate that ciliatine is incorporated into at least three lipids of the fowl liver.

Tamari et al[14] reported that the rat does not have the ability do decompose the C-P bond to phosphric acid. Therefore, these observations indicate that the fowl can incorporate ciliatine into liver phosphonolipids. But, it was not possible to identify this area with iodine vapor because of the low concentration of phosphonolipids.
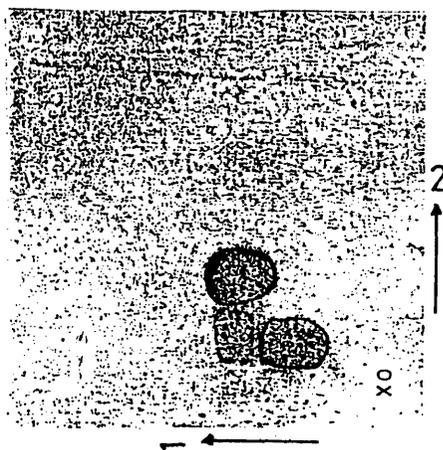

Fig. 1   Autoradiograms of ^{14}C-Ciliatine Incorporated into Phosphonolipids by Fowl Liver Homogenate. Solvent systems: No. 3 and No. 4

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

A 10 % fowl liver homogenate was prepared in 0.25M sucrose.
Nuclei and cell debris were removed by centrifugation for 1000
g×10 min. The incubation medium contained ATP: 5, 10^{-3}M;
CTP: 5, 10^{-4}M; MgCl$_2$: 5, 10^{-3}M; α, β-diglyceride: $5.3×10^{-3}$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-Ciliatine (38690 dpm/μgP) was added as indicated.
Incubations were performed at 37 °C in a shaking water bath.
The phospholipids was extracted as described in methods.

Incubation time (min)	Addition ^{14}C-ciliatine (μgP/tube)	Total radioactivity (dpm)	Incorporation (%)
15	46	0	0
15	230	38	$4×10^{-4}$
60	46	68	$3×10^{-3}$
60	230	26	$4×10^{-4}$

(2) Incorporation of Free ^{14}C-Ciliatine into Lipids by Fowl Liver Homogenate.

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

(3) Conversion of ^{14}C-Ciliatine to a Charcoal Adsorbable Compound by Fowl Liver
Homogenate.

The formation of nucleotide bound ciliatine by liver homogenate was then inves-
tigated. 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 described above and the formation of charcoal-held radioactivity was assayed. The
conversion of ^{14}C-ciliatine to a nucleotide derivative by fowl liver homogenate is
shown in Table 3.

When the ^{14}C-ciliatine was used as substrate, the formation rate was maximum
for 15 min., 0.7 % of nucleotide bound ciliatine were formed.

These results indicate that nucleotide-bound phosphonate analogs, CMP-^{14}C
-ciliatine had been formed, although these compounds were not isolated, and these
results indicate also that CMP-ciliatine is an intermediate in the phosphonolipid
synthesis.

Table 3.   Conversion of ^{14}C-Ciliatine to a Charcoal-adsorbable
Compound by Fowl Liver Homogenate.

The preparation of reaction medium and the incubations were
performed as described in the Table 2. The CMP-derivative was
separated as described in the methods.

Incubation time (min)	Addition ^{14}C-ciliatine (μgP/tube)	Total radioactivity (dpm)	Specific activity (dpm/μgP)	Conversion (%)
15	46	6648	174	0.37
15	230	62675	1843	0.70
60	46	7296	38	0.40
60	230	26016	195	0.29

(4)   Detection of Nucleotide-bound Ciliatine from the Reaction Mixture of Fowl Liver Homogenate and ^{14}C-Ciliatine.

The filtrate from the charcoal was suspended in 30 ml of 6N 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 Resenberg's reagent positive and migrated as ciliatine (Figure 2).

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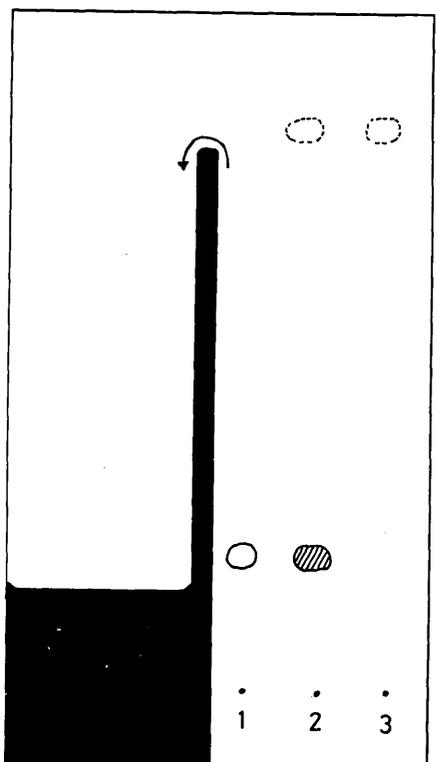

Fig. 2   Thin-layer Chromatogram of Water-soluble Phosphate Obtained from a Charcoal-adsorbable Compounds by Hydrolysis with 6N hydrochloric acid for 24 hrs at 120 °C.

Solvent system: n-butanol: acetic acid: water (4: 1: 2)
Sample 1: Authentic ciliatine
            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 4. 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 2. CMP-derivative (71864
dpm) was added to incubation mixture.

Incubation time (hr)	Total radioactivity (dpm)	Incorporation (%)
2	234	0.32
4	98	0.14

(5) Incorporation of Radioactivity from CMP-derivative into Phospholipids by Fowl
Liver Homogenate.

Table 4 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-ciliatine (see Table 2).

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

These studies are of importance, in that ciliatine 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.

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