Sci. Bull. Fac. Educ., Nagasaki Univ., No. 36, pp. 95~104 (1985)

Isolation and Identification of Ciliatine (2-aminoethylphosphonic acid) from Acid Soluble Fraction of Skim Milk Powder[1]

Masato TAMARI and Makoto KANDATSU*

Laboratory of Food and Nutrition, Faculty of Education,
Nagasaki University, Nagasaki 852, Japan
*Department of Agricultural Chemistry, Faculty of Agriculture,
The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
(Received Oct. 31, 1984)

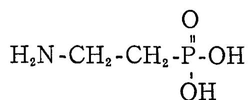
ABSTRACT

This investigation was carried out in order to demonstrate the occurrence of ciliatine in bovine milk powder. 10 mg of ciliatine was isolated from acid soluble fraction of skim milk powder (500 g) with a combination of ion exchange chromatographic techniques and was identified from the results of elementary analysis, infrared spectrum, melting point, chromatographic behaviors and ^{31}P-nuclear magnetic resonance spectrum.

The phosphonic acid was also detected in hydrolysates of chloroform-methanol soluble fraction (lipid fraction) and protein fraction of the whole milk powder. It has been demonstrated that the milk contains fairly high concentration of ciliatine. The results obtained here suggest that a man may ingest and absorb ciliatine through food chains like as dairy products.

INTRODUCTION

The first naturally occurring compound containing a carbon-phosphorus bond was 2-aminoethylphosphonic acid,



[1] This report was presented at the General Meeting of the Japanese Society of Nutrition and Food Science, held in Kumamoto, November 13, 1982.

(ciliatine), isolated by Horiguchi and Kandatsu[1] from tne lipid fraction of rumen ciliate protozoa. Since then this compound has been shown to be present either in the free state or in the bound to proteins or lipids in the lower and higher animals. Since the isolation of ciliatine, thirteen other aminophosphonic acids structurally related to ciliatine have been discovered in biological materials[2]. So for, nine phosphonate-containing lipids (termed phosphonolipid[3]) have been found as analogs of phosphatidylethanolamine, phosphatidylcholine and sphingoethanolamine from tissues of lower[4] and higher animals[5]. The distribution of the compound has been reviewed by Quin[6], and notable additional marine sources reported by Hori et al[7]. Quin[8] has also shown that ciliatine is incorporated into the insoluble protein of *Metridium dianthus*.

The biochemical role of ciliatine and the mechanism of its synthesis by living organisms are still unknown. Harkness[9] and Tamari et al[10] have shown that certain bacterial strains are able to utilize the phosphorus of ciliatine, as well as certain other aminoalkylphosphonic acids, for growth.

The ability to catabolize the carbon-phosphorus bond seems widespread among bacteria. It has also been shown that ciliatine added in a diet of rats[11] or injected intravenously into goats[12] was incorporated into the liver and other tissues of these animals. In view of the previously mentioned incorporation studies in animals, it is the quite possible for the widespread occurrence of ciliatine among mammalian animals. So far, there is still no detailed report on the isolation and crystalization of ciliatine from milk of ruminants and other animals, except a brief mention about the occurrence of ciliatine in the non protein fraction of skim milk powder by isolation in the 7 th International Congress of Biochemistry (1967)[24].

Therefore, our interest in mammalian metabolism of ciliatine has led to the present investigation on the detection and isolation of the ciliatine from milk powder which is well known as a suitable food for human.

MATERIALS AND METHODS

Chemicals : Ciliatine was prepared by the method described by Kosolapoff[13] and were purified by column chromatography and recrystallization. All reagents used were either of analytical grade or of the highest purity available.

Samples for analysis : Whole milk powder and skim milk powder were purchased from Morinaga Milk Industry Co., Ltd. and were stored at -20℃ until use.

Thin-layer and paper chromatography : The chromatographic separations were carried out either on thin-layer plate of Silica Gel F_{254} (Merck), 0.25 mm thick;

or on Whatman No. 3 paper (40 × 40 cm) by the ascending technique. The following solvent systems were used :

1) n-butanol : acetic acid : water (4 : 2 : 1, v/v)
2) Phenol : water (80 : 20, v/v)
3) 0.02 N acetic acid in 60% ehanol [14]
4) Isopropanol : acetic acid : 25% NH_4OH : water (5 : 2 : 4 : 3, v/v) [15]

Paper electrophoresis : Electrophoresis was carried out in a water-cooled (4–5℃) apparatus (Toyoroshi Co., Ltd., Type 11-13) under the following two conditions on Toyoroshi No. 514.

1) Voltage gradient of 40 volt per cm, for 30 min, in a buffer of a mixture of formic acid-acetic acid-water (2 : 8 : 56, v/v), pH 1.8. [16]
2) Voltage gradient of 40 volt per cm, for 30 min. in a buffer of a mixture of pyridine-acetic acid-water (10 : 1 : 89, v/v), pH 6.5. [16]

Ion-exchange resins : The resins employed were Dowex 50-X4 and Dowex 1-X8, 200 to 400 mesh, supplied by Dow Chemical Laboratories, U. S. A. Fine particles were removed by three cycles of settling and decanting. The cation-exchange resin (Dowex 50-X4) was purified by three cycles of treatment with 1 N NaOH and 1 N HCL, then washed with distilled water until free of chloride ion. The anion-exchange resin (Dowex 1-X 8) was converted into the acetate form by washing with 0.5% CH_3COONa until free of chloride ion, followed by batch treatment with 0.5 N acetic acid.

Reagents for detection : The ninhydrin reagent was 0.2% in acetone. The sprayed paper and thin-layer plate were placed, while still wet, in an oven at 95℃ for 2–3 min. This yielded an azure-blue spot with the phosphonic acid. The phosphonic group was detected with the reagent developed by Rosenberg [17] for orthophosphate and phosphate esters. This method utilizes a reduced vanadyl chloride solution in acetone for the reduction of the phosphomolybdate complex. Phosphonic acids yield transient green to blue green spots with this reagent.

Phosphorus analysis : Inorganic phosphate was estimated by the method of Chen et al [18], The organic phosphates were measured as inorganic phosphate after hydrolysis in 50 volumes of 6N HCL at 110℃ for 48 hrs; under these condition, the organophosphonic acids were resistant to hydrolysis. Total phosphorus was determined as inorganic phosphate after ashing of the samples with 6N H_2SO_4 for about 3 hr and with three drops of perchloric acid for an hour. Phosphorus in phosphonic acid (ciliatine) was determined as the difference between the total and the acid-labile phosphate.

Infrared spectra : Infrared spectra were determined on "Nujol mull" with a Model IR-S Infrared Spectrophotometer (Japan Spectroscopic Co., Ltd.).

Melting point : Melting point was determined with a melting point determinator, Melting Flash Type, preheated to 250℃.

The heating rate was 10-12℃ per min.

Detection of ciliatine by ^{31}P-NMR : The spectrometer was a JNM-FX 100 operating at 40.25 MHz for phosphorus with facilities for deuterium stabilization and fourier transform signal averaging. The ppm scale given was determined relative to an external standard of 85% orthophosphoric acid (zero ppm).

RESULTS

1. *Fractionation of ciliatine in whole milk powder.*

The sample (100 g) of the whole milk powder was stirred in 300 ml of acetone at room temperature by an efficient magetic stirrer. The suspension was filtered with a glass filter, and washed with 50 ml of acetone on the filter. The filtrate was evaporated to dryness under reduced pressure (acetone-soluble fraction). The acetone-insoluble residue was extracted four times with 600 ml of chloroform-methanol (2 : 1, v/v) for 3 hrs at 40℃. The four combined extracts were evaporated to dryness under reduced pressure (lipid fraction). The chloroform-methanol insoluble residue was suspended in 100 ml of 5% trichloroacetic acid (TCA) and the suspension was stirred at 30℃ for 2 hrs.

The suspension was then centrifuged for 10 min. at 14,000 rpm. The precipitate was re-extracted twice in the same conditions. The combined supernatant was evaporated to dryness under reduced pressure (TCA-soluble fraction). The residue was refluxed twice at 100℃ in 100 ml of 5% TCA for 15 min, and centrifuged for 15 min. at 4000 rpm, the supernatant discarded, and the residue was washed with 300 ml of ether, ethanol and hot ethanol successively to give a protein fraction.

A portion of the each fraction was hydrolysed with 6 N HCL as described in the experimental method. Water was added to the hydrolysate, and the mixture was extracted several times with ether. The aqueous layer was filtered, evaporated to dryness, and redissolved with a small amounts of water for phosphorus analysis. The results of ciliatine content of the acetone-soluble, lipid, TCA-soluble and protein fractions of the whole milk powder by phosphorus analysis are shown in Table 1.

The TCA-soluble fraction contained 69.1% of the ciliatine in the whole milk powder, and the amount of ciliatine of the TCA-soluble fraction was 18 and 2.5 times higher than in the lipid fraction and in the protein fraction, respectively.

Table 1. The Distribution of Ciliatine in Whole Milk Powder

Fraction	Ciliatine (mg) per 100 g WMP
Acetone-soluble fraction	0.04 (0.15)*
Lipid fraction	1.01 (3.87)
TCA-soluble fraction	18.04 (69.10)
Protein fraction	7.02 (26.88)

*( ), percent of ciliatine; WMP, whole milk powder

2. *Isolation and purification of ciliatione from the TCA-soluble fraction of the skim milk powder.*

Skim milk powder (500 g) was boiled in 2 liters of distilated water for 60 min. 3 liters of 10% TCA was added to the hot mixture, which was then agitated for 12 hrs at room temperature, and the mixture was centrifuged for 15 min. at 2300 rpm. The supernatant was evaporated to 1000 ml, the solution left for 12 hrs at 4℃, the precipitate discarded, and the supernatant was re-left twice in the same conditions. The three combined supernatants were extracted three times with 500 ml of ether to remove TCA, and the ether phase was discarded.

The aqueous phase was evaporated to 500 ml, adjusted to pH 6.0 by addition of 5% KOH, and evaporated to 250 ml. The solution was applied to a 5 × 80 cm column of Dowex 50-x4 (200-400 mesh, H^+form), and the column was washed with 500 ml of water. The column was then eluted with 8 liters of 0.3 N ammonia solution, and fractions (20 ml each) of the eluate were collected.

Tubes No. 257-323 contained the ninhydrin-positive and Rosenberg's reagent-positive material. The contents of these tubes were then pooled, evaporated to dryness, and redissolved with 100 ml of water. This solution was then applied to a 3 × 60 cm column of Dowex50-x 4 (200-400 mesh, H^+form), and the column was washed with 300 ml of water. The column was eluted with 2 liters of 0.3 N ammonia solution and each 8-ml fraction of the eluate was collected. Tubes No. 115-148 contained the ninhydrin-and Rosenberg's reagent-positive material. The contents of these tubes were then pooled and evaporated to dryness giving a pale brown solid which was redissolved with 50 ml of water.

This solution was then applied to a 1.5 × 60 cm column of Dowex 50-X4 (200-400 mesh, H^+form), and the column was washed with 100 ml of water. The column was then eluted with 1000 ml of 0.6 N hydrochloric acid, and each 10-ml fraction of the eluate was collected. Tubes No. 47-58 contained ninhydrin-positive and Rosenberg's reagent-positive material.

The contents of these tubes were then pooled, evaporated to dryness, giving

a pale brown solid and redissolved with 20 ml of water. This solution was decolorized with Norit, and evaporated to dryness. The dried matter was dissolved with 50 ml of water. This solution was then applied to a 0.9 × 50cm column of Dowex 50-X4 (200-400 mesh, H^{+}form) and the column was eluted with 300 ml of 0.3 N ammonia solution, and each 8-ml fraction of the eluate was collected. Tubes No. 18-22 contained ninhydrin-positive and Rosenberg's reagent-positive material.

The contents of these tubes were then pooled and evaporated to dryness. The dried matter was dissolved with 20 ml of water.

This solution was then applied to a 0.7 × 50cm column of Dowex 50-X 4 (200-400 mesh, H^{+}form) and the column was eluted with 250 ml of 0.6 N hydrochloric acid, and each 10-ml fraction of the eluate was collected. Tubes No. 8-10 was rich in phosphorus, and the phosphorus-containing substance was found to consist predominantly of ciliatine by paper-and thin layer-chromatography.

The contents of these tubes were pooled, evaporated to dryness, dissolved with 3 ml of 0.5 N acetic acid, and passed through a Dowex 1-X 8 (200-400 mesh, acetate form) column (2.5 × 30cm). The column was eluted with 200 ml of 0.5 N acetic acid, and fractions (10 ml) were collected. Tubes No. 5-6 was rich in phosphorus, and phosphorus-containing substance were found to consist predominantly of ciliatine by paper chromatography. The contents of these tubes were pooled, evaporated to dryness, dissolved in a small amount of 0.5 N acetic acid, and passed through a Dowex 1-X 8 (acetate form) column (0.9 × 2 cm). The column was eluted with 10 ml of 0.5 N acetic acid, and the eluate was evaporated to dryness. The residue was dissolved with 0.5 ml of water, and crystallization was induced by addition of ethanol to 50 % concentration. Recrystallization three times from the same solvent yielded about 10 mg of needle crystals which melted with decomposition at 280-281℃ (281-282℃, Kosolapoff [13]). Values of elementary analysis for the isolated material agreed closely with those calculated for ciliatine : found, C, 19.3; H, 6.2; N, 11.3; P, 24.9, calcd. for ciliatine, C, 19.2; H, 6.4; N, 11.2; P, 24.8.

3. *Identification of the isolated compound as ciliatine.*

Upon hydrolysis of a portion of the purified substance with 6 N hydrochloric acid at 120℃ for 48 hrs, in a sealed tube, neither change in the chromatographic behaviors of this substance nor liberation of inorganic phosphate was observed.

The stability of the compound to acid hydrolysis suggested the presence of a C-P bond. On comparison with synthetic compounds, the isolated compound behaved like ciliatine in paper chromatography developed with three different solvent systems, in thin-layer chromatography with two different solvent systems and in paper electrophoresis under two different conditions (Table 11).

Table 11. Paper (PC) and Thin-layer (TLC) Chromatographic and Paper Electrophoretic (PEP) properties of the Isolated Compound and Authentic Ciliatine

	Solvent systems						
	PC			TLC		PEP	
	1[c]	2	3	3	4	1	2
Isolated compound	0.25[a]	0.36	0.71	0.12	0.38	4.2[b]	-0.4
Authentic ciliatine	0.25	0.36	0.71	0.12	0.38	4.2	-0.4

a : Rf values on chromatogram
b : Migration distance (cm) toward anode with solvents.
c : Solvent compositions are given in the text.

On comparison with synthetic ciliatine, the melting points of the isolated material and the synthetic product were identical and there was no depression of the mixed melting point (280-281℃). The infrared spectra were virtually identical (Fig. 1). The spectrum showed an absorption band at 1180 cm^{-1} due to c-p bond, and was essentially identical with that of β-form crystal of ciliatine.

Fig. 2 shows the ^{31}P-NMR spectrum of orthophosphate phosphorus and phosphonate phosphorus (ciliatine). Spectrum A shows two absorption bands at 0 ppm and-18.2 ppm due to orthophosphate and authentic ciliatine, respectively.

This result show that there are the large differences in chemical shift between orthophosphate and phosphonate phosphorus.

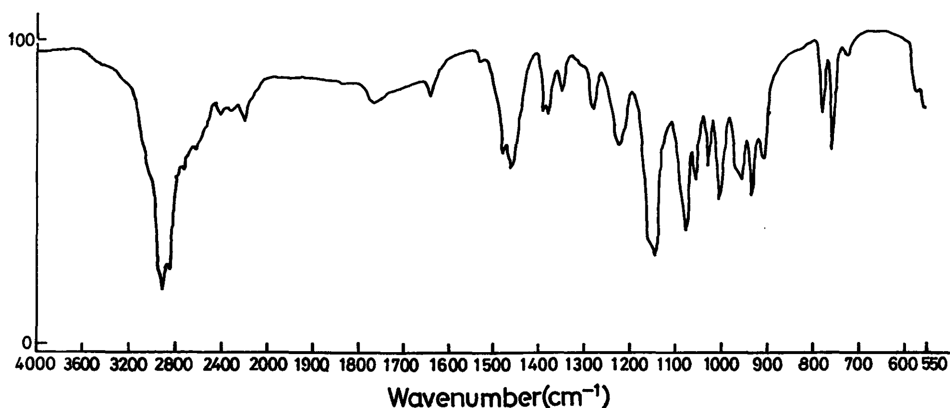


Fig. 1. Infrared Spectrum of Ciliatine Isolated from TCA-Soluble Extracts of the Skim Milk Powder with a Combination of Ion Exchange Chromatographic Technigues (Nujol Mull).

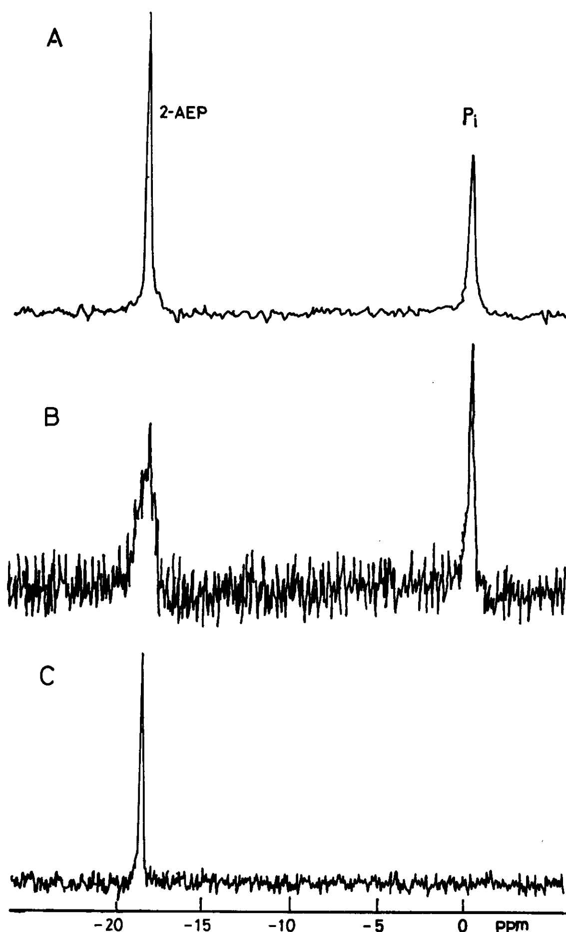


Fig. 2. The ^{31}P-NMR Spectra of TCA Soluble Fraction from the Skim Milk Powder.
The spectrometer was a JNM-FX 100 operating at 40.25 MHz for phosphorus with facilities for deuterium stabilization and fourier transform signal averaging.
The ppm scale given was determined relative to an external standard of 85% percent orthophosphoric acid (zero). Spectrum A was obtained from the marker ciliatine (−18.2 ppm) dissolved in 0.5 ml of 99.8 percent D_2O. Spectrum A : pulse number, 40; time constant, about 3 sec; total sweep width, 1000 hz; phosphorus concentration, 5 mg/0.5 ml total volume of 99.8% D_2O. Spectrum B was obtained from TCA soluble extracts of the skim milk powder. The sample was treated with ether to remove TCA. The sample was assayed in 20 percent D_2O and required 15 hours of accumulation time. Spectrum C was obtained from isolated material from TCA soluble extracts of the skim milk powder with a combination of ion exchange chromatographic techniques. The sample (about 1 mg) was assayed in 0.5 ml of 99.8 percent D_2O and required 2 hours of accumulation time.

Spectrum B shows the ^{31}P-NMR spectrum of the TCA-soluble fractions of the skim milk powder. The spectrum in phosphonate region appears to be essentially that of ciliatine.

Spectrum C showed that the chemical shift (-18.2 ppm) of the isolated compound was identical with that of the authentic ciliatine in the spectrum A.

DISCUSSION

Ciliatine is the first compound to be isolated from biological material ana has a covalent linkage between carbon and phosphorus (1). Since then, ciliatine and related alkylphosphonates have been detected in many invertebrates and some vertebrates species as components of lipids and as components of high-molecular-weight compounds (19).

Although lower animals can synthesize C-P compounds, mammals can not synthesize C-P compounds. From previous paper by Horiguchi et al[12, 20] dealing with the metabolism of ciliatine in goat, a possible occurrence of this compound in the bovine milk powder was suggested on the basis of the assumption that ciliatine-containing protozoa in the reticulo-rumen are digested and absorbed in the abomasum and the lower region of the alimentary tract in a manner comparable to that of the mono-gastric animals.

From the results obtained here, it has been demonstrated that the bovine milk powder contains fairly high concentration of ciliatine originating from rumen ciliate protozoa.

Recently the presence of ciliatine in the human tissues was reported[21, 22]. These results suggest that ciliatine originating from marine products may be found in many tissues of human, and that a man could ingest this compound through fishery products as well as dairy products[20].

On the other hand, so far, biological phosphonate phosphorus has been determined indirectly as the difference between total phosphorus and that released as orthophosphate upon prolonged acid hydrolysis. Recently there is a little reports on direct method for the determination and detection of biological phosphonate-phosphorus with ^{31}P-NMR[23, 24].

The present results show that the ^{31}P-NMR can be used for the detection of biological phosphate and phosphonate from the large differences in chemical shift between ester phosphorus and phosphonate phosphorus.

Acknowledgements

The authors wishes to express his thanks to Dr. A. Ōkubo of Department of Agricultural Chemistry of the University of Tokyo for his many valuable comments for recording the ^{31}P-NMR spectra. They are also grateful to Mr. M. Iwai for his helpful assistances to isolate ciliatine from skim milk powder.

REFERENCES

(1) M. Horiguchi and M. Kandatsu, Nature Lond., 184, 901 (1959)
(2) M. Tamari, Nippon Nogeikagaku Kaishi, 51, R 131 (1977)
(3) E. Baer and N. Z. Stanacev, J. Biol. Chem., 239, 3209 (1965)
(4) J. S. Kittredge and E. Robert, Science, 164, 37 (1969)
(5) S. Hasegawa, M. Tamari and M. Kametaka, J. Biochem., 80, 531 (1976)
(6) L. D. Quin, "In Topics in Phosphorus Chemistry", ed. by M. Grayson and E. J. Griffith, Interscience, New York, 4, 23 (1966)
(7) T. Hori, I. Arakawa and M. Sugita, J. Biochem., 62, 67 (1967)
(8) L. D. Quin, Biochemistry, 4, 324 (1965)
(9) D. R. Harkness, J. Bact., 92, 623 (1966)

(10) M. Tamari, M. Horiguchi and M. Kandatsu, J. Gen. Appl. Microbiol., 23, 49 (1977)
(11) M. Tamari, M. Horiguchi and M. Kandatsu, J. Agric. Chem. Soc. Japan, 45, 433 (1971)
(12) M. Kandatsu and M. Horiguchi, Agric. Biol. Chem., 29, 781 (1965)
(13) G. M. Kosolapoff, J. Am. Chem. Soc., 69, 2112 (1947)
(14) E. P. Kennedy, J. Biol. Chem., 22, 185 (1956)
(15) W. R. Snyder and J. H. Law, lipids, 5, 800 (1970)
(16) H. Shimizu, Y. Kakimoto, T. Nakajima, A. Kanazawa and I. Sano, Nature, 207, 1197 (1967)
(17) H. Rosenberg, J. Chromatog., 2, 487 (1959)
(18) P. S. Chen, T. Y. Toribara and H. Warner, Anal. Chem., 28, 1756 (1956)
(19) T. Hori and M. Horiguchi, C-P Kagobutsu No Seikagaku, Japan Scientific Societies Press, Tokyo.
(20) M. Kandatsu, M. Horiguchi and M. Tamari, (1967) Abstr. 7th Int. Congr. Biochem., Tokyo, 111, 449-450
(21) J. A. Alhadeff and G. D. Daves, Biochemistry, 9, 4866 (1970)
(22) J. A. Alhadeff and G. D. Daves, Biochim. Biophys. Acta, 244, 211 (1971)
(23) T. Glonek, T. O. Henderson, R. L. Hilderbrand and T. C. Myers, Science, 169, 192 (1970)
(24) M. V. Miceli, T. O. Henderson and T. C. Myers, Science, 209, 1245 (1980)