Determination of 2-Aminoethylphosphonic Acid by the Phenol-sodium Hypochlorite Method

By Haru KAMEYAMA and Shozaburo KITAOKA*

Shimane Women's Junior College, Matsue, Shimane

**Department of Agricultural Chemistry, College of Agriculture,
University of Osaka Prefecture, Sakai, Osaka*

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2-Aminoethylphosphonic acid (2-AEP) which occurs widely in various lower animals has been quantitatively analyzed either by the ninhydrin method or by the determination of phosphorus in the C-P bond.^{1,2)} De Koning,³⁾ however, reported that determination of 2-AEP by the reaction with ninhydrin gave a lower color yield relative to leucine on a molar basis, and that the color yield increased with the reaction time in the early stage, while as the reaction progressed the color was decomposed. From these results the ninhydrin method is not considered to be sufficiently sensitive, accurate and reproducible for the determination of 2-AEP. The method based on the determination of phosphorus in the C-P bond is not specific for 2-AEP, and such naturally occurring aminophosphonic acids as N-methyl-2-AEP and phosphonoalanine are inevitably included in the results. 2-AEP was recently isolated from human brain by gas-liquid chromatography and characterized by mass spectrometry.⁴⁾ But this method is somewhat troublesome, because it involves acylation and esterification.

Kitaoka and Nakano⁵⁾ have reported that 2-AEP gives a strongly positive color with the phenol and sodium hypochlorite reagents. The present paper reports experiments for the determination of 2-AEP by this method and its application to Shizimi (corb shell) lipid.

2-AEP was synthesized by the method of Kosolapoff,⁶⁾ except nitrogen was bubbled into a reaction mixture of N-(2-bromoethyl)-phthalimide and triethylphosphite⁷⁾ and purification was carried out by the use of an ion exchange resin (Amberlite IR-120, H⁺). N-Methyl derivatives of 2-AEP and phosphonoalanine were gifts from Dr. Tamari, University of Tokyo. Phenol (6%), sodium hypochlorite reagent (available chlorine 7.5%) and borate buffer (0.2 M, pH 9.0) used for the color reaction were prepared according to Kitaoka and Nakano.⁵⁾

To 0.1 ml of a 2-AEP solution in a test tube were added 0.2 ml of borate buffer and then 1.0 ml of the phenol reagent. After mixing thoroughly and cooling in ice water, 0.4 ml of the sodium hypochlorite reagent was added. The tube was shaken vigorously in ice water and placed in a boiling water bath for 10 min. It was then immediately cooled by placing in

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FIG. 1. Beer's Plots for the Reaction of 2-AEP with Phenol-hypochlorite Reagents.

ice water for 5 min. After adding 2.0 ml of 60% ethanol to the reaction mixture, the optical density at 630 mμ was measured with a Shimadzu-Baush and Lomb Spectronic 20 photometer. Plotting optical densities at different concentrations of 2-AEP gave a straight line in the range of 1~80 μg of 2-AEP as shown in Fig. 1.

With sample solutions containing 50 μg each of 2-AEP and some of its related compounds, colors were developed by the present method. The optical density at 630 mμ of 2-AEP was 0.5502, but N-methyl-, N-dimethyl- and N-trimethyl-2-AEP and phosphonoalanine were negative in this color reaction. The molecular extinction coefficients of 2-AEP, β-alanine and γ-aminobutyric acid in the present method

TABLE I. MOLECULAR EXTINCTION COEFFICIENTS OF 2-AEP, β-ALANINE AND γ-AMINOBTUTYRIC ACID

	Compounds		
	2-AEP	β-Ala ^{a)}	γ-ABA ^{b)}
Phenol-hypochlorite method (ϵ_{630})	5200	3440	4780
Ninhydrin method (ϵ_{570})	340	5160	17840

^{a)} β-Alanine, ^{b)} γ-Aminobutyric acid.

(ϵ_{630}) as well as in the ninhydrin method (ϵ_{570}) are shown in Table I. 2-AEP gave the ϵ_{630} value of 5200, but the ϵ_{570} was only 340. These results clearly show the high specificity and sensitivity of the phenol-sodium hypochlorite method in the determination of 2-AEP.

To test applicability of this method, 2-AEP in the lipid of Shizimi (carb shell), collected from Lake Shinzi, Matsue City, was determined and the result was compared with that by the determination of phosphorus in the C-P bond.

TABLE II. SEPARATION OF 2-AEP BY COLUMN CHROMATOGRAPHY

Fifty mg of a test sample was applied to a column and eluted with water. Recoveries of each component from the eluate were examined. 2-AEP, ethanolamine, serine, and choline were determined by the present method, ninhydrin or Glick's method.^{a)}

Column No.	Applied sample	Recovery
1	2-AEP	100%
2	ethanolamine	0
3	serine	0
4	choline	0
5	2-AEP	99
	ethanolamine	0
	serine	0
	choline	0

^{a)} D. Glick, "Methods of Biochemical Analysis," Vol. 1, Interscience Publishers, New York, 1954, p. 274.

Probable interference⁵⁾ by ethanolamine, serine and choline, the common constituents of naturally occurring lipids, in the determination of 2-AEP by the present method was eliminated by treatment with an ion exchange resin (Amberlite IR-120) column. Thus, as shown in Table II, when 2-AEP, ethanolamine, serine and choline, separately and in a mixture, were applied onto a column of the resin and eluted with water, only 2-AEP was recovered quantitatively in the eluate while none of the other compounds was detected in the eluate.

Lipid (1.0 g) was extracted with chloroform-methanol (2:1) from acetone powder of the edible part (73 g, wet wt.) of the shell-fish and hydrolyzed by refluxing with 0.2 N methanolic hydrochloric acid for 6 hr. The lipophilic phase was removed from the methanolysate by shaking with petroleum ether and the methanolic phase was concentrated. The residue was again hydrolyzed with 6 N hydrochloric acid for 24 hr at boiling point and the hydrolysate was filtered with a little charcoal and the filtrate was concentrated to a small amount. The residue was made up to 25 ml with water and the resultant solution was used as a sample solution for the determination of 2-AEP.

Five milliliters of the sample solution was applied onto a column (7×80 mm) of Amberlite IR-120 resin (H^+) which was eluted with 100 ml of water. The eluate was concentrated to a small amount and made up to 10 ml with water. 2-AEP contained in the eluate was determined by the present method. Thin layer and paper chromatographies of the eluate revealed the presence of 2-AEP in a sole spot positive with ninhydrin. For the determination of phosphorus of 2-AEP, 0.2 ml of the sample solution (containing about 80 μ g of 2-AEP) was passed through a column (7×55 mm) of Dowex 50 (H^+) resin to remove acid-labile phosphorus and washed with 5 ml of water. 2-AEP absorbed on the column was then

eluted with 5 ml of 1.2 N sulfuric acid, and after adding a few drops of perchloric acid the eluate was digested and the inorganic phosphorus released was determined according to the method of Chen *et al.*⁸⁾ The result was converted to the value of 2-AEP.

TABLE III. DETERMINATION OF 2-AEP IN SHIZIMI LIPID

	mg/1.0 g lipid	μ mole/1.0 g lipid
Phenol-hypochlorite method	10.99	87.90
Phosphorus method	10.77	86.09

The results of the determination of 2-AEP by both methods are shown in Table III. The amount of 2-AEP in 1 g of Shizimi lipid as obtained by the phenol-hypochlorite method was 87.90 μ moles which is in agreement with the value of 86.09 μ moles obtained by the determination of phosphorous in the C-P bond. From these results it is concluded that the colorimetric method by the phenol-sodium hypochlorite is convenient, specific and sensitive for the determination of 2-AEP, although it is necessary to carry out the pretreatment for removing interfering compounds affecting on the color reaction when applied to biological samples.

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