Biemann, K., Bommer, P., and Desiderio, D. M. (1964), *Tetrahedron Lett.*, 1725.
Burlingame, A. L. (1968), *Advan. Mass Spectrom. 4*, 15.
Capella, P., and Zorzut, C. M. (1968), *Anal. Chem. 40*, 1458.
Chorney, W., Scully, N. J., Crespi, H. L., and Katz, J. J., (1960), *Biochim. Biophys. Acta 37*, 280.
Crespi, H. L., Archer, S. M., and Katz, J. J. (1959), *Nature (London) 184*, 729.
Dinh-Nguyên, N., and Stenhagen, E., (1966), *Acta Chem. Scand. 20*, 1423.
Eglinton, G., Hunneman, D. H., and McCormick, A. (1968), *Org. Mass Spectrom. 1*, 593.
Hallgren, B., Ryhage, R., and Stenhagen, E. (1959), *Acta Chem. Scand. 13*, 845.
Holman, R. T., and Rahm, J. J. (1966), *Progr. Chem. Fats Other Lipids 9*, 79.
Hulanicka, D., Erwin, J., and Bloch, K. (1964), *J. Biol. Chem. 239*, 2778.
Klenk, E., and Knipprath, W. (1959), *Hoppe-Seyler's Z. Physiol. Chem. 317*, 243.
McCloskey, J. A. (1970), *in* Topics in Lipid Chemistry, Vol. 1, Gunstone, F. D., Ed., London, Logos Press, p 375.
McCloskey, J. A., and Law, J. H. (1967), *Lipids 2*, 225.
McCloskey, J. A., Lawson, A. M., and Leemans, F. A. J. M. (1967), *Chem. Commun.*, 285.
McCloskey, J. A., and McClelland, M. J. (1965), *J. Amer. Chem. Soc. 87*, 5090.
Rokop, S., Gajda, L., Parmerter, S., Crespi, H. L., and Katz, J. J. (1969), *Biochim. Biophys. Acta 191*, 707.
Ryhage, R., and Stenhagen, E. (1959), *Ark. Kemi 13*, 523.
Ryhage, R., and Stenhagen, E. (1960a), *Ark. Kemi 15*, 291.
Ryhage, R., and Stenhagen, E. (1960b), *Ark. Kemi 15*, 333.
Ryhage, R., and Stenhagen, E. (1963), *in* Mass Spectrometry of Organic Ions, McLafferty, F. W., Ed., New York, N. Y., Academic, p 399.
Saur, W. K., Crespi, H. L., Halevi, E. A., and Katzl J. J. (1968), *Biochemistry 7*, 3529.
Sen Gupta, A. K., and Peters, H. (1966), *Fette Seifen Anstrichm. 68*, 349.
Spiteller, G., Spiteller-Friedmann, M., and Houriet, R. (1966), *Monatsh. Chem. 97*, 121.
Sweeley, C. C., Elliott, W. H., Fries, I., and Ryhage, R. (1966), *Anal. Chem. 38*, 1549.
Thompson, J. F. (1963), Biological Effects of Deuterium, New York, N. Y., Pergamon.
Waller, G. R., Sartry, S. D., and Kinneberg, K. (1969), *J. Chromatogr. Sci. 7*, 577.
Williams, A. J., Morse, A. T., and Stuart, R. S. (1966), *Can. J. Microbiol. 12*, 1167.
Wolff, R. E., Wolff, G., and McCloskey, J. A. (1966), *Tetrahedron 22*, 3093.

Occurrence of 2-Aminoethylphosphonic Acid in Human Brain*

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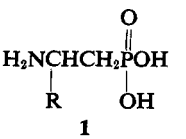
ABSTRACT: The occurrence of 2-aminoethylphosphonic acid (AEP) in acid hydrolysates of nonpolar lipid (hexane soluble) and proteinaceous residue (hexane–methanol insoluble) fractions of human brain tissue has been unambiguously established. This was accomplished using a new, highly sensitive technique for analysis of AEP in biological materials. This method involves acylation and esterification of hydrolysates of tissue extracts, detection and isolation by gas–liquid chromatography, and unambiguous characterization by mass spectrometry.

The presence of 2-aminoethylphosphonic acid (**1**, R = H) in hydrolysates of human brain has been established using a new, highly sensitive and unambiguous technique. The occurrence in human brain of AEP[1] which possesses the biologically unusual carbon–phosphorus bond, suggests that it and perhaps other, closely related, naturally occurring phosphonic acids (Kittredge and Roberts, 1969; Quin, 1967), may have important, but as yet undiscovered, functions in human biochemistry.

Following its initial isolation from ciliated protozoa of sheep rumen (Horiguchi and Kandatsu, 1959), AEP and four closely related compounds have been isolated from a variety of natural sources—principally marine invertebrates and microorganisms (Kittredge and Roberts, 1969; Quin, 1967). Surprisingly, no systematic study of the occurrence of aminoethylphosphonic acids in higher animals has been made, although the presence of AEP in goat liver (Kandatsu and Horiguchi, 1965) and bovine brain (Shimizu *et al.*, 1965) and of trimethylaminoethylphosphonate (**2**) in human aorta (Bishop, 1968) has been reported.



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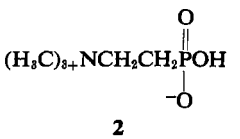

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[1] Abbreviation used is: AEP, 2-aminoethylphosphonic acid.

Experimental Section

General Comments. Infrared spectra were determined with a Perkin-Elmer 337 grating spectrophotometer. Nuclear magnetic resonance spectra were determined with a Varian HA-100 spectrometer. Mass spectra were obtained with a CEC Model 21-110B mass spectrometer at 100° probe temperature and 70 eV. Amino acid analyses were done on a Beckman 120C Analyzer using the method of Moore *et al.* (1958). Melting points were taken on a microscope hot stage and are uncorrected. Gas–liquid chromatography was carried out using a F & M Model 810 gas chromatograph with a flame ionization detector. A prepoured glass column (6 ft × 0.25 in.) with 10% GE SE30 on a A-W Chromosorb G (Applied Science) was used under the following conditions: H_2 pressure of 18 psig; air pressure of 20 psig; N_2 flow rate of 60 cc/min; injector temperature of 220°; flame ionization detector temperature of 315°. Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Ion-exchange chromatography was accomplished using a 2 × 27 cm column of Dowex 1-X8, 200–400 mesh, acetate form.

2-Acetamidoethylphosphonic Acid (**3**). To 42.1 mg of 2-aminoethylphosphonic acid[2] (Kosolapoff, 1947) was added 8 ml of acetic acid–acetic anhydride (1:1, v/v). The resulting mixture was stirred at room temperature until complete solution was obtained (approximately 2 hr). The solution was then filtered and the volatile reagents were removed. The resulting residue was crystallized from methanol to yield 31.6 mg (75%) of 2-acetamidoethylphosphonic acid (**3**); mp 172–180°; nuclear magnetic resonance (given in τ values throughout) spectra (Me_2SO-d_6), 2.19, broad (NH); 6.82, multiplet (N-CH_2); 8.24, singlet ($CH_3C{=}O$); 8.32, multiplet (P-CH_2); infrared spectrum (KBr) 1590 cm^{-1} (C=O).

Anal. Calcd for $C_4H_{10}NO_4P \cdot 0.5H_2O$: C, 27.3; H, 6.26; N, 7.96. Found: C, 27.5; H, 6.27; N, 7.90.

Dimethyl-2-acetamidoethylphosphonate (**4**). To a solution of 30.0 mg of 2-acetamidoethylphosphonic acid (**3**) in 5 ml of methanol was added excess diazomethane in ether. The solvents were evaporated using a stream of nitrogen to yield a viscous liquid, 25.8 mg (86%) of dimethyl-2-acetamidoethylphosphonate (**4**). The nuclear magnetic resonance spectra showed ($CDCl_3$), 3.44, broad (NH); 6.24, doublet (6 H, OCH_3); 6.50, multiplet (N-CH_2); 7.98 multiplet (P-CH_2); 8.20, singlet ($CH_3C{=}O$); infrared spectrum (smear), 1655 cm^{-1} (C=O); mass spectrum: see Figure 1.

Anal. Calcd for $C_6H_{14}NO_4P$: C, 36.9; H, 7.19; N, 7.18. Found: C, 36.9; H, 7.37; N, 7.08.

Model Studies. A mixture of 2.0 mg of AEP and 30.0 mg of each of 5 amino acids (Glu, Val, Phe, Thr, and Asp) in 6 ml of acetic acid–acetic anhydride (1:1) was stirred for 3 hr. The solvent was evaporated, the residue was dissolved in 50 ml of methanol and a 5-ml aliquot was treated with excess diazomethane in ether. The resulting mixture was evaporated, the residue was dissolved in 1 ml of chloroform and subjected to gas–liquid chromatographic analysis using the gas–liquid chromatographic column and conditions described under General Comments. The AEP derivative, **4**, was readily separated from the amino acid derivatives at isothermal column temperatures of 170, 180, or 200° exhibiting retention times of 9.6, 7.4, and 4.4 min, respectively (see Figure 1A). Various programmed column temperature regimes were equally effective or, in some cases superior. Comparison of these gas–liquid chromatographic results with those obtained using standard solutions of **4** indicated that essentially quantitative conversion of AEP to the volatile derivative **4** had been achieved.

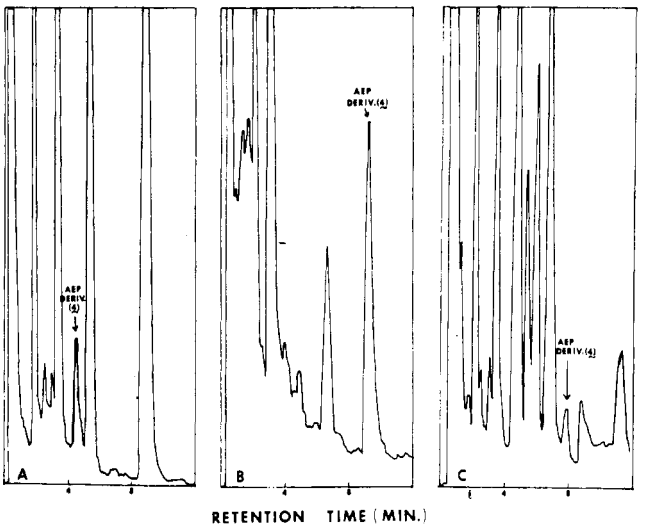


FIGURE 1: Typical gas–liquid chromatograms of complex mixtures showing separation of AEP derivative **4** obtained using instrumentation and conditions as described (general comments). (A) Mixture of derivatized AEP and amino acids (see model studies); column 200° isothermal; AEP derivative retention time = 4.4 min. (B) Derivatized hydrolysate of nonpolar lipid (hexane soluble) fraction; column 170° isothermal; AEP derivative retention time = 9.6 min. (C) Derivatized hydrolysate of proteinaceous residue (hexane–methanol insoluble) fraction; column 180° isothermal; AEP derivative retention time = 7.4 min.

Fractionation of Brain Tissue. An adult human brain[3] was homogenized in a Waring blender and subsequently lyophilized. The lyophilized tissue (36.7 g) was extracted with 3.5 l. of redistilled hexane by shaking mechanically overnight. The hexane extract was removed, dried over sodium sulfate, and the solvent was removed to yield 12.5 g of residue (nonpolar lipids). The hexane-insoluble residue was extracted using 3 l. of methanol for 12 hr. The methanol extract was separated from the hexane–methanol-insoluble material (14.3 g of proteinaceous residue). Removal of solvent from the methanol extract produced 7.8 g of polar lipids.

Analysis of Nonpolar Lipid (Hexane Soluble) Fraction for AEP. A portion (8 g) of the hexane-soluble material was hydrolyzed by heating for 19 hr in 500 ml of 24% hydrobromic acid under reflux. The cooled hydrolysate was diluted with 500 ml of water and extracted with 2.5 l. of chloroform to remove lipids. The aqueous portion of the hydrolysate was evaporated under reduced pressure to yield 3 g of ninhydrin-positive residue which was fractionated on a Dowex 1-X8, 200–400 mesh column in the acetate form. Elution was carried out with distilled water; the compound of interest was eluted

[2] Commercially available from Calbiochem, Los Angeles, Calif.

[3] The brain was that of a 38-year-old caucasian male who died as a result of a crushing chest injury. Postmortem examination showed no evidence of systemic disease. The brain was removed, frozen, and stored (3 years) until used.

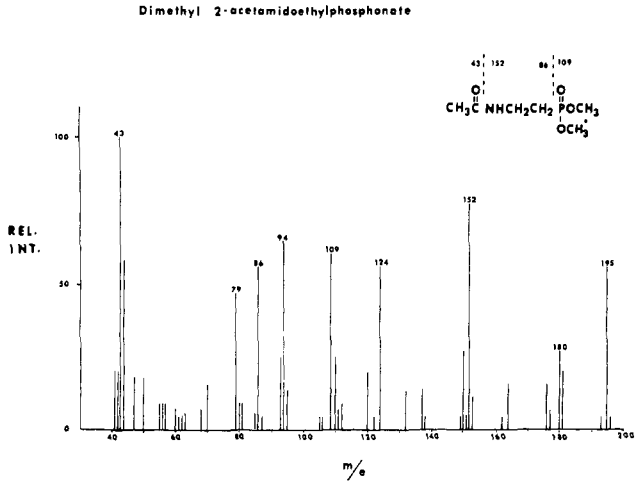


FIGURE 2: Mass spectrum of dimethyl 2-acetamidoethylphosphonate (4).

in the first 50 ml (fraction I). This fraction was ninhydrin positive and contained a compound which was indistinguishable from authentic AEP by descending paper chromatography (Whatman No. 1) in the following solvent systems: (a) 1-butanol–acetic acid–H_2O (4:1:4, v/v), R_F 0.38; (b) isopropyl alcohol–formic acid–H_2O (8:1:1, v/v), R_F 0.11. Also cochromatography by ascending paper chromatography was exhibited in the following solvent systems: (c) methanol–pyridine–H_2O (17:1:5, v/v), R_F 0.43; (d) pyridine–H_2O (65:55, v/v), R_F 0.42. An aliquot of fraction I gave a negative test for P_i (Chen *et al.*, 1956); a second aliquot, following oxidation with 70% perchloric acid (Hirata and Appleman, 1959), gave a positive phosphate test indicating that fraction I contained one or more compounds possessing a C–P bond.

A third aliquot was evaporated, brought up to 1 ml with pH 2.2 citrate buffer and examined using the automatic amino acid anlyzer. Using a 55-cm column a band was eluted (28 min) which corresponded to that of authentic AEP (Kittredge and Hughes, 1964; Chou and Scherbaum, 1966; Hori *et al.*, 1967).

The remainder of fraction I was derivatized in the manner described (see model studies) and the resulting mixture was examined by gas–liquid chromatography. A compound was observed which exhibited retention times identical with those of synthetic dimethyl 2-acetamidoethylphosphonate (**4**) using six different temperature regimes (*e.g.*, see Figure 1B). This compound was isolated by preparative gas–liquid chromatography using a column temperature of 170° (eight 10-μl injections). A mass spectrum of the collected material exhibited a parent ion, *m/e* 195.0650 (calcd for **4**, 195.0654) and fragment ions identical with those observed in a spectrum of synthetic **4** (Figure 2).

Analysis of Polar Lipid (Methanol Soluble) and Proteinaceous Residue (Hexane–Methanol Insoluble) Fractions for AEP. Samples (2 g) of each of the fractions were hydrolyzed using 24% hydrobromic acid and extracted with chloroform as described for the nonpolar lipid (hexane soluble) fraction. The respective aqueous phases were evaporated to dryness and derivatized for gas–liquid chromatographic analysis as described. A peak corresponding to **4** was observed in the chromatogram for the proteinaceous residue (Figure 1C). This material was collected from the gas–liquid chromatographic column (thirteen 10-μl injections) and a mass spectrum was obtained which exhibited a molecular ion and a fragment pattern identical with that shown in Figure 2. Gas–liquid chromatographic analysis of the derivatized mixture obtained from the polar lipid (methanol soluble) fraction hydrolysate exhibited no peak with a retention time assignable to **4**.

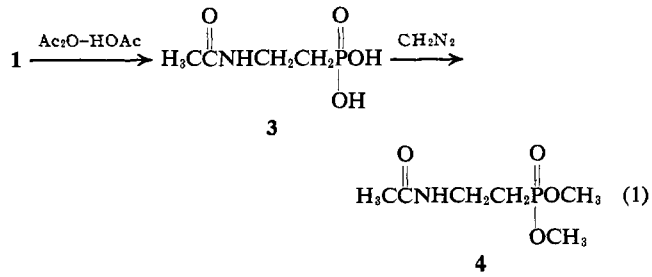
TABLE I: Concentration of 2-Aminoethylphosphonic Acid (AEP) in Human Brain Fractions.

Fraction	Concn (mg of AEP/g of Fraction)
Nonpolar lipids, 12.5 g (hexane soluble)	~0.4
Polar lipids, 7.8 g (methanol soluble)	Not detected
Proteinaceous residue, 14.3 g (hexane–methanol insoluble)	~1

Concentration of AEP in Fractions. An estimate of the concentrations of the AEP derivative **4** in the nonpolar lipid and proteinaceous residue fractions was made by comparing the areas of the appropriate gas chromatographic peaks with peak areas obtained using known amounts of authentic **4**. The assumption was made that complete conversion of AEP into **4** had been achieved (see Model Studies). The results, corrected to indicate concentration of AEP rather than of **4**, are shown in Table I.

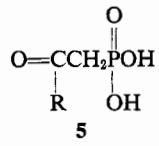
Results and Discussion

In order to establish the presence of AEP in bovine brain by isolation, Shimizu and coworkers (1965) processed 28.5 kg of tissue. Quite clearly, a prerequisite for an extensive investigation of the occurrence of AEP in nature is the availability of a sensitive method for its determination. We have developed a new procedure which utilizes two highly sensitive instrumental techniques—gas–liquid chromatography and mass spectrometry. Since each of these techniques requires volatile samples for analysis, it is necessary to derivatize mixtures to be examined for the presence of AEP. We accomplished this by carrying out sequential acylation and methylation reactions (eq 1). Dimethyl 2-acetamidoethylphosphonate (**4**) obtained in this way is a colorless viscous oil suitable for gas–liquid chromatographic and mass spectrometric analysis.[4] The mass spectrum of **4** (Figure 2) exhibits an intense parent ion and characteristic fragmentations which make identification straightforward.



(1)

[4] Recently Karlsson (1967) has reported the use of a trimethylsilyl derivative of AEP in a similar procedure.



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To assess the suitability of the technique for detection of AEP in complex mixtures, a model study was carried out. When AEP (<1%) in a mixture of amino acids was treated as indicated (eq 1) conversion into **4** was essentially complete and the resulting mixture was readily resolved by gas–liquid chromatography (Figure 1A).

With our method adequately developed we undertook a study of human brain tissue. Since previous workers had shown AEP to be contained in phospholipid (polar lipid) fractions and in proteinaceous material (Quin, 1964, 1965), we elected to use an initial hexane extraction which was expected to remove considerable amounts of material containing no AEP. Contrary to our expectations, however, analysis of this extract showed a significant amount of AEP to be present. Equally surprising AEP was not detected in the methanol-soluble fraction thought to contain most of the phospholipids (Ansell and Hawthorne, 1964; Entenman, 1957). The fraction richest in AEP (Table I), however, was the proteinaceous residue in agreement with the results for beef brain (Shimizu *et al.*, 1965).

The discovery that AEP is present in both lipid and proteinaceous fractions of human brain, as well as in goat liver (Kandatsu and Horiguchi, 1965) and beef brain (Shimizu *et al.*, 1965) suggests that a possible role for AEP in mammalian biochemistry should be considered. In this regard, the observation that AEP and its biosynthetic precursor in *Tetrahymena* (Trebst and Geike, 1967; Warren, 1968; Liang and Rosenberg, 1968; Horiguchi *et al.*, 1968), 2-amino-3-phosphonopropionic acid (**1**, R = COOH) undergo enzymatic transaminations in the presence of amine acceptors to yield the corresponding carbonyl derivatives **5** (R = H, COOH) (Roberts *et al.*, 1968; La Nauze and Rosenberg, 1968) is potentially very important. The carbonyl compounds **5** (R = H, COOH) are isoelectronic with known biological (high-energy) metaphosphate-transfer agents (*e.g.*, phosphoenolpyruvate). The possibility that AEP (with the intermediacy of the corresponding carbonyl derivative **5** (R = H)) is involved in highly specific enzymatic transfers of high-energy phosphate is an intriguing one.

References

Ansell, G. B., and Hawthorne, J. N. (1964), Phospholipids, New York, N. Y., Elsevier, Chapter 3.

Bishop, S. H. (1968), 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept.

Chen, P. S., Jr., Toribara, T. Y., and Huber, W. (1956), *Anal. Chem. 28*, 1756.

Chou, S. C., and Scherbaum, O. H. (1966), *Exp. Cell Res. 45*, 31.

Entenman, C. (1957), *Methods Enzymol. 3*, 299.

Hirata, A. A., and Appleman, D. (1959), *Anal. Chem. 31*, 2097.

Hori, T., Arakawa, I., and Sugita, M. (1967), *J. Biochem. (Tokyo) 62*, 67.

Horiguchi, M., and Kandatsu, M. (1959), *Nature (London) 184*, 901.

Horiguchi, M., Kittredge, J. S., and Roberts, E. (1968), *Biochim. Biophys. Acta 165*, 164.

Kandatsu, M., and Horiguchi, M. (1965), *Agr. Biol. Chem. 29*, 781.

Karlsson, K. A. (1970), *Biochem. Biophys. Res. Commun. 39*, 847.

Kittredge, J. S., and Hughes, R. R. (1964), *Biochemistry 3*, 991.

Kittredge, J. S., and Roberts, E. (1969), *Science 164*, 37.

Kosolapoff, G. M. (1947), *J. Amer. Chem. Soc. 69*, 2112.

La Nauze, J., and Rosenberg, H. (1968), *Biochim. Biophys. Acta 165*, 438.

Liang, C. R., and Rosenberg, H. (1968), *Biochim. Biophys. Acta 156*, 437.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Biochem. 30*, 1185.

Quin, L. D. (1964), *Science 144*, 1133.

Quin, L. D. (1965), *Biochemistry 4*, 324.

Quin, L. D. (1967), *Topics Phosphorus Chem. 4*, 23.

Roberts, E., Simonsen, D. G., Horiguchi, M., and Kittredge, J. S. (1968), *Science 159*, 886.

Shimizu, H., Kakimoto, Y., Nakajima, T., Kanazawa, A., and Sano, I. (1965), *Nature (London) 207*, 1197.

Trebst, A., and Geike, F. (1967), *Z. Naturforsch. 22 B*, 989.

Warren, W. A. (1968), *Biochim. Biophys. Acta 156*, 340.