

- (London) *B156*, 521.
- Frenster, J. H. (1965), *Nature* 206, 1269.
- Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol.* 12, 829.
- Girard, M., Latham, H., Penman, S., and Darnell, J. E. (1965), *J. Mol. Biol.* 11, 187.
- Harris, H. (1963), *Progr. Nucl. Acid Res.* 2, 19.
- Hoyer, B. H., McCarthy, B. J., and Bolton, E. T. (1964), *Science* 144, 959.
- Huang, R. C. C., and Bonner, J. (1965), *Proc. Natl. Acad. Sci. U. S.* 54, 960.
- Johnson, T. C., and Holland, J. J. (1965), *J. Cell Biol.* 27, 565.
- Marmur, J. (1961), *J. Mol. Biol.* 3, 208.
- McCarthy, B. J., and Hoyer, B. H. (1964), *Proc. Natl. Acad. Sci. U. S.* 52, 915.
- Penman, S. (1966), *J. Mol. Biol.* 17, 117.
- Penman, S., Scherrer, K., Becker, Y., and Darnell, J. E. (1963), *Proc. Natl. Acad. Sci. U. S.* 49, 654.
- Prescott, D. M. (1964), *Progr. Nucl. Acid Res. Mol. Biol.* 3, 33.
- Prescott, D. M., and Bender, M. A. (1962), *Exptl. Cell Res.* 26, 260.
- Watts, J. W. (1964), *Biochem. J.* 93, 306.

## Isolation and Characterization of the *N*-Methyl Derivatives of 2-Aminoethylphosphonic Acid from the Sea Anemone, *Anthopleura xanthogrammica*\*

J. S. Kittredge, A. F. Isbell, and R. R. Hughes

**ABSTRACT:** The phosphonic acid analogs of choline phosphate (trimethyl-2-phosphonoethylammonium hydroxide inner salt) and 2-methylaminoethylphosphonic acid have been isolated in crystalline form from the ethanolic extracts of the sea anemone, *Anthopleura xanthogrammica*. The presence of a small amount of 2-dimethylaminoethylphosphonic acid was also detected.

The two aminophosphonic acids previously isolated, ciliatine (2-aminoethylphosphonic acid) (Horiguchi and Kandatsu, 1959, 1960; Kittredge *et al.*, 1962) and phosphonoalanine (2-amino-3-phosphonopropionic acid) (Kittredge and Hughes, 1964), may be considered to be the phosphonic acid analogs of  $\beta$ -alanine and aspartic acid, or their structural similarity to the phosphate esters of ethanolamine and serine may be emphasized, *i.e.*, they are desoxyethanol aminophosphate and desoxyserine phosphate. Ciliatine occurs in phospholipids (Kittredge *et al.*, 1962; Rouser *et al.*, 1963; Hori *et al.*, 1964; de Koning, 1966). A survey of 19 species of *Coelenterata* for phosphonic acids (Kittredge, 1965) revealed a number of new phosphonic acids; among these were several ninhydrin-negative zwitterions. The possibility that one of the compounds

The three *N*-methyl derivatives of 2-aminoethylphosphonic acid have been synthesized and the structures of the isolated phosphonic acids have been established by comparison with the synthetic compounds. The biological occurrence of the phosphonic acid analogs of ethanolamine phosphate, serine phosphate, and choline phosphate, the three major constituents of phospholipids, has now been established.

detected might be desoxycholine phosphate<sup>1</sup> was examined.

### Materials and Methods

*Anthopleura xanthogrammica*. Approximately 4.8 kg wet weight of this sea anemone was collected intertidally near Scripps Institution of Oceanography, La Jolla, Calif. They were drained briefly, freed of much of the adhering sand and shells, and diced into 6 l. of 95% ethanol.

*Synthetic 2-Trimethylaminoethylphosphonic Acid*. We are indebted to Dr. A. F. Rosenthal, Long Island Jewish Hospital, for a gift of this compound (Rosenthal and Geyer, 1958).

*Ion-Exchange Resins*. The resins employed for the

\* From the Department of Biochemistry, City of Hope Medical Center, Duarte, California (J. S. K. and R. R. H.), and the Department of Chemistry, Texas A&M University, College Station, Texas (A. F. I.). Received September 15, 1966. This work was partially supported by a grant (NONR-3001[00]) from the Office of Naval Research.

<sup>1</sup> Trimethyl-2-phosphonoethylammonium hydroxide inner salt. For clarity we have considered the *N*-methyl derivatives as a series and have used the names: 2-aminoethylphosphonic acid, 2-methylaminoethylphosphonic acid, 2-dimethylaminoethylphosphonic acid, and 2-trimethylaminoethylphosphonic acid betaine.

isolation were Bio-Rad AG 1X8 and AG 50WX8, 200–400 mesh. Initially the resins were freed of fines and recycled (Kittredge *et al.*, 1962). Later batches proved to be satisfactory as received. The AG 1 resin was purchased in the formate form and converted to the bicarbonate or acetate form on the column and rinsed with degassed water immediately before use.

**Paper Chromatography.** Ascending chromatography with a phenol–water (4:1) system was used to monitor the fractionation. All of the phosphonic acids tested yield a transient blue-green color with the molybdate reagent of Rosenberg (1959).

**Paper electrophoresis** was conducted on water-cooled platens (E-C Apparatus Co.). The electrolytes employed were (1) 1.2 N formic acid; (2) acetic acid (25.6 ml/l.) and sodium acetate (13.6 g/l.); and (3) boric acid (5 g/l.) and sodium borate (5 g/l.).

**Elemental Analysis.** The microelemental analysis for C, H, N, and P was performed by Galbraith Laboratories, Knoxville, Tenn.

**Infrared Spectra.** The spectra of the synthetic and isolated phosphonic acids were recorded in KBr pellets on a Perkin-Elmer Model 421 grating spectrophotometer. We are grateful to Mr. A. J. Bauman for preparing these spectra.

**Nuclear Magnetic Resonance (Nmr) Spectra.** The proton nmr spectra were examined in deuterium oxide with a Varian Model A-60 spectrometer.

**Quantitative Ninhydrin Analysis.** The organic solvent system of Troll and Cannon (1953) was employed.

#### *Synthesis of N-Methyl Derivatives of 2-Aminoethylphosphonic Acid*

**2-Methylaminoethylphosphonic Acid.** Monomethylamine (24.5 g, 0.79 mole) was dissolved in 200 ml of anhydrous ether. This solution was chilled in an ice bath and 49.0 g (0.2 mole) of diethyl 2-bromoethylphosphonate was added quickly. The reagents were thoroughly mixed and the tightly stoppered solution was allowed to warm slowly to room temperature overnight. About 3 min after mixing, the solution became cloudy and began to deposit an insoluble white solid. By the next day there were two layers. The upper layer was decanted from the smaller more viscous lower layer. The viscous material was extracted four times with 50-ml volumes of anhydrous ether. After four extractions a solid residue remained, which was discarded. The combined ether extracts were evaporated to dryness under vacuum at room temperature leaving 34.1 g (87.5% yield) of a colorless oil. The infrared spectrum of this oil was similar to that of diethyl 2-bromoethylphosphonate except that the reaction product had a weak band at  $3.02\ \mu$  and a very weak band at  $6.08\ \mu$ . The first band probably corresponded to an NH group and the second band may be a C=C absorption caused by the presence of a small amount of diethyl vinylphosphonate resulting from a dehydrobromination reaction.

This crude oil was heated under reflux with 200 ml of 48% HBr for 18 hr. The solution was evaporated to dryness, and the residue was dissolved in 150 ml of

water, applied to a  $2 \times 85$  cm column of Dowex 50 ( $H^+$ ) resin, and eluted with water. The acidic front was discarded and further elution with deionized water yielded a product in about 2 l. of eluate. This product was detected by the blue cupric chelate formed when a drop of eluate was added to the blue suspension formed by adding a slight excess of sodium bicarbonate solution to 0.1 M cupric sulfate solution. When this eluate was evaporated to dryness and the solid residue was dissolved in a small volume of hot water and diluted with methanol, needle crystals separated from the cloudy solution upon chilling. The first crop of crystals weighed 13.9 g (50.0% yield) and a small second crop was recovered by concentrating the filtrate. The crystals melted at  $291^\circ$  dec. Titration produced a moderate break at approximately pH 4.1 (isoelectric point) and a sharper break at approximately pH 9.0. This gave a neutralization equivalent of 137.5 (theory 139.0).<sup>2</sup>

**2-Dimethylaminoethylphosphonic Acid.** A mixture containing 18.75 g (0.15 mole) of 2-aminoethylphosphonic acid (Kosolapoff, 1947), 24.6 ml (0.33 mole) of 37% formaldehyde, 1.0 g of 5% palladium on carbon, and 200 ml of water was shaken under an initial hydrogen pressure of 4 atm at room temperature. Within 19.5 hr 91.1% of the calculated amount of hydrogen was absorbed, 0.5 g more of catalyst was added, and in a total of 25 hr 95.8% of the theoretical amount of hydrogen was absorbed. The mixture was filtered and the filtrate was evaporated to dryness. The semisolid residue was dissolved in 150 ml of water and applied to a column of Dowex 50 ( $H^+$ ) resin. Elution with water removed an acidic front followed by 2 l. of eluate which gave a negative ninhydrin reaction but formed a blue cupric chelate. The eluate was evaporated to dryness leaving a residue which was dissolved in a small amount of water. The addition of 400 ml of methanol and chilling at  $-10^\circ$  overnight yielded 10.0 g of pale yellow crystals which appeared to be hygroscopic. Concentration of the filtrate and dilution with methanol allowed the recovery of additional amounts of this product. On drying in a vacuum oven at  $100^\circ$ , the product melted at  $249.5^\circ$  dec and no longer appeared hygroscopic. The titration curve had a break at pH 3.4 (isoelectric point) and a second break at approximately pH 8.4. The neutralization equivalent was 153.3 (theory 153.0).

**Anal.** Calcd for  $C_4H_{12}NO_3P$ : P, 20.23; N, 9.15. Found: P, 20.21, 20.23; N, 8.95, 8.93.

**2-Trimethylaminoethylphosphonic Acid.** Into a 250-ml erlenmeyer flask was placed 9.7 g (0.063 mole) of 2-dimethylaminoethylphosphonic acid, freshly precipitated and thoroughly washed silver oxide from 51 g (0.3 mole) of silver nitrate, 100 ml of water, 100 ml of alcohol, and 14.2 g (0.1 mole) of methyl iodide. Stirring this mixture caused an immediate rise in temperature, and after stirring for 20 min an excess of hydrochloric acid and a quantity of filter aid were

<sup>2</sup> The analysis of this compound is given in Table I.

added and the mixture was filtered. The clear filtrate was evaporated to dryness leaving 12 g of white solid. This solid was dissolved in 100 ml of water and applied to a column of Dowex 21K ( $\text{OH}^-$ ) resin. After washing the resin thoroughly with deionized water, the betaine was eluted with 0.1 N acetic acid. This compound was detected in the eluate with a refractive index monitor (Nester-Faust Co.). The eluate was evaporated to dryness and the solid residue was recrystallized from 95% ethanol. The well-defined needle crystals melted at 252° dec. Myers and Jibril (1957) reported this compound to be a hemihydrate from alcohol, mp 250–252. Rosenthal (1958) states that this compound crystallizes from alcohol as the dihydrate but becomes the monohydrate when dried over phosphorus pentoxide at 50° under vacuum and becomes anhydrous when the drying temperature is 80°. On drying our crystals at 100° and 30-mm pressure for 20 hr we obtained a product which gave a neutralization equivalent of 169.5 (theory 167). This compound is hygroscopic and will liquefy on exposure to humid air. When the material is dried over  $\text{CaCl}_2$  at room temperature the monohydrate is obtained, which yielded a neutralization equivalent of 184.9 (theory 185.2).

#### Isolation

The sea anemones were homogenized in a Waring blender in the alcohol in which they were preserved. The homogenate was filtered through a Celite bed and the filter cake was reextracted twice with 20 l. of 70% ethanol. The combined filtrates were concentrated in a rotary evaporator to remove the ethanol and the bulk of the lipids was removed with three extractions with diethyl ether. The ether was back-extracted with water and the combined aqueous phases were concentrated to remove the dissolved ether. Hydrolysis of the aqueous extract with 6 N HCl in sealed tubes at 110° for 48 hr released all of the esterified phosphate. The hydrochloric acid was removed by concentration to near-dryness in a rotary evaporator, diluted with water, and re-concentrated. The hydrolysate was decolorized by boiling briefly with charcoal (Norit A) and filtered through glass filter paper (Hurlbut 984 H).

The extract was desalted on an AG 50W ( $\text{H}^+$ ) resin column (11.5 × 33 cm). Each column accommodated one-half of the hydrolysate. The column was washed with 5000 ml of water and eluted with 3 N  $\text{NH}_4\text{OH}$ . The ammonia fronts (1500 ml each) were combined and concentrated.

The desalted extract was fractionated on an AG 50W ( $\text{NH}_4^+$ ) column (5 × 46.5 cm) made up in pH 11.4 buffer (20 ml of concentrated  $\text{NH}_4\text{OH}/1000$  ml) and eluted with the same buffer. One-fifth of the total extract was fractionated on each column. This column allowed the recovery of a very basic phosphonic acid which has not yet been characterized and also removed much of the pigment and amines from the extract. The neutral and acidic amino acids and the two *N*-methylaminoethylphosphonic acids were eluted at the front (310 ml).

The combined fronts from the pH 11.4 columns were concentrated to remove the buffer, applied an AG 1 ( $\text{HCO}_3^-$ ) column (5 × 47.5 cm), and eluted with water (17,500 ml). This column removed the acidic amino acids and the remaining pigments from the extract.

The concentrated extract (150 ml) from the bicarbonate column was then fractionated in 30-ml aliquots on an AG 1 ( $\text{Ac}^-$ ) resin column (5 × 100 cm). Since the acidic amino acids were removed on the bicarbonate column, this column may be used repeatedly without regenerating the resin. The column was eluted with 0.01 N acetic acid. The front contained all of the neutral amino acids and the betaine (880–1070-ml effluent). Following this a long peak composed of 2-aminoethylphosphonic acid and the mono-*N*-methyl derivative was eluted (1070–4400 ml). The monomethyl derivative tends to precede the aminoethylphosphonic acid in this peak. During the monitoring of the fractionation the first few tubes of this peak were found to contain a small amount of another phosphonic acid which migrated during paper chromatography with synthetic 2-dimethylaminoethylphosphonic acid.

Isolation of the phosphonates was accomplished on an AG 50W ( $\text{H}^+$ ) resin column (2.5 × 80 cm) eluted with 1.5 N HCl. The combined peaks from the AG 1 ( $\text{Ac}^-$ ) columns containing the monomethyl derivative were concentrated and made to 140-ml volume and 1.5 N in HCl. The betaine-containing peaks were combined and made to 100 ml, 1.5 N HCl. Each was fractionated in 10-ml aliquots. The three aminoethylphosphonic acids were eluted from this column in the following volumes: 2-aminoethylphosphonic acid, 510–570 ml; 2-methylaminoethylphosphonic acid, 590–645 ml; trimethylaminoethylphosphonic acid, 645–705 ml. The isolation of the trimethyl derivative from the neutral amino acids yielded additional monomethyl compound contaminated with serine and threonine, while the trimethyl peaks were contaminated with threonine and glycine. The appropriate fractions containing the two *N*-methylaminoethyl compounds were combined and the hydrochloric acid was removed by concentration in the rotary evaporator, dilution, and repeated concentration. The residual acidity was neutralized with ammonium hydroxide. The contaminating amino acids were removed from the two extracts on AG 1 ( $\text{Ac}^-$ ) columns. The columns were made up in water and, after application of the extract, were washed with 500 ml of water. The monomethyl derivative was eluted with 0.01 N acetic acid. It was found that the betaine was held more firmly by the resin when applied in a relatively pure state and 0.5 N acetic acid was required to elute it.

The fractions from the final AG 1 column of the monomethyl derivative were concentrated to 5 ml, a trace of color was removed by adding a few drops of a boiling suspension of Norit A and filtering, and crystallization was induced by adding ethanol to 50% concentration. After two recrystallizations the yield was 376 mg of fine white needles.

The trimethylaminoethylphosphonic acid was simi-

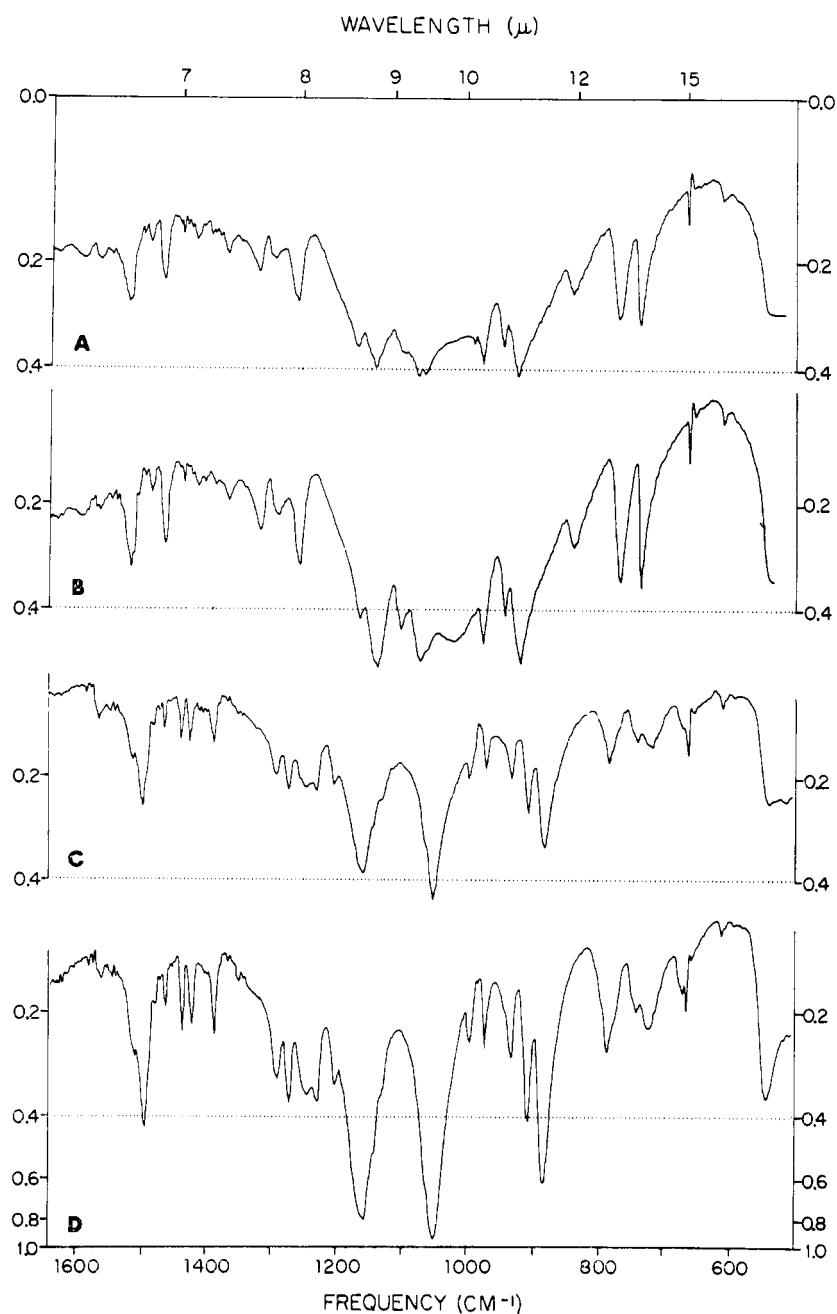


FIGURE 1: Infrared spectra of synthetic 2-methylaminoethylphosphonic acid (A), natural 2-methylaminoethylphosphonic acid (B), synthetic 2-trimethylaminoethylphosphonic acid (C), and natural 2-trimethylaminoethylphosphonic acid (D).

larly concentrated and decolorized. The filtrate was evaporated to dryness and taken up in 3 ml of hot ethanol. A residue of fine white needles remained. The supernatant was withdrawn and crystallization was induced by adding acetone to 50% concentration. Three recrystallizations from hot ethanol yielded 92 mg of small cuboidal crystals. The filtrates were combined and a second crop of crystals was recovered by adding acetone. The ethanol-insoluble material proved to be the monomethyl derivative.

The elementary analyses of each of the isolated compounds and of the synthetic compounds are given in Table I. The infrared spectra of the synthetic and the isolated compounds are shown in Figure 1. In each case the two are identical.

The nuclear magnetic resonance (nmr) spectra were also identical and are shown in Figure 2. The triplets due to spin-spin interaction between the phosphorus and the  $\alpha$  and the  $\beta$  protons yield degenerate four- and five-part peaks. The *N*-methyl protons yield a

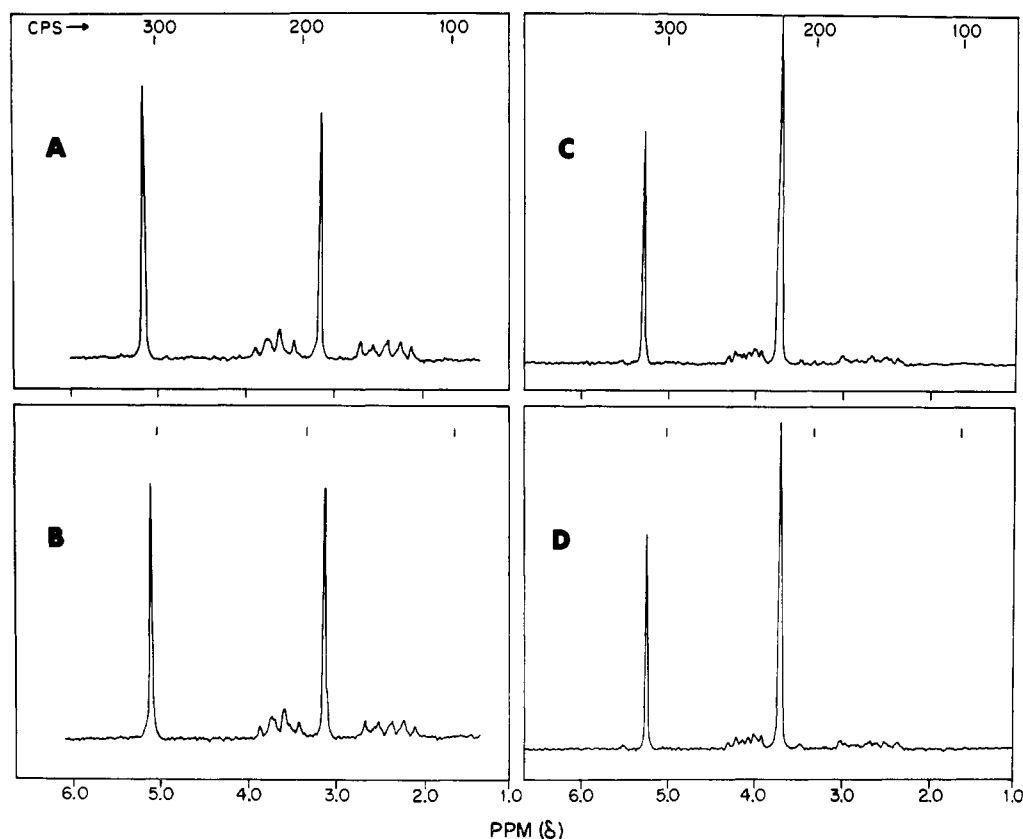


FIGURE 2: Proton magnetic resonance spectra of synthetic 2-methylaminoethylphosphonic acid (A), natural 2-methylaminoethylphosphonic acid (B), synthetic 2-trimethylaminoethylphosphonic acid (C), and natural 2-trimethylaminoethylphosphonic acid (D). External tetramethylsilane reference, uncontrolled pH.

TABLE I: Elementary Analyses.

	C	H	N	P
2-Methylaminoethylphosphonic Acid ( $C_3H_{10}NO_3P$ )				
Calculated (%)	25.90	7.25	10.07	22.27
Natural (%)	25.73	7.19	10.17	22.36
Synthetic (%)	26.00	7.20	10.21	22.16
	C	H	N	P
2-Trimethylaminoethylphosphonic Acid ( $C_5H_{14}NO_3P \cdot H_2O$ )				
Calculated (%)	32.43	8.71	7.57	16.73
Natural (%)	31.66	8.72	7.96	17.30
Synthetic (%)	32.44	8.74	7.74	16.77

strong peak and the HDO peak represents the exchangeable protons. Aliquots of both of the isolated compounds were subjected to vigorous hydrolytic conditions (6 N HCl, 110°, 48 hr) and no release of orthophosphate could be detected.

The 2-methylaminoethylphosphonic acid had been

observed to yield a weak ninhydrin reaction on paper chromatograms. Quantitative ninhydrin analysis gave a color yield only 4% of that produced by leucine. This compound also is less sensitive to the molybdate reagent of Rosenberg (1959) than is 2-aminoethylphosphonic acid, yielding a transient spot of about one-half the intensity of that shown by the latter compound.

Table II lists the  $R_F$  values of aminophosphonic acids in several developing systems. Choline phosphate and desoxycholine phosphate can be separated by paper chromatography in an isopropyl alcohol-trichloroacetic acid-ammonia-water developing system and also with a *t*-butyl alcohol-methyl ethyl ketone-diethylamine-water system; however, the best system found was paper electrophoresis in a buffer containing 25.6 ml of acetic acid and 13.6 g of sodium acetate per l. (pH 4.0). In this system choline phosphate migrates at 0.019 cm<sup>2</sup>/v per hr, while the phosphonate analog migrates at 0.022 cm<sup>2</sup>/v per hr.

An aliquot of the ether extract was hydrolyzed under reflux in 6 N HCl for 48 hr, the HCl was removed, and the nonsaponifiable material was extracted with ether. The aqueous hydrolysate was fractionated on an AG 50W (H<sup>+</sup>) column and the ammonia front was

TABLE II:  $R_F$  Values of Several Phosphorus-Containing Compounds.

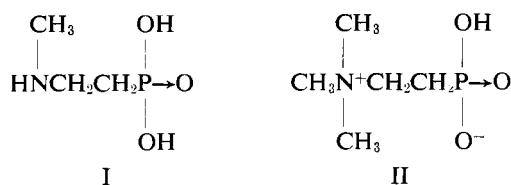
Compound	Developing System						
	A	B	C	D	E	F	G
PAI <sup>a</sup>	0.07 s <sup>b</sup>	0.08	0.34 s	0.43	0.13	0.38	s
AEP	0.36	0.21	0.57	0.56	0.18	0.36	0.38
MMAEP	0.68	0.42	0.74	0.62	0.22	0.37	0.53
DMAEP	0.82	0.65	0.85	0.60	0.22	0.44	0.57
TMAEP	0.90	0.73	0.89	0.51	0.21	0.32	0.57
Choline-PO <sub>4</sub>	0.88	0.73	0.88	0.46	0.21	0.25	s

<sup>a</sup> PAI, phosphonoalanine; AEP, 2-aminoethylphosphonic acid; MMAEP, 2-methylaminoethylphosphonic acid; DMAEP, 2-dimethylaminoethylphosphonic acid; TMAEP, 2-trimethylaminoethylphosphonic acid; choline-PO<sub>4</sub>, choline phosphate. Systems: (A) phenol (88%)-water (4:1), (B) phenol (88%)-ethanol-ammonia-water (12:4:1:3); (C) phenol (88%)-acetic acid-ethanol-water (17:4:4:3); (D) isopropyl alcohol-trichloroacetic acid-ammonia-water (750:50:3:250, v/w/v/v); (E) *n*-butyl alcohol-acetic acid-water (12:3:5); (F) *t*-butyl alcohol-methyl ethyl ketone-diethylamine-water (10:5:1:10); and (G) methanol-pyridine-water (20:1:5). Systems A, B, E, F, and G are from Smith (1960). <sup>b</sup> s, streaks.

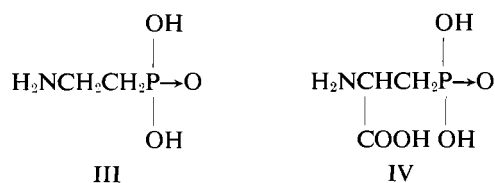
fractionated on an AG 1 (Ac<sup>-</sup>) column. Examination of the eluate by paper chromatography revealed considerable 2-aminoethylphosphonic acid and the monoethyl derivative but only a trace of the trimethyl derivative.

#### Discussion

The above criteria establish the structures of the two new naturally occurring phosphonic acids as 2-methylaminoethylphosphonic acid (I) and 2-trimethylaminoethylphosphonic acid betaine (II).



The probable presence of a small amount of the dimethyl derivative was demonstrated by paper chromatography. These, together with the two previously characterized naturally occurring phosphonic acids, ciliate (III) and phosphonoalanine (IV), constitute the nucleus of a new field of biochemistry.



The covalent carbon to phosphorus bond is strong (65 kcal/mole; Hudson, 1964) and completely resistant to phosphatases, though the work of Zeleznick *et al.*

(1963) indicates the possible occurrence in *Escherichia coli* of an enzyme capable of splitting this bond. However, his work might indicate only oxidative catabolism of the carbon chain of the phosphonic acids.

Segal (1965) has proposed a route to the biosynthesis of these phosphonic acids through a phosphoramidic acid rearrangement, but there is no experimental evidence for any scheme of biosynthesis. The presence of compounds containing a C-P bond has been established in members of five animal phyla ranging from *Protozoa* to *Chordata* (Horiguchi, 1966). These compounds are not minor constituents of the tissues of animals; in many animals from 5 to 13% of the total phosphorus in the organism is contained in phosphonic acids: in the sea anemone, *Metridium dianthus*, ciliate constitutes 1% of the tissue dry weight (Quin, 1965). Its presence in proteins (Quin, 1964, 1965; Rosenberg, 1964) as well as in phospholipids has been established. Liang and Rosenberg (1966) have demonstrated the synthesis of the phosphonic acid analog of cytidine diphosphate ethanolamine by *Tetrahymena* supernatant and the intermediate nature of this compound in the biosynthesis of the analog of cephalin. The biological role of these phosphonic acids is entirely unknown, but it is possible that they may provide polar functions on membrane lipids that are approximately equivalent to the corresponding phosphate esters and are entirely resistant to phosphatases.

#### References

- de Koning, A. J. (1966), *Nature* 210, 113.
- Hori, T., Itasaka, O., Inoue, H., and Yamada, K. (1964), *J. Biochem. (Tokyo)* 56, 477.
- Horiguchi, M. (1966), *Nippon Nogeikagaku Kaishi* 40, R25.
- Horiguchi, M., and Kandatsu, M. (1959), *Nature* 184, 901.

- Horiguchi, M., and Kandatsu, M. (1960), *Bull. Agr. Chem. Soc. Japan* 24, 565.
- Hudson, R. F. (1964), *Pure Appl. Chem.* 9, 371.
- Kittredge, J. S. (1965), Ph.D. Thesis, University of California, La Jolla, Calif.
- Kittredge, J. S., and Hughes, R. R. (1964), *Biochemistry* 3, 991.
- Kittredge, J. S., Roberts, E., and Simonsen, D. G. (1962), *Biochemistry* 1, 624.
- Kosolapoff, G. M. (1947), *J. Am. Chem. Soc.* 69, 2112.
- Liang, C. R., and Rosenberg, H. (1966), *Biochim. Biophys. Acta* 125, 548.
- Myers, T. C., and Jibril, A. O. (1957), *J. Org. Chem.* 22, 180.
- Quin, L. D. (1964), *Science* 144, 1133.
- Quin, L. D. (1965), *Biochemistry* 4, 324.
- Rosenberg, H. (1959), *J. Chromatog.* 2, 487.
- Rosenberg, H. (1964), *Nature* 203, 299.
- Rosenthal, A. F., and Geyer, R. P. (1958), *J. Am. Chem. Soc.* 80, 5240.
- Rouser, G., Kritchevsky, G., Heller, D., and Lieber, E. (1963), *J. Am. Oil Chemists' Soc.* 40, 425.
- Segal, W. (1965), *Nature* 208, 1284.
- Smith, I., Ed. (1960), in *Chromatographic and Electrophoretic Techniques*, New York, N. Y., Interscience, p 84.
- Troll, W., and Cannon, R. K. (1953), *J. Biol. Chem.* 200, 803.
- Zelevnick, L. D., Meyers, T. C., and Titchener, E. B. (1963), *Biochim. Biophys. Acta* 78, 546.

## The Reactions of Sulfatide with Metallic Cations in Aqueous Systems\*

Morris B. Abramson, Robert Katzman, Robert Curci, and Clarence E. Wilson

**ABSTRACT:** Sulfatide when converted to the sodium salt could be dispersed in water by ultrasonic radiation to form stable systems. Titrations and reactions with metallic cations showed the strongly acidic behavior of this lipid. Addition of univalent and divalent metal chlorides produced increases in turbidity and coagulation showing the relative affinity of the sulfate group for the cations was  $\text{Ca} > \text{Mg} > \text{K} > \text{Na} > \text{Li}$ . Analysis of the lipid coagulated by adding salts or mixtures of salts showed a stoichiometric relation of lipid to

cation where each  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  bridges two lipid molecules. Selective binding of  $\text{K} > \text{Na}$  and  $\text{Ca} > \text{Mg}$  is found by turbidimetric measurements and by analysis of coagula.

Mixed dispersions of sulfatide with either lecithin or cerebroside show cation association by sulfatide with inclusion of neutral lipid in the coagula. Similarities are shown by acidic lipids and cation-exchange resins with analogous acid groups in their reactions with cations.

Theories of ion transport across biologic membranes as well as the establishment of membrane potentials assume specific binding of metallic cations at negative-charge sites on the membrane surface. Investigations in this laboratory directed toward the determination of such specificities have already shown the relative order of binding in aqueous systems of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{TMA}^{+1}$  by the acidic phospholipids, phosphatidic acid, and phosphatidylserine. The binding of cations by acidic phospholipids was postulated to be an intermediate step in membrane ion transport by Hokin and Hokin (1960), although now the relationship of the lipid to transport is con-

sidered to be more complex (Hokin, 1966). However, Tobias *et al.* (1962) have implicated the binding of cations by phospholipids in the physical changes occurring in the membrane in transport and potential phenomena.

Lipids with ionized sulfate groups constitute an important component of membranes in the central nervous system. These sulfate groups are more strongly acidic than the lipid phosphate or carboxyl groups. In the present paper, we give data on the ionic characteristics of sulfatide and measurements of the interaction of the ionized sulfate group of this lipid with alkali and alkaline earth cations in aqueous systems. Whereas the phospholipids have been shown to bind  $\text{Na}^+$  more strongly than  $\text{K}^+$ , we have found that aqueous dispersions of sulfatide react more strongly with  $\text{K}^+$  than  $\text{Na}^+$ . This result was anticipated by the finding that sulfatide can be purified by precipitation as the potassium salt (Blix, 1933; Lees *et al.*, 1959). An

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<sup>1</sup> Abbreviation used:  $\text{TMA}^+$ , tetramethylammonium ion.