The Occurrence of Free 2-Aminoethylphosphonic Acid in the Sea Anemone, *Anthopleura elegantissima**

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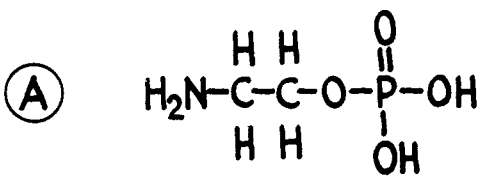
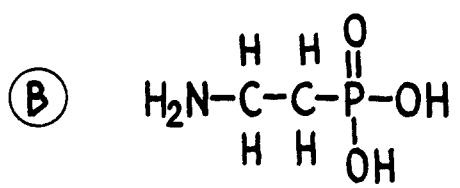
Received March 28, 1962

Free 2-aminoethylphosphonic acid has been isolated in crystalline form from the sea anemone, *Anthopleura elegantissima*, and conclusively identified. The free glycerol ester of this phosphonic acid also was isolated. Large amounts of the phosphonic acid were liberated on saponification of crude lipid extracts of the anemone. From this it was concluded that there may exist in this organism phospholipids in which 2-aminoethyl phosphate is replaced by 2-aminoethylphosphonic acid, the only compound isolated from biological material to date which possesses a carbon-to-phosphorus bond.

As part of a continuing program in this laboratory on free or easily extractable ninhydrin-reactive constituents of living material (Roberts and Simonsen, 1960), a survey was made of the tissues of a number of taxonomically diverse marine invertebrates (Kittredge *et al.*, 1962). Conventional, two-dimensional phenol-lutidine paper chromatograms of extracts of the sea anemone, *Anthopleura elegantissima*, revealed considerable quantities of a material which migrated like 2-aminoethyl phosphate (formula A) (Awapara *et al.*, 1950) or the phosphodiester of L-serine and ethanolamine (Roberts and Lowe, 1954). The present paper reports the identification of this compound as 2-aminoethylphosphonic acid (formula B).





Materials and Methods

Paper Chromatography.—Conventional, two-dimensional descending phenol-lutidine chromatograms on Whatman No. 1 paper (18 × 22 in.; 46 × 56 cm) were employed in the survey. Small scale (7 × 7 in.; 18 × 18 cm) ascending chromatograms were used for comparison and monitoring. One-dimensional lutidine chromatography on 3 MM paper with a heavy "stripe" application was used for the initial isolation.

Paper Electrophoresis.—Electrophoresis was carried out in a water-cooled unit (E-C Apparatus Company) on Whatman No. 1 paper at 950 v for 4 hours in 0.05 N sodium formate buffer, *p*H 3.5, or 0.07 M sodium citrate–barbital buffer, *p*H 8.6.

Ion-Exchange Resins.—The resins employed were AG 50W-X4 and AG 1-X4, 200 to 400 mesh, as supplied by Bio-Rad Laboratories, Richmond, Calif. Fines were removed by three cycles of settling and decanting. The cation-exchange resin (AG 50W-X4) was purified by three cycles of treatment with 1 N NaOH and 4 N HCl, then washed with distilled water until chloride free. The anion-exchange resin (AG 1-X4) was converted to the acetate form by elution with 1% Na_2CO_3 until chloride free, followed by batch treatment with 12 N acetic acid.

Phosphorus Analyses. — Phosphorus analyses were performed, after wet-ashing of the samples with a sulfuric acid–nitric acid mixture, by a colorimetric procedure employing a modified Fiske-SubbaRow ammonium molybdate reagent (Chen *et al.*, 1956).

Elemental Analysis.—The analysis of the crystalline compound was performed by Elek Micro Analytical Laboratories, Los Angeles, Calif.

Synthesis.—Synthetic 2-aminoethylphosphonic acid was prepared by the procedure of Kosolapoff (1947) by Isotope Specialties Company, Burbank, Calif.

Infrared Spectra.—Infrared spectra in KBr pellets containing approximately 1.42% of the test material were determined on a Beckman IR-4 spectrophotometer by Dr. Gene Kritchevsky of our staff.

Equivalent Weight.—Aqueous solutions were titrated with tetraethylammonium hydroxide in a Radiometer Titrigraph.

Enzymes.—Alkaline and acid phosphatase preparations were obtained from Worthington Biochemical Corporation, Freehold, N. J., and venom from the rattlesnake, *Crotalus adamanteus*, was purchased from Ross Allen's Reptile Institute,

* This work was partially supported by Grant No. Nonr-3001(00) from the Office of Naval Research.

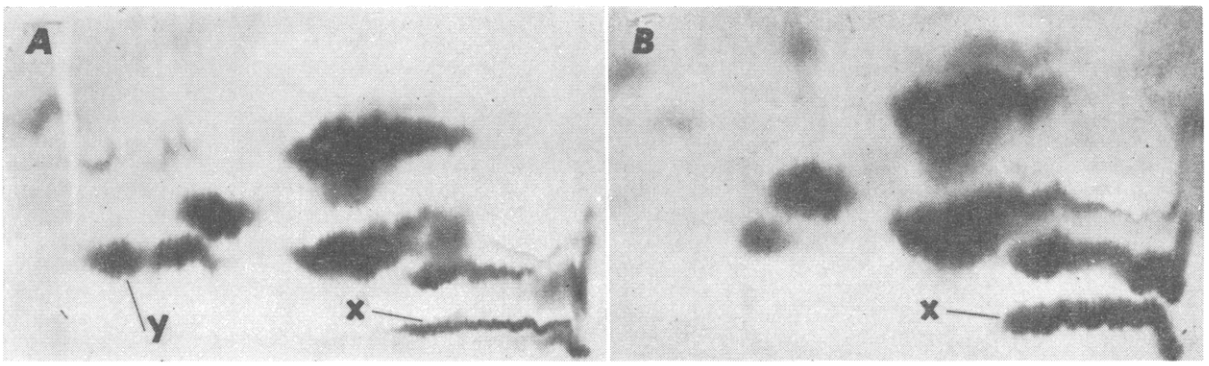


FIG. 1.—Two-dimensional chromatograms of the free amino acids of the sea anemone, *A. elegantissima*. Phenol direction, right to left; lutidine direction, bottom to top. *A*, ethanolic extract; *B*, hydrolyzed extract. Unknown constituents, *X* and *Y*.

Silver Springs, Fla., and was used as a source of phosphodiesterase.

Melting Point.—Determinations were performed on a Fisher-Johns hot stage, preheated to 250°. The heating rate was 10–12° per minute.

RESULTS

Ninhydrin-Reactive Constituents of Alcoholic Extracts of Sea Anemones.—A large number of two-dimensional chromatograms were made of extracts of whole anemones collected at different times of the year as well as of the tentacles and pedal discs separately. In all of the chromatograms there were found to be relatively high concentrations of a variety of ninhydrin-reactive constituents ordinarily found in tissues of both vertebrate and invertebrate organisms (Figure 1a). Two new detectable constituents (Figure 1a, *X* and *Y*) were selected for further study. Spot *Y*, which appeared in the position close to that of glycerylphosphorylethanolamine (Norman and Dawson, 1953), always disappeared on hydrolysis with 6 N HCl for 24 hours in a sealed tube at 100°, and a corresponding increase in the quantity of *X* was noted (Figure 1*b*).

The above hydrolytic conditions have been found sufficient to destroy 2-aminoethyl phosphate and the phosphodiester of L-serine and ethanolamine, substances which migrate to approximately the same position in the phenol-lutidine chromatographic system. It was, therefore, suspected that *X* was a compound not previously found in the easily extractable form in any of the numerous tissue extracts of either terrestrial or marine organisms which have been examined in this laboratory.

Small-scale isolations of both *X* and *Y* were achieved by suitable combinations of preparative one-dimensional chromatograms. It was found that *X* was remarkably stable to acid hydrolysis and that hydrolysis of *Y* gave rise to *X*. At this point of the investigation the presence of phosphorus in *X* was not suspected since tests for orthophosphate after hydrolysis were uniformly negative. Comparison of the movement on paper of *X* with a large number of acid-stable ninhydrin-reactive substances which have been reported to migrate similarly in the phenol-lutidine solvent system employed showed that the unknown substance was not identical with any of the compounds with which it was compared. Paper electrophoresis in acid and alkaline buffers showed the unknown material to be a weakly acidic ampholyte.

Isolation of Compound X by Column Chromatographic Procedures.—At the site of collection 4.2 kg of fresh anemones were drained briefly and diced, and sufficient ethanol was added so that the final concentration of ethanol was 70%. Upon return to the laboratory, the material was homogenized in a large Waring blendor and filtered. The filter cake was rehomogenized twice with three times its volume of 70% ethanol. The filtrate was reduced to one tenth of the original volume in a rotary evaporator and extracted twice with equal volumes of ethyl ether. The aqueous-ethanolic extract was desalted in portions on 4.5 × 30 cm columns of cation-exchange resin (AG 50W-X4 H^+). The columns were washed with 2 liters of water and the acidic and neutral amino acids eluted with 2 N NH_4OH. The eluate was evaporated to dryness. Since the new compound occurs both free and in a bound form, the total extract was taken up in 4 N HCl and hydrolyzed for 24 hours at 100° in sealed tubes. The hydrochloric acid was removed in a rotary evaporator and finally over sodium hydroxide *in vacuo*. The hydrolysate was taken up in water and considerable amounts of "fishy-smelling" amines and pigments were removed by a second application to columns of AG 50W-X4 H^+ and elution with 2 N NH_4OH. The eluate containing the neutral and acidic amino acids was concentrated to dryness and taken up in 20 ml of 0.5 N acetic acid and applied to a 4.5 × 13.5 cm column of anion-exchange resin (AG 1-X4 in the acetate form). The column was eluted with 0.5 N acetic acid at 170 drops per minute. The first 60 ml of eluate was discarded, and the neutral amino acids and the new compound were found in the next 135 ml of eluate while the more acidic compounds were

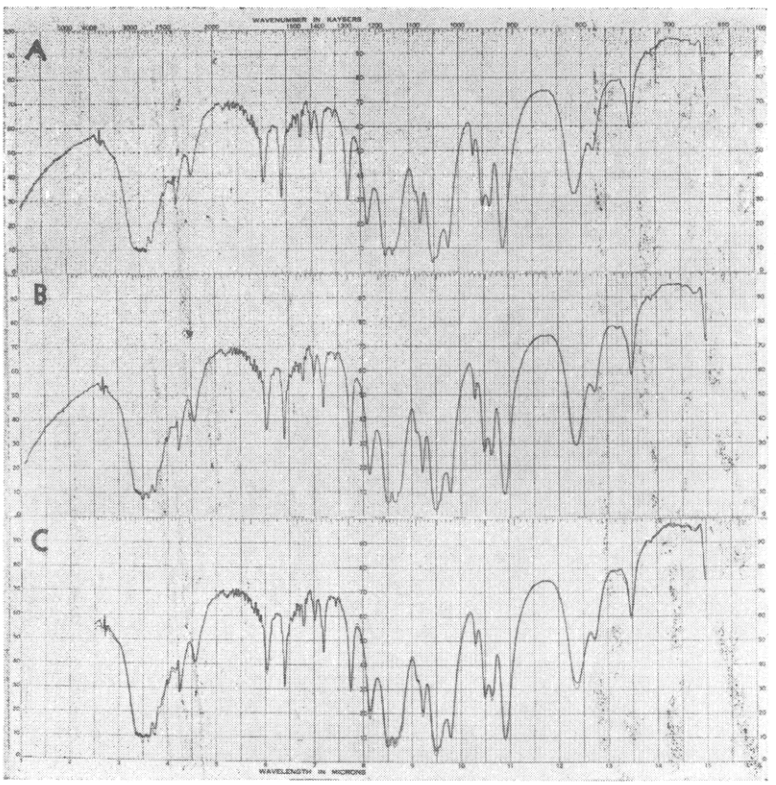


FIG. 2.—Infrared spectra of natural and synthetic 2-aminoethylphosphonic acid. *A*, natural product, 1.43% in KBr; *B*, synthetic, 1.41% in KBr; *C*, superimposed spectra, natural: heavy trace, synthetic: light trace.

retained on the column. After removal of the 0.5 N acetic acid the residue (5.035 g) was taken up in 15 ml of 0.01 N acetic acid. Five milliliters were applied to a 4.3 × 100 cm column of AG 1-X4 in the acetate form. The column was eluted with 0.01 N acetic acid at 72 drops per minute. All of the contaminating amino acids were eluted in the first 795 ml, and the new compound was eluted from 1055 ml to 1625 ml. The remaining extract was fractionated in two similar runs without regeneration of the resin.

After removal of the acetic acid the residue was dissolved in 0.55 ml of water and ethanol was added to 50% concentration. Three crystallizations yielded 184.4 mg of rhombic crystals which melted with decomposition at 280–281° (uncorrected) (281–282°, Kosolapoff, 1950).

The Unknown Compound is 2-Aminoethylphosphonic Acid.—Analysis of the recrystallized compound gave the following percentage composition: C, 20.0; H, 6.2; N, 11.2; P, 26.3. That calculated for $C_2H_8NPO_3$ is: C, 19.2; H, 6.4; N, 11.2; P, 24.8. In keeping with the above empirical formula with the molecular weight of 125, an equivalent weight of 123 was obtained by electrometric titration. The entire titration curve showing only one inflection was virtually identical to that subsequently found to have been reported for "2-aminoethane phosphonic acid" (Horiguchi and Kandatsu, 1960). The pK_a found by the latter workers was 6.4, while that from our curve was 6.23.

The existence of a carbon-to-phosphorus bond was suggested by the stability of the compound to hydrolysis. No orthophosphate was liberated by incubation with acid or alkaline phosphatases and no degradation of the compound was observed by snake venom phosphodiesterase, even when incubation with the respective enzymes was carried out under optimal conditions for as long as 6 hours. Nor was any liberation of phosphate observed when hydrolysis was performed in a sealed tube with 8 N HCl at 150° for 48 hours. The wet-ashing procedure employed, however, gave the high phosphorus values reported above.

All of the above data were compatible with the simplest structure for the compound being either 1-aminoethylphosphonic acid or 2-aminoethylphosphonic acid. It was decided that the 2-aminoethylphosphonic acid structure was the most likely because no copper chelation by the compound took place when a solution was shaken with copper phosphate suspension. Under the conditions employed α-amino acids chelate copper, but ω-amino acids do not (Baxter and Roberts, 1958).

On comparison with synthetic 2-aminoethylphosphonic acid, 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. 2*a*, *b*, and *c*).

Isolation of Compound Y.—A small amount of compound *Y* (see Figure 1*a*) was isolated by column chromatography. The compound proved to be unstable during desalting on AG 50W-X4 H^+. A fresh ethanolic extract was passed through a 4.5 × 38 cm mixed bed of AG 1-X1 in the OH^- form, and AG 50W-X4 in the H^+ form, and was washed with 1500 ml of water. Elution was carried out with 0.2 M ammonium acetate. The eluate from 1000 ml to 1560 ml was found to contain taurine and compound *Y* and a small amount of 2-aminoethylphosphonic acid, the latter probably originating from the hydrolysis of *Y* on the column. This fraction was concentrated in a rotary evaporator and the residue was taken up in 20.5 ml of 0.1 M formic acid and applied to a 4 × 99 cm column of AG 1-X4 resin in the acetate form. The column was contained in a water jacket and the fractionation was conducted at 2–3°. Elution was performed with 0.1 M formic acid. Taurine was eluted from 975 ml to 1150 ml, two minor ninhydrin-reacting fractions at 2575 ml to 2625 ml and 3375 ml to 3575 ml, and compound *Y* at 6650 ml to 7400 ml. The fraction containing *Y* was neutralized with 1 N NH_4OH and reduced to a small volume in a rotary evaporator, and the ammonium formate-acetate was removed under vacuum in the presence of NaOH pellets and sulfuric acid.

Compound Y Contains 2-Aminoethylphosphonic Acid and Glycerol.—A portion of the residue (0.1 mg) was hydrolyzed with 0.1 ml of 1.5 N HCl in a sealed tube at 100° for 2 hours. After removal of the HCl the hydrolysate was taken up in 0.1 ml of water and chromatographed one-dimensionally on Whatman No. 1 paper with ethyl acetate–pyridine–water (2:1:2) together with known amounts of glycerol. The papers were stained with $AgNO_3$ (Smith, 1954). Lutidine chromatograms were run with known amounts of 2-aminoethylphosphonic acid. The results showed that compound *Y* contains 2-aminoethylphosphonic acid and glycerol in approximately equimolar amounts. Compound *Y* has, therefore, been tentatively identified as the glycerol ester of 2-aminoethylphosphonic acid. Definite proof awaits isolation of larger quantities of the compound.

2-Aminoethylphosphonic Acid Probably is a Constituent of the Phospholipids of the Anemone.—A portion of the ether originally employed to delipidate the 70% ethanolic extract of the anemones was washed with water and evaporated to dryness. The residue was saponified with 1.5 N NaOH in 50% ethanol for 16 hours at 50°. The alkali was neutralized with an equivalent amount of AG 50-X4 H^+ resin. The total neutralized suspension was poured on a short column of the same resin, washed with water, and eluted with 2 N NH_4OH. Two-dimensional chromatography of the residue of the eluate in phenol-lutidine revealed a large amount of 2-aminoethylphosphonic acid together with small amounts of other ninhydrin-reactive compounds.

Discussion

2-Aminoethylphosphonic acid is the first compound to have been isolated from biological material which has a covalent linkage between carbon and phosphorus. The only previous report of the presence of this substance was the isolation from a hydrolysate of a "proteolipid-like" extract of ciliates found in sheep rumen (Horiguchi and Kandatsu, 1959, 1960). A problem of immediate concern with regard to the isolation of this substance from sea anemones is whether the compound actually is made by the anemones or furnished to them by symbiotic algae usually associated with them, the zooxanthellae. This problem can be approached either by studying pure cultures of the algae or by largely freeing the anemones from the symbiotic microorganisms by maintaining them in the dark for relatively prolonged periods. However, the isolation of the phosphonic acid from the pedal discs of the sea anemones, tissue which does not contain zooxanethellae, indicates that the phosphonic acid occurs in the anemone tissues, and therefore may be presumed to be utilized by them.

The fixation of phosphorus into a covalent linkage with a carbon residue may enable the organisms containing such compounds to retain an adequate supply of phosphorus for phospholipid metabolism in a marine environment in which phosphorus can become limiting.

A survey is being conducted to establish the distribution of this phosphonic acid throughout the Coelenterata. Special emphasis will be made to examine tropical members of the phylum, especially the orders Siphonophora, Semaeostomeae, and Alconaria, which are more likely to have to contend with low concentrations of phosphate in their environment.

The present results showed that the C-P linkage in 2-aminoethylphosphonic acid is not attacked by phosphatases or by snake venom. An intensive search is being made for enzymes which can split this type of bond. Enzymes involved in the metabolism of 2-aminoethylphosphonic acid should be sought not only in the anemones and their associated microorganisms, but also in the Nudibranciata, some species of which feed exclusively on hydroids or sea anemones. It also would be of interest to study the metabolism of 2-aminoethylphosphonic acid in crabs, starfish, and certain fish which feed on the anemones as well as on other organisms.

Attempts are under way to isolate and characterize the phospholipids which might contain 2-aminoethylphosphonic acid and the corresponding quaternary ammonium compound. A comparison of the chemical and metabolic properties of these materials with those compounds which normally contain 2-aminoethyl phosphate or

choline phosphate should prove to be of great interest. It is also being determined whether, upon administration to rats and mice, 2-aminoethylphosphonic acid can be incorporated into the phospholipids in the place of 2-aminoethyl phosphate.

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The Enzymic Production of 2,5-D-Threo-diketohexose*

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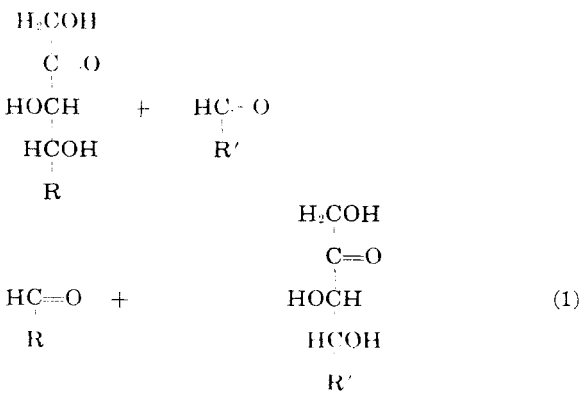
Received February 9, 1962

The enzymic synthesis of 2,5-D-threo-diketohexose has been achieved. The compound has been obtained as a product of a transaldolase reaction between fructose 6-phosphate and hydroxypyruvic aldehyde. The nature of the compound has been established by paper chromatography after reduction to the corresponding alcohols and by periodate oxidation of the isolated product before and after $NaBH_4$ reduction. Further identification was provided by the susceptibility of the reduction product to oxidation by an enzyme of known stereospecificity.

The enzyme transaldolase catalyzes an exchange reaction in which a dihydroxyacetone group is transferred from a ketose donor to an aldehyde acceptor. The reaction may be represented as follows:



(1)

As indicated in equation (1), both the substrate and the product which is formed must have hydroxyls in the *trans* configuration in positions 3 and 4 (Bonsignore, 1959). In addition to erythrose 4-phosphate and glyceraldehyde 3-phosphate, which were the first acceptors described for this reaction (Horecker and Seegmiller, 1953), other aldehydes have been recognized to react, including D-ribose 5-phosphate (Racker, 1955–1956), L-glyceraldehyde 3-phosphate (Venkataraman *et al.*, 1960), D-erythrose (Prandini and Lopes do Rosario, 1960), D-glyceraldehyde (Bonsignore *et al.*, 1959), and formaldehyde (Venkataraman and Racker, 1961). Some of the products of the transaldolase reaction are familiar metabolites. Others, like octulose 8-phosphate, which is formed when ribose 5-phosphate is the acceptor, were first synthesized by this enzymic reaction and only later demonstrated in biological materials (Charlson and Richtmyer, 1960).

Except for formaldehyde all of the known acceptors for transaldolase possess a hydroxyl group in the position adjacent to the aldehyde group. We have now found that hydroxypyruvic aldehyde, which does not possess such a hydroxyl group, may also act as an acceptor for transaldolase. The 2,5-diketohexose which is formed has been characterized as 2,5-D-threo-diketohexose, possessing the configuration of the hydroxyl groups in positions 3 and 4 which would be expected from the known specificity of trans-

* Supported by a grant from the United States Public Health Service (A 5228).