

The Occurrence of α -Amino- β -phosphonopropionic Acid in the Zoanthid, *Zoanthus sociatus*, and the Ciliate, *Tetrahymena pyriformis**

J. S. KITTRIDGE† AND R. R. HUGHES

From the Department of Biochemistry,
City of Hope Medical Center, Duarte, Calif.

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A new amino acid, α -amino- β -phosphonopropionic acid, was first detected in aqueous-ethanolic extracts of the Zoanthid, *Zoanthus sociatus*, by ion-exchange chromatography and paper electrophoresis. The identity of the natural and the synthetic amino acid was established by isolating the labeled amino acid from the hydrolysate of an aqueous-ethanolic extract of the ciliate, *Tetrahymena pyriformis*, which had been cultured in media containing [32 P]phosphate, crystallizing it in the presence of the synthetic amino acid and demonstrating a constant specific activity through four serial crystallizations. The labeled and the synthetic compound also exhibited complete coincidence during paper chromatography, paper electrophoresis, and ion-exchange chromatography.

The first compound containing a covalent carbon-to-phosphorus bond to be isolated from biological material was β -aminoethylphosphonic acid (Horiguchi and Kandatsu, 1959, 1960). The same compound and its glycerol ester were found in sea anemones (Kittredge *et al.*, 1962). Later Kandatsu and Horiguchi (1962) isolated β -aminoethylphosphonic acid from *Tetrahymena pyriformis* and showed that about 13% of the total phosphorus of this ciliate was in the phosphonic acid. Kittredge *et al.* (1962) had demonstrated that saponification of the crude lipid extract of the sea anemone released significant amounts of the aminoethylphosphonic acid. Rousser *et al.* (1963) undertook the isolation of this new phosphonic acid-containing lipid, which proved to be a ceramide.

With the demonstration of a covalent phosphorus-to-carbon bond in a compound found in two phyla, *Protozoa* and *Coelenterata*, one might expect that the enzymatic ability to synthesize this bond would be exhibited in other simple compounds. Of immediate interest was the possibility that the phosphonic acid, α -amino- β -phosphonopropionic acid, may occur as the precursor of β -aminoethylphosphonic acid, since the biosynthetic route to many amines is through the decarboxylation of the corresponding amino acid. An unsuccessful search was made for this compound in ethanolic extracts of the sea anemone, *Anthopleura elegantissima*. Analogy with the decarboxylation of phosphatidylserine to phosphatidylethanolamine and subsequent hydrolysis to free ethanolamine phosphate (Borkenhagen *et al.*, 1961) suggested the fractionation of sea anemone lipids. A chloroform-methanol extract was fractionated on a DEAE-cellulose column by gradient elution and the "phosphatidylserine" fraction was hydrolyzed and examined for the suspected new amino acid without success.

With the availability of synthetic α -amino- β -phosphonopropionic acid (Chambers and Isbell, 1964; Chavane, 1949) we were able to develop the ion-exchange column-chromatographic procedures for its isolation, and the paper-chromatographic and paper-electrophoretic techniques necessary for monitoring the fractionation. The present paper reports the detection and the isolation of this new amino acid.

MATERIALS AND METHODS

Tetrahymena pyriformis W 14.—The initial culture, kindly provided by Dr. J. B. Loefer, was carried in

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auxenic culture. The media contained Difco proteose-peptone No. 4, 20 g; Difco yeast extract, 5 g; and dextrose, 8.5 g/liter. Stock cultures were carried in 10 ml of media in culture tubes and were transferred weekly. Batch cultures were grown in 350 ml of media in 1000-ml Erlenmeyer flasks inoculated with 2 ml of stock culture. These were grown on a rotary shaker at 37° and were harvested near the end of the log phase of growth at 36 hours. The cells were harvested by chilling to 3–4°, anaesthetizing the organisms with CO₂, and centrifuging in a swing-bucket centrifuge at 1000 rpm. The medium was aspirated immediately and 25 ml of chilled ethanol was added to the pellet.

Corals.—Five species of corals and zoanthids were kindly collected and identified by Dr. F. M. Bayer of the University of Miami Marine Laboratory. Dr. E. F. Corcoran aided materially in making them available to us.

Synthetic α -Amino- β -phosphonopropionic Acid.—This work would not have been possible without the kind gift of the synthetic amino acid by Dr. A. F. Isbell of the Department of Chemistry of the A. and M. College of Texas. The synthesis of this compound will be published elsewhere. (Chambers and Isbell, 1964).

Paper Chromatography.—A rapid method for monitoring column fractions has been devised. A flat spiral of 5-mm glass cane with just sufficient intercoil clearance for filter paper, the inner end extended 12 cm on the axis for a handle, and three 5-mm teats for legs will support an 11.5 × 56-cm coil of filter paper in a 600-ml beaker. Thirty fractions may be spotted 1.5 cm from one edge. Solvent (25 ml) is placed in the beaker and an aluminum-foil cover is crimped on. A 10-cm development may be completed rapidly and, as column fractions seldom contain complex mixtures, location of components, an evaluation of their purity, and an approximate identification of the components may be obtained.

The solvents used were those of Smith (1960, p. 84) with the addition of an acidic high-water-capacity solvent; *t*-butyl alcohol-methyl ethyl ketone-formic acid (90.8%)-water, 10:5:1:10 (v/v).

Chromatography to determine the coincidence of migration of isotopically labeled isolate and the synthetic compound was carried out by descending development in well-equilibrated chromatography cabinets on 56-cm strips of Whatman 3MM filter paper.

Paper Electrophoresis.—The most reliable technique for detecting the new amino acid was electrophoresis in 1 N formic acid buffer. Migration for 3.5 hours at 19 v/cm in a water-cooled (2–3°) apparatus (E-C Apparatus Co.) was sufficient to differentiate it from all

of the ninhydrin-reactive compounds with which it was compared. In determining the coincidence of migration with the synthetic compound the migration was continued for 7 hours. Electrophoresis was also carried out at pH 5.3 in a pyridine-acetic acid-water buffer (10 ml of pyridine and 4 ml of acetic acid per liter) at 19 v/cm for 1.5 hours.

Location Reagents.—The ninhydrin reagent was 0.2% (w/v) in 95% ethanol-acetic acid-collidine, 16:3:1 (v/v). The sprayed paper was placed, while still wet, in an oven at 95° for 2–3 minutes. This yielded an azure-blue spot with the phosphonic acid. The ninhydrin-treated papers were preserved by washing with cupric nitrate (saturated aqueous solution of $\text{Cu}(\text{NO}_3)_2$ -acetone-10% HNO_3 , 5:500:1).

The phosphonic group was detected with the dip reagent developed by Rosenberg (1959) 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.

Ion-Exchange Resins.—The resins employed were AG50W-X8, 200–400 mesh, and AG1-X10, 100–200 mesh, as supplied by Bio-Rad Laboratories, Richmond, Calif. Fines were removed by three cycles of settling. The cation-exchange resin was conditioned by three cycles of treatment with three bed volumes each of 1 N NaOH and 4 N HCl, with water washes to neutrality. The volume of the final acid wash was doubled and the washing was continued until the effluent was free of chloride. The anion-exchange resin was converted to the formate or the acetate form through the bicarbonate form by eluting with saturated NaHCO_3 until chloride free, washing to neutrality, batch treatment with 4 N formic acid or acetic acid, and then washing with degassed water until neutral.

Radioactivity.—The activity of suitable aliquots was determined by drying the sample on 3-cm aluminum planchets with filter-paper disks and counting the radioactivity in a Nuclear-Chicago Model C-11 gas-flow counter. The count rates were corrected for background and decay, but not for self-absorption, coincidence, or efficiency of the detector.

The location of radioactivity on paper chromatograms and paper electrophoresis was determined by scanning the strips with a Nuclear-Chicago Model C100B gas-flow counter equipped with a count-rate meter (Model 1620B) and a strip recorder (Model R-1000).

Quantitative Amino Acid Analysis.—Quantitative ninhydrin analysis of duplicate 0.2-ml aliquots was conducted in an organic-solvent system according to the method of Troll and Cannan (1953).

EXPERIMENTAL

Detection in Zoanthid.—The new amino acid was first detected in an ethanolic extract of the zoanthid *Zoanthus sociatus*. The zoanthid, contained in about 20 volumes of 70% ethanol in which it had been preserved, was decalcified with HCl and then homogenized briefly in a Virtis blender. The extract was filtered and the lipids were removed by extraction with diethyl ether. The aqueous phase was concentrated in a rotary evaporator and then hydrolyzed in 6 N HCl at 110° for 24 hours. The acid was removed by concentrating repeatedly in the evaporator. The extract was fractionated on a Dowex-50 H^+ column (2.5 × 20 cm) made up in 1 N formic acid. The column was washed with 100 ml of 1 N formic acid and then eluted with water. The phosphonic acid was detected in the frac-

tions at the water front. Comparison of the isolated material with the synthetic compound by paper chromatography in several developing systems indicated their identity. The isolated acid was rehydrolyzed with no detectable release of phosphorus. Electrophoresis followed by cutting the filter-paper strip in half along the center and staining one half with ninhydrin and the other with the molybdate reagent demonstrated the coincidence of migration of the amino group and the phosphonic group. Hydrolysis of the lipid extract, isolation of the water-soluble substances from the hydrolysate, followed by chromatography and paper electrophoresis indicated the presence of only β -aminoethylphosphonic acid.

Isolation from Tetrahymena.—The difficulty of obtaining sufficient amounts of the zoanthid, together with the rather tedious task of decalcifying and homogenizing, prompted an investigation of the ciliate, *Tetrahymena pyriformis*. Neither the acid-soluble (0.5 N HClO_4) fraction nor the chloroform-methanol (2:1) extract of about 4 g of cells yielded any detectable α -amino- β -phosphonopropionic acid. However, when two 350-ml-batch cultures were allowed to deplete the phosphate in the simple medium for 35 hours and then were allowed to take up ^{32}P -labeled phosphate for 1 hour the ethanolic extract yielded a nonhydrolyzable labeled fraction which migrated on electrophoresis with the synthetic compound. Further trials revealed that the addition of chilled ethanol to the pellet in the centrifuge tubes immediately after aspirating the media to stop any enzymatic breakdown increased the yield.

Four flasks were inoculated and cultured for 33 hours at which time 2.5 mc of carrier-free radioactive orthophosphate was added aseptically to each culture. The flasks were replaced on the shaker for a further 3 hours and the cells were harvested after 36 hours. The yield was 40.5 g wet weight of cells. The cells were homogenized with 20 volumes of 70% ethanol and filtered through a Celite bed, and the filter cake was re-extracted twice with 10 volumes of ethanol. The combined filtrate was concentrated to about 20 ml on a rotary evaporator at 40°. The extract was hydrolyzed in 500 ml of 6 N HCl under reflux for 24 hours, concentrated to about 10 ml in the rotary evaporator, diluted to about 100 ml, and concentrated twice again to remove much of the HCl. The hydrolysate was diluted to 50 ml, decolorized with Norit, filtered through glass filter paper (Hurlbut No. 9844 ultra filter), and concentrated to 10 ml.

The crude hydrolysate was fractionated on a Dowex-50 H^+ cation-exchange-resin column, (4.2 × 16 cm) made up in 1 N formic acid. The column was washed with 1 N formic acid until the yellow front came off, then the column was eluted with water. Ten-ml fractions were collected. Most of the phosphate came off of the column at the front (100 through 120 ml of eluate) but phosphate always trails on Dowex-50 H^+ . Fractions 30 through 57 (0.2 ml each) were plated on planchets and radioactivity was counted. Fractions 30 through 43 averaged 2000 cpm/fraction. The pH of the eluate increased after fraction 42. Figure 1 is a plot of the radioactivity of the eluate and indicates the peak of activity found in the eluate from 440 through 530 ml. The total peak was pooled and concentrated to 0.5 ml.

To remove the residual phosphate ion the peak from the Dowex-50 H^+ column was fractionated on a column of Dowex-1 in the formate form. The column (0.7 × 44.5 cm) was made up in 1 N formic acid. The sample was transferred and washed in with 1 N formic acid and the column was washed with 72.5 ml of 1 N formic acid. Three fractions of 20 ml each were collected and

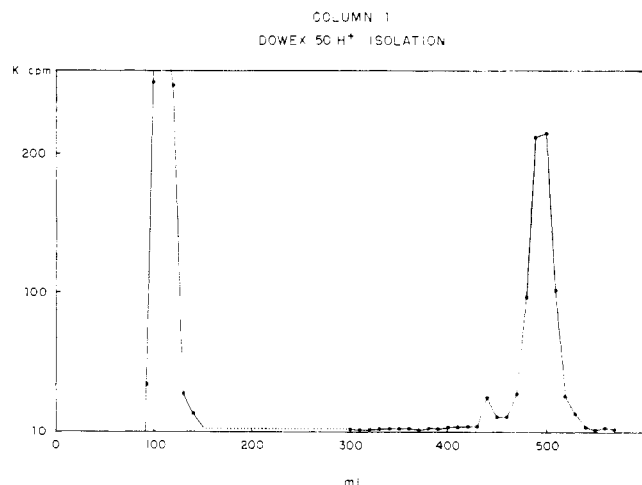


FIG. 1.—Isolation of acidic amino acid from hydrolysate of ethanolic extract. The column (Dowex-50 H^+) was made up in 1 N formic acid and washed with 1 N formic acid and eluted with water. The first peak is phosphate and the second peak contains the amino acid with nonhydrolyzable radioactive phosphorus.

then 2-ml fractions were collected. The column was eluted with 0.3 M ammonium formate. Fifty μ l of the even fractions 4 through 28 were plated for counting. Figure 2 indicates that phosphonic acid was found in the eluate from 60 through 106 ml. To confirm that the phosphate contamination was fractionating properly, fractions 30–84 (0.2 ml each) were plated and radioactivity was counted. Slight peaks of activity were noted at 136 and 164 ml eluate and the phosphate peak was found in the fractions expected, peaking at 200 ml eluate. This activity also migrated with phosphate during electrophoresis in 1 N formic acid buffer. The total peak was pooled and concentrated on the rotary evaporator to 2 ml, and the ammonia was removed by passing the extract through a 1×15 -cm column of Dowex-50 H^+ and washing with water. Most of the formic acid was removed by concentrating the eluate repeatedly on the rotary evaporator to about 1 ml. It was noted during preliminary isolations that the aminophosphonic acid tends to form a difficultly soluble residue, possibly an anhydride, if the extract is taken to dryness in the evaporator.

The isolated material was transferred to a 50-ml volumetric flask and diluted to volume. Two 0.5-ml aliquots were plated on planchets for counting and two 0.5-ml aliquots were transferred to 10-ml volumetric flasks for quantitative ninhydrin analysis.

Crystallization with Synthetic Amino Acid.—The crystallization was carried out in a 9×45 -mm test tube that was calibrated and scribed for volume in 0.2-ml increments. Recrystallized synthetic α -amino- β -phosphonopropionic acid (44.6 mg) was transferred to the crystallization tube. The volume of the solution of the isolated phosphonic acid was reduced to about 0.5 ml and this was transferred to the crystallization tube with several rinses. The volume in the tube was reduced to about 0.3 ml between each transfer with a jet of nitrogen while the tube was in a boiling-water bath. The walls were rinsed and the volume in the tube was diluted to 0.75 ml with water. Crystallization was induced by chilling and adding ethanol slowly with stirring until cloudiness appeared (about 30–40% ethanol) and then diluting to 50% ethanol. Crystallization was allowed to take place for 1 hour in an ice bath. The crystals were filtered on a Willstaetter button supporting a 1-cm disk of Whatman 41H filter

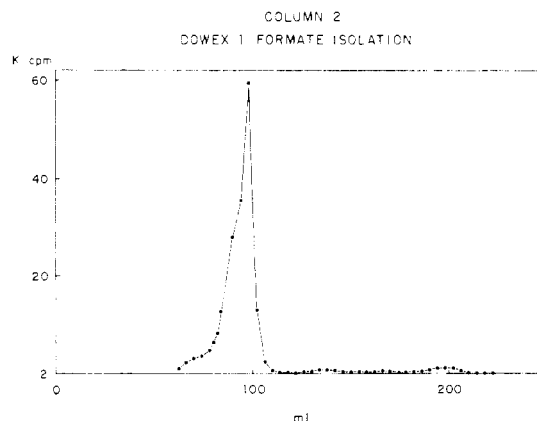


FIG. 2.—Purification of the acidic amino acid on an anion-exchange resin (Dowex-1 formate). The column was made up and washed with 1 N formic acid and eluted with 0.3 M ammonium formate. The small peak at 200 ml is phosphate.

paper and washed with chilled 50% ethanol and absolute ethanol. Under these conditions it was found that about 20% of synthetic α -amino- β -phosphonopropionic acid remained in the filtrate. The crystals were redissolved from the filter with water and diluted to 50 ml, two 1-ml aliquots were plated for counting, and triplicate 0.1-ml aliquots were pipetted into 10-ml volumetric flasks for ninhydrin analysis.

The total volume was again reduced to about 0.5 ml and transferred to the crystallization tube using the same procedures. The volume of water and ethanol and dilution volume before aliquoting were suitably reduced for each crystallization so that the concentrations remained approximately the same. The crystallization was repeated four times. The radioactivity of the aliquots was determined by counting the radioactivity of the planchets serially ten times at 12,800 events per count.

Failure to Release Orthophosphate by Hydrolysis from Crystals.—To demonstrate that all of the phosphate was indeed nonhydrolyzable, and therefore covalently bonded, one-fifth of the fourth crop of crystals was rehydrolyzed in 6 N HCl at 110° for 24 hours. The HCl was removed by repeated concentration in the rotary evaporator, 4.2 mg of cold phosphate was added, and the solution was concentrated to about 1 ml and made 1 N in formic acid. This was subjected to fractionation on a Dowex-50 H^+ column, 0.7×22 cm, made up in 1 N formic acid. The column was washed with 25 ml of 1 N formic acid and then eluted with water. The phosphate peak was in fractions 6 and 7. Figure 3 shows that the α -amino- β -phosphonopropionic acid was eluted in a peak from 48 through 66 ml of eluate with complete coincidence of the ninhydrin-reactive material and the activity. A slight amount of activity preceded the phosphate peak which represented 1.1% of the total activity.

Paper Electrophoresis and Paper Chromatography.—Further proof of the identity of the isolated material and the synthetic phosphonic acid was gained by subjecting aliquots of the fourth crop to paper electrophoresis at two pH values and to paper chromatography in four different developing systems selected for their diverse fractionating abilities, and by gradient elution from an anion-exchange column. After development, the paper chromatograms and paper electrophoresis strips were sprayed with the ninhydrin reagent and heated, and the colored spots were preserved with the $Cu(NO_3)_2$ wash. Each was then coded for rematching with the standard runs and cut into strips suitable for

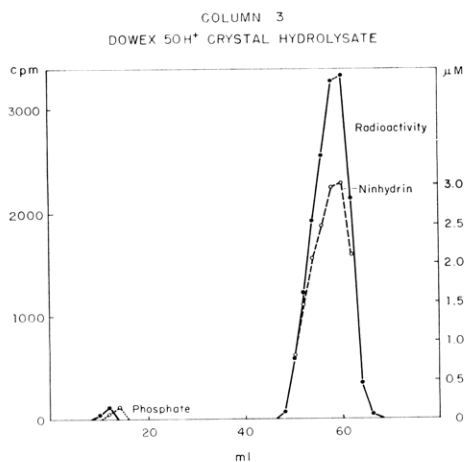


FIG. 3.—The recrystallized amino acid was hydrolyzed in 6 N HCl at 110° for 24 hours. Fractionation on a Dowex-50 H⁺ column made up in 1 N formic acid, washed with formic acid, and eluted with water indicated the stability to hydrolysis of the phosphorus-to-carbon bond. The 1.1% of the activity eluted in front of the cold phosphate added to the hydrolysate probably is an oxidative-breakdown product of the amino acid.

the gas-flow counter. In Figure 4 the records from the strip counter are shown with each of the paper-electrophoresis and paper-chromatography runs.

Gradient Elution.—The remainder of the fourth crop from the crystallization was subjected to the more subtle fractionating power of a gradient-elution scheme. The column was Dowex-1 in the formate form (0.7 × 43.2 cm) made up in 0.5 N formic acid. The sample was applied in 1 ml 0.5 N formic acid and rinsed in with 3 × 1 ml of 0.5 N formic acid. The elution was started with 50 ml of 0.5 N formic acid in the mixing chamber and 50 ml of 0.5 N ammonium formate in the reservoir. Two-ml fractions were collected and 0.5 ml of each was plated for counting. Figure 5 shows that the activity was found in a peak from 54 through 70 ml eluate. The ammonia was removed from each of these fractions by passing them through 0.7 × 5-cm columns of Dowex-50 H⁺ with three bed volumes of water wash. They were then concentrated repeatedly to remove the formic acid and diluted to volume, and aliquots were withdrawn for quantitative ninhydrin analysis. The ninhydrin peak corresponded precisely with the peak of activity.

Isolation of β -Aminoethylphosphonic Acid.—The β -aminoethylphosphonic acid in the original ethanolic extract was retained on the initial Dowex-50 H⁺ column. The column was eluted with 3 N ammonium hydroxide and the front, containing all the neutral and acidic amino acids, was pooled and concentrated repeatedly in the rotary evaporator to remove the ammonia. This was applied to a column of Dowex-1 in the acetate form which had been exhaustively washed with degassed water. The column was eluted with 0.001 N acetic acid and 4-ml fractions were collected. The neutral amino acids were eluted at the front (fractions 10–17) and the aminoethylphosphonic acid in fractions 29 through 40. The total peak was pooled and concentrated to 1 ml, and the aminoethylphosphonic acid was crystallized by adding ethanol and chilling. The crystals were filtered off, washed, dissolved in water, and diluted to a volume of 25 ml from which aliquots were withdrawn for quantitative ninhydrin analysis and counting.

Bound α -Amino- β -phosphonopropionic Acid.—The cell residue from the filter pad after ethanolic extraction

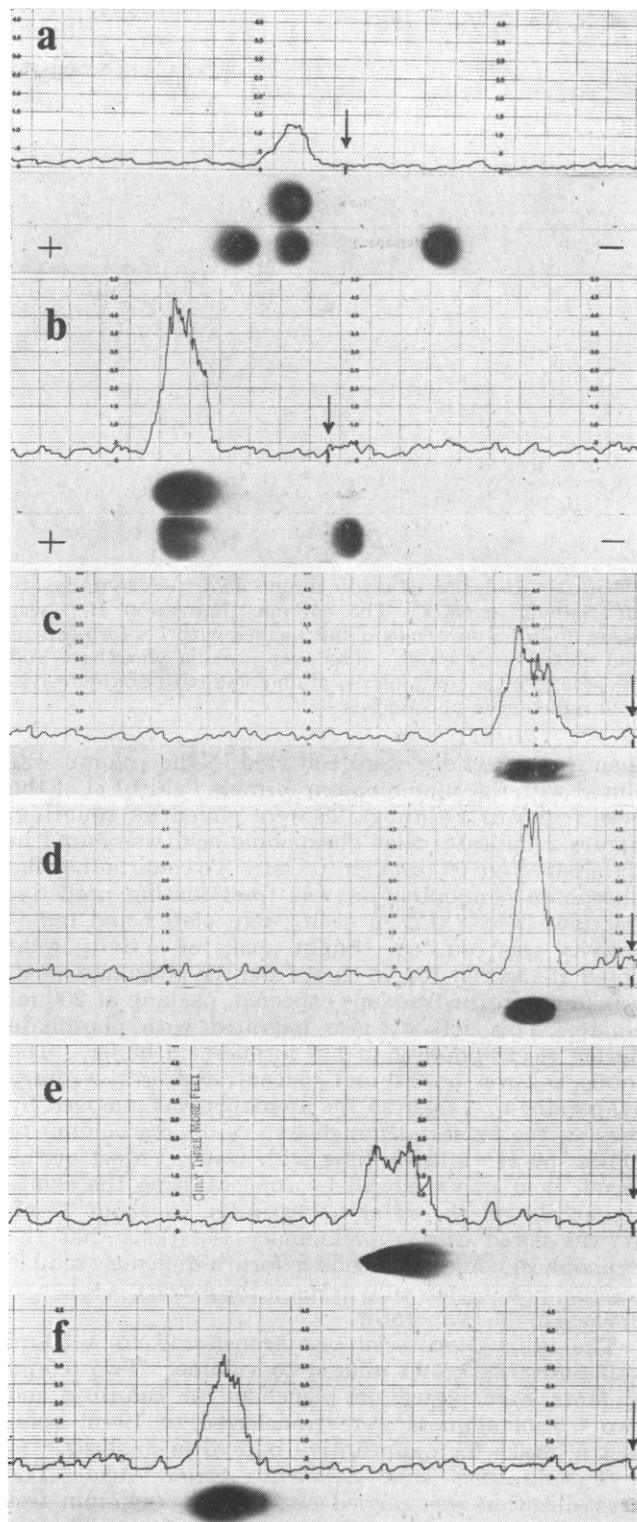


FIG. 4.—Paper electrophoresis and paper chromatography. (a) Electrophoresis in 1 N formic acid buffer at 19 v/cm for 7 hours. The trace indicates the distribution of radioactivity along the upper strip. The upper spot is an aliquot after crystallization; the lower spots are standards (from left to right), serine phosphate, α -amino- β -phosphonopropionic acid, β -aminoethylphosphonic acid. (b) Electrophoresis, pH 5.3 buffer (pyridine, 10 ml; acetic acid, 4 ml/liter) at 19 v/cm for 1.5 hours. Upper and lower as in (a). Serine phosphate and α -amino- β -phosphonopropionic acid migrate together at this pH. (c)–(f) Paper chromatography of aliquots of the fourth crop of crystals. Developed with (c) water-saturated phenol; (d) butanol-acetic acid-water, 12:3:5 (v/v); (e) *t*-butyl alcohol-methyl ethyl ketone-diethylamine-water, 10:5:1:10 (v/v); (f) *t*-butyl alcohol-methyl ethyl ketone-formic acid (90.8%)–water, 10:5:1:10 (v/v). All papers were sprayed with ninhydrin and preserved with Cu(NO₃)₂ wash.

TABLE I
SPECIFIC ACTIVITY FROM SUCCESSIVE RECRYSTALLIZATIONS

	1st	Crystallization 2nd	3rd	4th
Radioactivity (cpm)	152,100	134,100	116,760	92,380
Amino acid (μ M)	146	126	110	90
cpm/ μ M	1,042	1,065	1,062	1,027

was hydrolyzed and the hydrolysate was fractionated on similar ion-exchange columns.

RESULTS

The results of the assays of the aliquots of each of the successive crops of crystals from the mixture of synthetic α -amino- β -phosphonopropionic acid and the isolated aminophosphonic acid are shown in Table I. The specific activity of each crop of crystals was constant within the limits of error of the assay technique (the aliquot volumes for the ninhydrin assay were the principal source of error). Further, there is no trend of change of specific activity throughout the series.

The isolated radioactive compound resisted hydrolysis, both during isolation and after crystallization. It is felt that the 1.1% of the activity which emerged at the front of the final Dowex-50⁺ column represents oxidative breakdown of the α -amino acid. The coincidence of the elution of radioactivity and ninhydrin-reactive material, both from this column and from the more selective gradient elution of the Dowex-1-formate column, further support the identities of the isolated and the synthetic amino acid.

The coincidence of migration of the radioactivity and of the ninhydrin-reactive material during paper chromatography in four developing systems and during electrophoresis at two pH values is illustrated in Figure 4. The standards run with the electrophoresis include β -aminoethylphosphonic acid, the only other compound containing nonhydrolyzable phosphorus which has been isolated from biological material, and serine phosphate, the phosphate ester analog of α -amino- β -phosphonopropionic acid. In the 1 N formic acid buffer β -aminoethylphosphonic acid migrates cathodically while serine phosphate migrates anodically at almost twice the rate that α -amino- β -phosphonopropionic acid does. At pH 5.3 serine phosphate and α -amino- β -phosphonopropionic acid migrate together.

The assay of the aliquots from the pooled peak of the initial Dowex-1-formate column indicated that there was 18 μ M of α -amino- β -phosphonopropionic acid isolated with a specific activity of 17,450 cpm/ μ M. The hydrolysis of the cell residue yielded approximately 5 μ M of the phosphonic acid. Since preliminary experiments in which the lipids were extracted with chloroform-methanol (2:1) had failed to yield any activity migrating with α -amino- β -phosphonopropionic acid when the water-soluble fraction of the lipid hydrolysate was examined, the rather large amount of α -amino- β -phosphonopropionic acid liberated on hydrolyzing the cell residue may indicate that this new amino acid can occur in peptide linkage. It is felt that an attempt to isolate acidic peptides containing the new amino acid from a mild hydrolysis of the cell residue is the most promising technique for investigating this possibility.

The β -aminoethylphosphonic acid peak from the Dowex-1-acetate column yielded 130 μ M of this phosphonic acid with a specific activity of 53,200 cpm/ μ M. While the higher specific activity of the β -aminoethylphosphonic acid tends to negate the original assumption

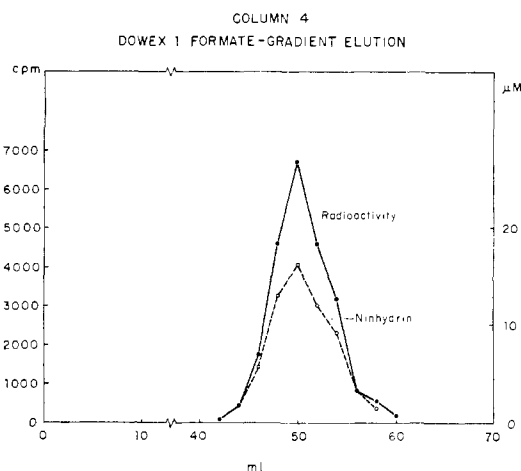


FIG. 5.—Gradient elution of the product of recrystallization of the synthetic and the isolated amino acid. The column was Dowex-1 in the formate form, made up in 0.5 N formic acid and eluted with 50 ml of 0.5 N formic acid in the mixing chamber and 0.5 N ammonium formate in the reservoir.

tion that α -amino- β -phosphonopropionic acid may be the precursor, the extremely long labeling period together with the fact that there may be several pools of free and bound forms of each phosphonic acid leaves this question open for further investigation.

The combined results are submitted as evidence for the occurrence in biological systems of a new aminophosphonic acid, which by all criteria examined behaves identically with the synthetic α -amino- β -phosphonopropionic acid.

In the course of a survey of the distribution of phosphonic acids in the *Coelenterata* (to be published later) we have isolated eight phosphonic acids, five of which are ninhydrin positive. It is felt that the ability to form the stable phosphorus-to-carbon bond must represent an important biosynthetic advantage to these organisms. As the field is entirely uninvestigated, one can only guess at possible functions of the phosphonic acids. However, the two phyla in which these compounds have been found live in aqueous environments and would be exposed to all of the cations present in natural waters. Since the two pK values of phosphonic acids are higher than the pK values of the corresponding phosphate ester analogs (Chavane, 1949), these compounds, especially the aminophosphonic acids, may provide the organism with a more selective ability to cope with the wide ranges in the concentrations of cations to which they may be exposed.

In the case of the present aminophosphonic acid, which may be considered to be the phosphonic acid analog of either serine phosphate or aspartic acid, we may surmise that it may exist in peptide linkage and furnish an acidic site in proteins. The liberation of considerable α -amino- β -phosphonopropionic acid on hydrolysis of the alcohol-extracted cell residue lends strength to this possibility. It may also exist in phospholipids, as does the β -aminoethylphosphonic acid, although to date it has not been detected in crude lipid extracts of *Tetrahymena* or *Zoanthus*.

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Variations in Affinities of Antibodies during the Immune Response*

HERMAN N. EISEN AND GREGORY W. SISKIND†

From the Department of Microbiology,
 Washington University School of Medicine, St. Louis, Missouri

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The affinities of anti-2,4-dinitrophenyl (DNP) antibodies for a variety of 2,4-dinitrobenzenes have been measured by fluorescence quenching and the values obtained have been verified in representative instances by equilibrium dialysis. All populations of anti-DNP molecules examined, whether isolated from pooled sera or from single bleedings of individual rabbits, were heterogeneous in respect to affinity for dinitrobenzenes. The heterogeneity in virtually every instance could be described by the Sips distribution function over a wide range of binding data, and each titration was thus characterized by an average intrinsic association constant (K_s), and by a heterogeneity index (α). Fractional precipitation of antibodies from serum, by addition of limiting amounts of antigen, yielded from the serum of individual rabbits anti-DNP populations that differed as much as 10,000-fold in K_s . Changes in affinity for ϵ -DNP-lysine were followed by isolating antibodies from sera at various times after a single injection of DNP-bovine- γ -globulin. The K_s increased progressively with time after immunization and the rate of increase was most conspicuous when small quantities of DNP-bovine- γ -globulin were injected initially. The rise in K_s was markedly delayed when large doses of antigen were injected. Antibody populations isolated early after immunization had nearly the same low affinity for both ϵ -DNP-L-lysine and for 2,4-dinitroaniline, and it is inferred that their specific binding sites are poorly adapted to the dinitroanilino group and insensitive to the norleucine moiety of ϵ -DNP-lysine. In contrast, antibodies isolated late after immunization bind ϵ -DNP-lysine strongly, and from their interactions with a variety of dinitrobenzenes it is inferred that their binding sites, on the average, are just about large enough to accommodate ϵ -DNP-L-lysine. Complex formation was enthalpy driven: ΔH° values ranged from -8 to -20 kcal mole $^{-1}$ for different antibody-ligand pairs, and ΔS° values ranged from -5 to -30 entropy units mole $^{-1}$. When representative 2,4-dinitrobenzenes were bound by anti-DNP molecules, bathochromic and hypochromic spectral shifts were observed; in the case of antibody-bound 2,4-dinitrotoluene a new absorption peak appeared at 300 m μ . The possibility is raised that charge-transfer contributes to the stability of antibody-dinitrophenyl complexes.

A fundamental difference between antibodies and enzymes is manifested in the specific binding of their respective ligands. Enzyme preparations of a given specificity generally behave as though composed of homogeneous molecules with uniform capacity to bind their substrate. Antisera of a given specificity, on the other hand, invariably seem to contain antibodies with diverse affinities for any appropriate ligand (e.g., Landsteiner and van der Scheer, 1936; Heidelberger and Kendall, 1935; Pauling *et al.*, 1944). The heterogeneity of antibodies in respect to affinity, which is implied by the properties of antisera, has been confirmed in studies of the reversible binding of haptens

by purified antibodies (e.g., Eisen and Karush, 1949; Karush, 1956; Nisonoff and Pressman, 1958). In view of this heterogeneity, average association constants have been determined on the assumption that the specific binding sites in any population of antibody molecules are characterized by a normal distribution function in respect to affinities (Pauling *et al.*, 1944; Karush, 1956). The average values thus found for a variety of antibody-hapten systems have, until recently, been remarkably similar, ranging from about 10^5 to 10^6 liters mole $^{-1}$ (Karush, 1962; Eisen and Pearce, 1962), and the uniformity of these values has been interpreted (e.g., by Nisonoff *et al.*, 1959a) as evidence in support of Pauling's theory of antibody formation. According to this theory (Pauling, 1940), antibodies of exceptionally high affinity for antigen would be infrequent since, during biosynthesis, their dissociation from hypothetical antigen-templates would be improbable, whereas antibodies of unusually low affinity would escape detection and isolation.

Within the past few years, however, two studies of the interaction between ϵ -DNP-lysine¹ and antibodies specific for the 2,4-dinitrophenyl group have given

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† Present address: Department of Biology, Harvard University, Boston, Mass. Supported by a U. S. Public Health Service Research Training Fellowship administered by the Department of Medicine, New York University College of Medicine.

¹ Abbreviations used in this work: DNP, 2,4-dinitrophenyl; B γ G, bovine γ -globulin; Tris, tris(hydroxymethyl)aminomethane. Where ϵ -DNP-L-lysine appears unqualified by L or D it represents ϵ -DNP-L-lysine.