Isolation and Characterization of a Phosphonic Acid Rich Glycoprotein Preparation from *Metridium dianthus*†

Richard L. Hilderbrand,‡ Thomas O. Henderson,* Thomas Glonek, and Terrell C. Myers

ABSTRACT: In studies on the phosphonate and phosphate–composition of the sea anemone *Metridium dianthus*, 78% of the phosphorus present in the hot-water extract of delipidated homogenates was found by ^{31}P nuclear magnetic resonance (nmr) to be phosphonate–phosphorus. Chromatography on this material yielded a phosphonate-rich glycoprotein preparation. The preparation contained 11% aminoethylphosphonic acid (referred to as the phosphonic acid), 22% neutral sugars, 4% hexosamines, and 40% protein (isoelectric point of 3.7); no orthophosphates were present. Amino acid analysis showed the presence of high relative amounts of threonine, serine, aspartic acid, glutamic acid, and hexosamines (glucosamine and galactosamine) in addition to the phosphonic acid (28% of the total ninhydrin reactive material). An effective Stoke's radius equal to or greater than that of a globular portein of molecular weight 1.5×10^6 daltons was indicated by Bio-Gel A 1.5m filtration; however, ultracentrifugation indicated a Stoke's radius equivalent to 1.5×10^5 daltons. A similar preparation was obtained by a procedure involving extraction of homogenized animals with lithium diiodosalicylate. This preparation had an amino acid, neutral sugar, hexosamine, and phosphonic acid composition similar to the preparation described above but contained phosphate–phosphorus in the same concentration as phosphonate–phosphorus.

The discovery of biological phosphonates in 1959 by Horiguchi and Kandatsu (1959) led to the subsequent identification of aminoethylphosphonic acid ($NH_2CH_2CH_2PO_3H_2$) as the major alkylphosphonate molecule in lipids of various species of lower marine invertebrates. Concomitant with identification of the phosphonolipids were reports of the phosphonic acid in hydrolysates of insoluble protein from the sea anemone *Metridium dianthus* (Quin, 1964), from the protozoan *Tetrahymena pyriformis* (Rosenberg, 1964), and from bovine brain (Shimizu *et al.*, 1965). Phosphonates have since been reported in several mammalian tissues, including human brain (Alhadeff and Daves, 1970) and atherosclerotic plaques (Alam and Bishop, 1968). Although the phosphonic acid has been reported in insoluble proteinaceous residues, water-soluble biological polymers containing the phosphonic acid have only recently been isolated. Simultaneously, we (Hilderbrand *et al.*, 1971) and Kirkpatrick and Bishop (1971a) reported the isolation of such macromolecules from *M. dianthus* and *Metridium senile*, respectively. More recently, Korn *et al.* (1973) isolated a phosphonate-rich preparation from the membranes of the amoeba *Acanthamoeba castellanii* which they call a phosphonoglycan.

During a series of studies on the application of phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P nmr) to the determination of phosphonate–phosphorus in biological materials (Glonek *et al.*, 1970; Henderson *et al.*, 1972), we observed the presence of phosphonate in a hot H_2O extract of delipidated *M. dianthus* homogenates. Sephadex G-100 gel filtration yielded a voided fraction which contained 1.7% phosphonate–phosphorus and little or no phosphate–phosphorus. Amino acid analysis verified the presence of the phosphonic acid and also indicated significant proportions of glutamic acid, aspartic acid, serine, threonine, and hexosamine (Hilderbrand *et al.*, 1971), the amounts of the latter being consistent with the composition of known glycoproteins (Winterburn and Phelps, 1972; Spiro, 1970).

Recently, a similar procedure has yielded a polydisperse, high molecular weight (*ca.* 7 S) glycoprotein fraction which is rich in the phosphonic acid (11.4% dry weight). The following is a report of the isolation of this fraction as well as evidence that phosphonoglycoproteins can be extracted by a specific glycoprotein extraction procedure utilizing lithium 3,5-diiodosalicylate and phenol (Marchesi and Andrews, 1971).

Materials and Methods

Large *M. dianthus* anemones (12 animals, 570 g wet weight) (Marine Biological Laboratory, Woods Hole, Mass.) were homogenized in 1 l. of 0.9% KCl using a Sorvall Omni-Mixer and lyophilized. The dry powder was extracted exhaustively with $CHCl_3$–MeOH (2:1, 1500 ml) and filtered; the extraction was repeated using $CHCl_3$–MeOH (1:1, 1200 ml). The residue was washed with 200 ml of acetone, air-dried, and extracted in 1 l. of boiling distilled H_2O for 10 min. Following Buchner filtration the material solubilized by hot H_2O was dialyzed for 24 hr against three 4-l. changes of H_2O and lyophilized (fraction 1; 2.93 g; see Figure 1 and Table I).

DEAE-cellulose (Whatmann DE-23), precycled according to manufacturer's instructions, was equilibrated with 0.04 M

* To whom correspondence should be addressed in the Department of Biological Chemistry.

† From the Department of Biological Chemistry and the Research Resources Laboratory, University of Illinois at the Medical Center, Chicago, Illinois 60612. *Received May 29, 1973.* This work was supported by the General Research support grant awarded to the University of Illinois College of Medicine, a grant from the Research Board of the Graduate College, University of Illinois at the Medical Center, and Grants USPHS-11702 and USPHS-NS-9354. R. L. H. received support from U. S. Public Health Service Training Grant 00471. This work is based upon a dissertation submitted by R. L. H. to the Graduate College of the University of Illinois at the Medical Center, in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biological Chemistry.

‡ Present address: University of California, Berkeley, School of Public Health, Naval Biomedical Research Laboratory, Naval Supply Center, Oakland, California 94625.

TABLE I: Composition of Principal Fractions from *M. dianthus*.[a]

Fraction	Dry Weight (mg)	Phosphorus (mg)	Phosphorus (%)	Relative % of Total Phosphorus as[b] P–C	Relative % of Total Phosphorus as[b] PO_4^{3-}	Phosphonic Acid (mg)	Phosphonic Acid (%)	Neutral Sugars (mg)	Neutral Sugars (%)	Protein (mg)	Protein (%)	Molar Ratio of Phosphonic Acid to Neutral Sugars	Fraction Accounted for
1	2930	20.5	0.7	78	22	84	2.86	199	6.8	1942	66.3	0.463	76.0
2	440	6.16	1.4	100	0	25	5.68	36.5	8.3	275	62.5	0.985	77.9
5	179	2.51	1.4	100	0	10	5.59	14.3	8.0	73.7	41.2	1.010	56.1
10	77	2.16	2.8	100	0	8.6	11.17	16.7	21.7	33.9	44.0	0.790	79.7
Gly-2	97	2.23	2.3	50	50	4.5	4.6	9.2	9.5	40.7	42.0	0.706	58.4

[a] The fractions listed were obtained as shown in the flow diagram in Figure 1 and described in Materials and Methods. [b] Calculation is based on ^{31}P nmr data. P–C represents phosphonate–phosphorus; PO_4^{3-} represents phosphate–phosphorus.

Tris-glycine buffer (pH 9.3) and a 2.2 × 45 cm column bed was poured in the same buffer. One thousand milligrams of fraction 1 was dissolved in 10 ml of the equilibration buffer and centrifuged at 6000*g* for 10 min to remove insoluble material (100 mg). The fraction 1 supernatant solution was divided into three equal volumes which were applied in three similar runs to the column bed and eluted using 50 ml of equilibration buffer, followed by a 400 ml of 1 M NaCl in buffer. The column effluent was monitored at 280 nm and 10-ml fractions were collected; two fractions were obtained, designated fraction 2 (440 mg) and 3 (240 mg), respectively, in order of their elution (Figure 2A). Fraction 2 was desalted by dialysis against water and gel filtration on Sephadex G-10 and rechromatographed as described below. Fraction 3 was not fractionated further.

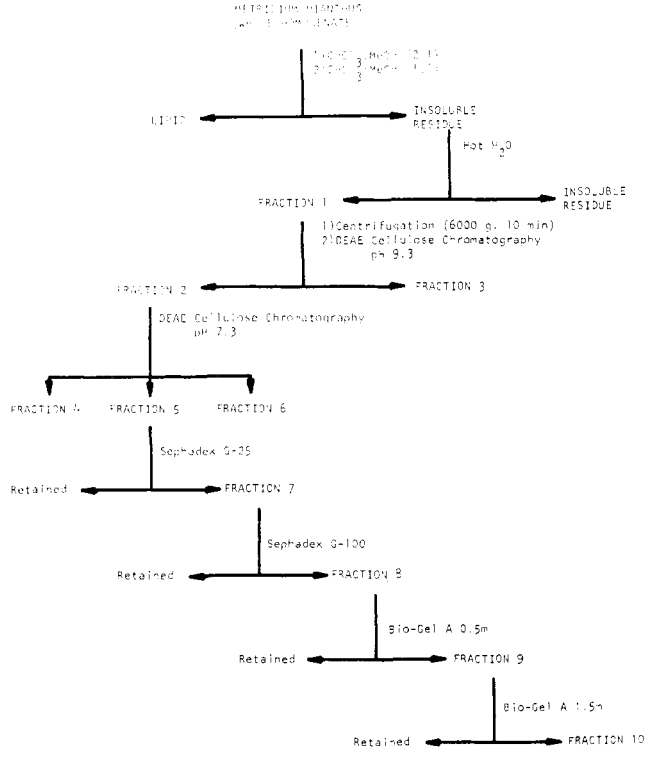


FIGURE 1: A flow diagram of the fractionation of *Metridium dianthus* which yielded a high molecular weight glycoprotein fraction enriched with 2-aminoethylphosphonic acid (fraction 10).

A second DEAE-cellulose column (2.5 × 22 cm), equilibrated with 0.04 M Tris-HCl (pH 7.3), was prepared to which a 300-mg aliquot of fraction 2 was applied and eluted using 100 ml of equilibration buffer, followed by a 500 ml 0–5% ammonium carbonate linear gradient in buffer. Three components, designated fractions 4 (33 mg), 5 (179 mg), and 6

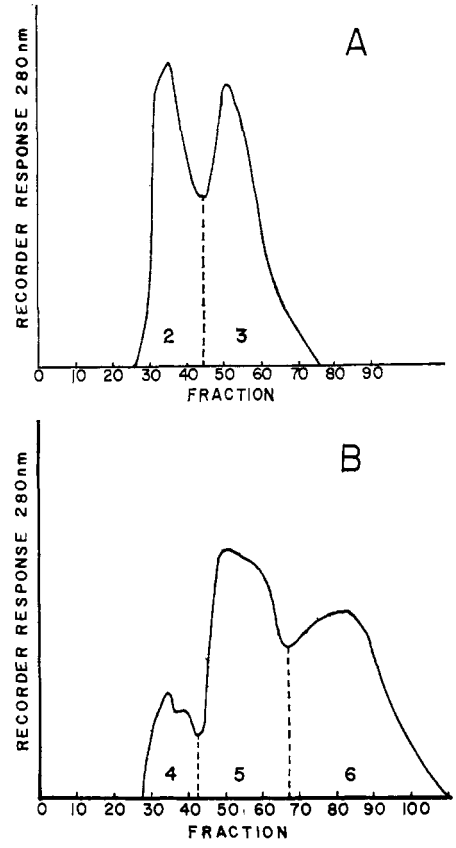


FIGURE 2: Elution profiles from DEAE-cellulose chromatography: (A) fraction 1 (see Figure 1 and Materials and Methods) gave rise to peaks 2 and 3 (corresponding to fractions 2 and 3 of Figure 1) when chromatographed with a 0–1 M NaCl linear gradient at pH 9.3; (B) fraction 2, from A, above, gave rise to peaks 4, 5, and 6 (corresponding to fraction 4, 5, and 6 of Figure 1) when chromatographed with a 0–5% ammonium carbonate linear gradient at pH 7.3. In both A and B, the dotted vertical lines indicate the arbitrary division of the indicated peaks.

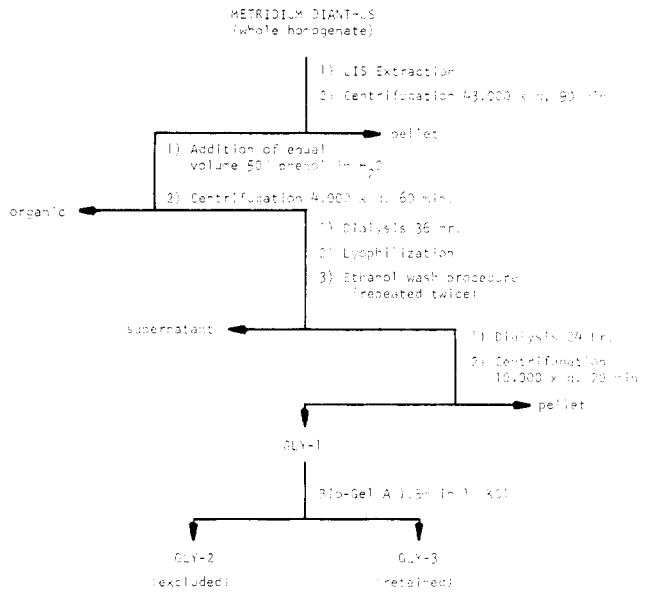


FIGURE 3: A flow diagram of the lithium diiodosalicylate–phenol–ethanol fractionation (as described by Marchesi and Andrews, 1971) which produced glycoprotein fraction enriched in 2-aminoethylphosphonic acid (Gly-2).

(45 mg), were obtained (Figure 2B). Fraction 5 was desalted as described for fraction 2.

Following evidence from an exploratory fractionation that the phosphonate–phosphorus was enriched in a high molecular weight material (Hilderbrand *et al.*, 1971), fraction 5 was fractionated by gel filtration using Sephadex G-25, Sephadex G-100, Bio-Gel A 0.5m, and Bio-Gel A 1.5m. The excluded fraction from each column was chromatographed on the next larger gel, yielding, finally, fraction 10 (77 mg), a phosphonate-rich fraction which was eluted with the void volume of Bio-Gel A 1.5m (exclusion limit of 1.5×10^6 daltons for globular materials).

The individual fractions were examined for phosphonate–phosphorus by ^{31}P nmr as described by Glonek *et al.* (1970) and Henderson *et al.* (1972). The basic instrumentation was a Bruker HFX-5 nmr spectrometer operating at 36.4 MHz for ^{31}P with a Fabri-Tek 1064 instrument computer for signal averaging.

Electrofocusing was done in an LKB Model 8101 ampholine column equipped with an LKB power supply. The solutions were prepared to manufacturer's specifications with the anode at the bottom of the apparatus and H_2SO_4 in the anode solution. An aliquot of fraction 10 (6.7 mg) was mixed in the light solution of the sucrose gradient for a pH 3–10 equilibration. The temperature was maintained at 4° and the column equilibrated (48 hr) at 0.09 W. Two milliliter fractions were collected at a flow rate of 1 ml/min and the pH, phosphorus concentration, and absorbance at 280 nm of individual fractions determined.

A 10-mg/ml solution of fraction 10 (0.4 ml) was run in H_2O in a Beckman Model E analytical ultracentrifuge using schlieren optics to obtain the sedimentation pattern and constant (56,100 rpm, 88 min).

Amino acid analyses were carried out using a Spinco Model 120 C analyzer. A sample of approximately 2 mg dry weight, with added internal standards norleucine and 2-amino-3-guanidopropionic acid, was hydrolyzed in 6 N HCl (1 ml) for 24 hr *in vacuo* at toluene reflux temperature. The HCl was evaporated and the sample dissolved in 1 ml of 0.2 N citrate buffer (pH 2.2) filtered through glass wool, and analyzed (Moore *et al.*, 1958). Amino acids were eluted using citrate buffer and a discontinuous gradient (pH 3.25–4.25).

Hydrolyzable phosphorus was determined by the method of Snyder and Law (1970); total phosphorus by the methods of Chen *et al.* (1956) or Kirkpatrick and Bishop (1971b); neutral carbohydrate by orcinol–H_2SO_4 employing galactose: mannose (1:1) as a standard (Winzler, 1955); protein by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Fluorodinitrobenzene derivatives of the phosphonic acid (N_2ph–aminoethylphosphonic acid) were made at pH 9 by the addition of 5% fluorodinitrobenzene in 100% ethanol to aqueous solutions. The N_2ph–aminoethylphosphonic acid was extracted into ethyl acetate from the reaction mixture after adjusting the pH to 1 (Quin, 1967), and identified by thin-layer chromatography. Silica gel G thin-layer plates were developed in butanol–pyridine–acetic acid (80:20:2) (Brenner *et al.*, 1969) and $CHCl_3$–MeOH–17% NH_4OH (4:4:2). Unhydrolyzed glycoprotein samples were derivatized both prior and after hydrolysis to determine if the amino group of the phosphonic acid was free.

To test for the possibility that aggregates or subunit structures were responsible for the gel filtration behavior described above, a 15-mg sample of fraction 9 was reduced with 7.7 mg of dithiothreitol in the dark for 2 hr under N_2 and alkylated with 18.6 mg of iodoacetic acid in 8 M urea. The reagent blank and a 15.4-mg control sample (not reduced or alkylated) were prepared in the same manner and eluted in turn on a calibrated Bio-Gel A 0.5m column equilibrated in 8 M urea. A similar experiment was run on an aliquot of fraction 10 by reducing the material with 0.05% dithiothreitol in 8 M urea and applying the preparation to a Bio-Gel A 1.5m column equilibrated with the same solution as eluent.

A specific glycoprotein extraction procedure using lithium 3,4-diiodosalicylate (Marchesi and Andrews, 1971) was used to obtain a glycoprotein fraction from 100 g of *M. dianthus*. The procedure employed a phenol extraction step and ethanol washes as described by Marchesi and Andrews (1971). A flow sheet for the procedure is shown in Figure 3. An aliquot (162 mg) of the final glycoprotein extract (516 mg) was fractionated on Bio-Gel A 1.5m to give a high molecular weight material which was designated fraction Gly-2. Analytical ultracentrifugation was carried out as described above, except 0.04 M Tris-glycine (pH 9.3) was used as the solvent and reference. Amino acid analysis was carried out on a 2.3-mg aliquot.

Results

The analysis of fraction 1 (hot H_2O soluble; refer to Figure 1 for flow diagram) demonstrated the occurrence of phosphonate in this fraction. The ^{31}P nmr spectrum showed the characteristic resonance bands (phosphonate–phosphorus between −20 and −15 ppm, and phosphate–phosphorus between −5 and +5 ppm) which are obtained from a sample of this type (Glonek *et al.*, 1970; Henderson *et al.*, 1972). For typical spectra see Figure 4. Very little fine structure was seen though extensive signal averaging was employed; however, integration of the spectrum yielded the relative proportions of nuclei absorbing in each region (Table I).

Following verification of the presence of phosphonate–phosphorus in fraction 1, aliquots were analyzed for phosphonate–phosphorus by the colorimetric method and for neutral carbohydrate (Table I). In the sample the phosphonate corresponds to 2.86% aminoethylphosphonic acid (the

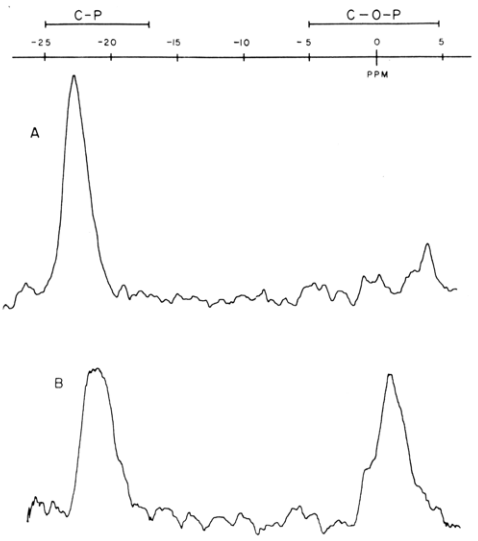


FIGURE 4: Phosphorus-31 nuclear magnetic resonance spectra of glycoprotein fractions from *Metridium dianthus*. Spectrum A was obtained from fraction 10; spectrum B from fraction Gly-2. The resonances in the region from −5 to +5 ppm arise from orthophosphoric acid and its esters, whereas resonances in the region from −25 to −15 arise from alkylphosphonates.

smallest known naturally occurring phosphonate) or 83.8 mg of aminoethylphosphonic acid in the total fraction 1.

After DEAE-cellulose chromatography (pH 9.3) (Figure 2A), significant differences between fractions 2 and 3 were observed by ^{31}P nmr. Fraction 2 was enriched in phosphonate–phosphorus and contained no detectable phosphate–phosphorus whereas fraction 3 contained equal amounts of phosphonate–phosphorus and phosphate–phosphorus. These data together with the per cent total phosphorus as determined colorimetrically showed fraction 2 to contain 1.4% phosphonate–phosphorus and fraction 3 to contain 0.9% phosphonate–phosphorus and an equal amount of phosphate–phosphorus. Amino acid analysis of fraction 2 showed that aminoethylphosphonate was the sole detectable phosphonate subunit and accounted for 20.8% of the ninhydrin reactive material. There was no evidence for the occurrence of 2-amino-3-phosphonopropionic acid (phosphonoalanine).

Further chromatography of fraction 2 on DEAE-cellulose (pH 7.3) provided fractions 4, 5, and 6 (Figure 2B). Fraction 5 contained the bulk of the phosphonate–phosphorus (10 mg of the phosphonic acid) and was fractionated further as described below.

Earlier work in our laboratory (Hilderbrand *et al.*, 1971) showed that gel filtration gave high molecular weight fractions enriched in phosphonate–phosphorus; accordingly, fraction 5 was applied to Sephadex G-25 and the bulk of the sample (fraction 7, 172 mg) was eluted with the void volume. Fraction 7 was subjected to analytical ultracentrifugation which showed the presence of two polydisperse components. The major peak sedimented rapidly (*ca.* 7 S, calculated to the top of the peak); the minor peak sedimented slowly, and a meaningful sedimentation constant could not be determined.

Fraction 7 was successively eluted from Sephadex G-100, Bio-Gel A 0.5m, and Bio-Gel A 1.5m, providing fractions 8, 9, and 10, respectively. Each time the bulk of the material eluted with the void volume. Throughout this procedure ^{31}P nmr of the excluded material showed only the presence of a broad phosphonate–phosphorus resonance signal. Analytical ultracentrifugation showed that although gel filtration had decreased the relative amount of the less rapidly sedimenting material, elimination was not complete. The sedimentation pattern of fraction 10 is shown in Figure 5.

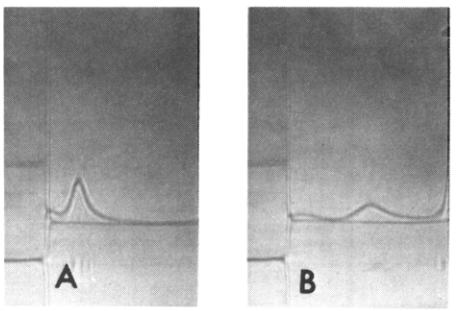


FIGURE 5: Analytical ultracentrifugal patterns obtained with fraction 10 (0.4 ml of 1-mg/ml sample was run). Photograph A was taken when the rotor reached 56,100 rpm; photograph B was taken 32 min later.

Reduction and alkylation experiments demonstrated that only a small amount of material (<10%) was dissociated by the procedures and that the contribution of aggregate or subunit structures was of little significance to the behavior or structure of the high molecular weight material (Figure 6). Similar results were obtained with an aliquot of a similar preparation upon treatment with 1% sodium dodecyl sulfate and subsequent gel filtration on Bio-Gel A 0.5m equilibrated and eluted with 1% sodium dodecyl sulfate (R. L. Hilderbrand and T. O. Henderson, unpublished observations).

Amino acid analysis verified the presence of the phosphonic acid (28% of the ninhydrin reactive material) in fraction 10, and demonstrated the presence of high relative amounts of aspartic acid, glutamic acid, serine, and threonine. Glucosamine and galactosamine, in a ratio of 2:1, made up 10% of the ninhydrin reactive material (see Tables I and II).

TABLE II: Amino Acid Composition of Fractions 10 and Gly-2.

Amino Acid	Mole %[a]	
	Fraction 10	Fraction Gly-2
Aspartic	5.87	4.35
Threonine	5.55	6.23
Serine	4.33	9.58
Proline	4.67	3.12
Glutamic	5.00	4.94
Glycine	5.76	10.05
Alanine	4.25	4.52
Valine	5.01	5.88
Methionine	2.60	0.77
Isoleucine	2.58	2.00
Leucine	3.82	3.06
Tyrosine	1.60	
Phenylalanine	1.97	
Aminoethylphosphonate	28.35	27.42
Lysine	4.70	3.82
Histidine	1.25	1.18
Arginine	2.22	1.30
Glucosamine	6.85	8.88
Galactosamine	3.33	2.94

[a] Based on total ninhydrin-reactive material. The values reported are not corrected for any destruction which may have occurred during acid hydrolysis.

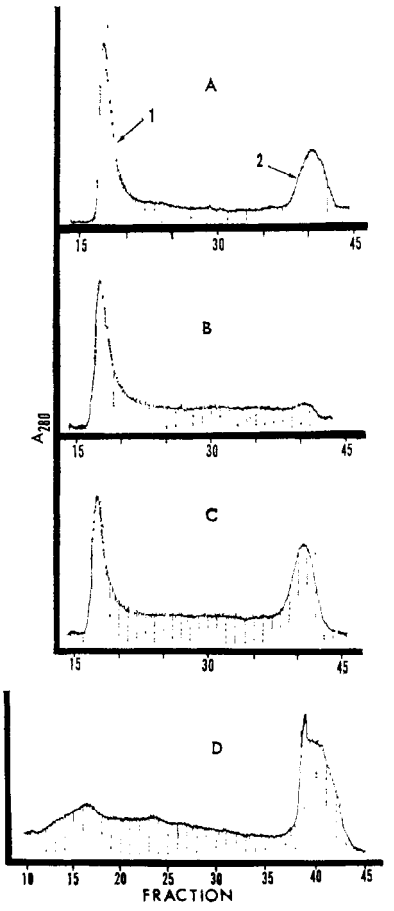


FIGURE 6. Elution profiles obtained on reduction and alkylation of fraction 9 (see Figure 1) with dithiothreitol and iodoacetic acid as described in Materials and Methods. The same Bio-Gel A 0.5m column (2.5 × 70 cm) was used for each of the chromatographic runs, and 8 M urea was used as the eluent in all cases. (A) Profile of Blue Dextran (peak 1) and cyanocobalamine (peak 2) which indicated the relative elution volumes of completely excluded and retained materials, respectively. (B) Profile of control aliquot (15.4 mg) of fraction 9. (C) Profile of reduced and alkylated aliquot (15.0 mg) of fraction 9. (D) Profile of reagent blank (dithiothreitol and iodoacetic acid).

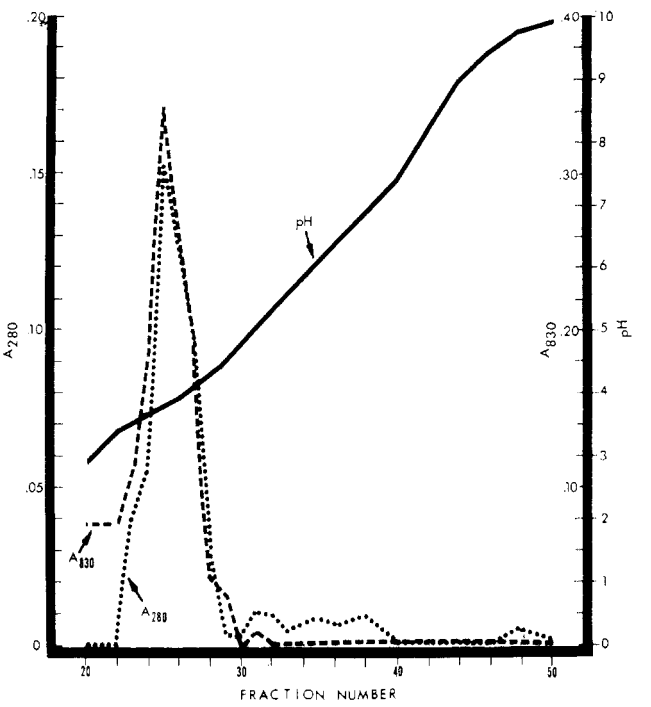


FIGURE 7: Isoelectric focusing profile of fraction 10 (see Figure 1): (—) the pH of the fraction, (- - - - -) the phosphorus content (determined as described by Kirkpatrick and Bishop, 1971b), and (· · · ·) the absorbancy at 280 nm, reflecting protein concentration of the fractions.

The isoelectric point of fraction 10 as determined by electrofocusing was between 3.5 and 4.0. Figure 7 shows the pH gradient, protein concentration (absorbance at 280 nm), and phosphorus concentration (absorbance at 830 nm). The phosphonic acid was recovered from the material in tubes 22–28 by hydrolysis of the material and derivatization with fluorodinitrobenzene. N_2ph–aminoethylphosphonic acid was identified as being present in the hydrolysate by thin-layer chromatography as described in Methods. The phosphorus values given in Figure 7 are the averages of two determinations using 0.1-ml aliquots of the odd-numbered tubes.

During the course of this study, a procedure reported to be specific for the extraction of glycoproteins from erythrocytes appeared (Marchesi and Andrews, 1971). Since our data suggested that fraction 10 was glycoprotein in nature, we applied the complete procedure (as shown in the flow sheet in Figure 3) to homogenates of *M. dianthus*. The high molecular weight fraction Gly-2 obtained by this method gave an analytical ultracentrifugation sedimentation pattern which was similar to that of fraction 10 and had a sedimentation constant of about 6.2. Amino acid analysis (Table II) indicated that aminoethylphosphonate makes up 27.4% of the ninhydrin reactive material, which is comparable to the phosphonic acid content of fraction 10. The phosphonic acid in unhydrolyzed Gly-2 did not form a N_2Ph derivative. In contrast to fraction 10, ^{31}P nmr analysis of Gly-2 (Figure 4B) shows a resonance band from phosphate phosphorus as well as from phosphonate phosphorus with the two bands in a 1:1 ratio.

Discussion

This research was initiated at a time when little was known of the occurrence, metabolism, or detection of biological phosphonates, and the original goal of applying ^{31}P nmr to the detection of such compounds led ultimately to the isolation and characterization of the water-soluble phosphonate-rich glycoprotein preparation of this study. Automated amino acid analysis was also used to determine the phosphonic acid and phosphonoalanine which could be eluted separately using a common buffer system. Aminoethylphosphonate was found to be the only phosphonate subunit present in the hot water-soluble material. In the case of fraction 10 this single aminoalkylphosphonate accounted for 28% of the ninhydrin reactive material with the combined totals for the phosphonic acid, aspartic acid, and glutamic acid accounting for 39% of such material. Since the pK values of the side-chain carboxyl groups of aspartic acid and glutamic acid are 3.8 and 4.2 respectively, they cannot account for the low pI of the material (about 3.7 as determined by isoelectric focusing, Figure 7). Since the pK of a monoester of the phosphonic acid is about 2, the observed pI indicates there is at least one unesterified position in the bound phosphonic acid.

Experiments designed to further fractionate fraction 10 into discrete macromolecular species by disc gel electrophoresis could not be carried out because of the inability of the material to penetrate the gels. The gels investigated included polyacrylamide gels with different degrees of cross-linking, sodium dodecyl sulfate–polyacrylamide gels, and polyacrylamide

agarose composite gels under a variety of conditions (*e.g.*, high pH, low pH, and reversed polarity). However, as shown in Figure 7, we were able to demonstrate that the protein (absorbance at 280 nm) and phosphonate–phosphorus in fraction 10 migrated together on isoelectric focusing. Carbohydrate analysis of the material was not possible because of interference from the sucrose and ampholytes of the isoelectric focusing column.

Characterization of the high molecular weight material indicates that it is glycoprotein-like in nature. Throughout the procedure the hexosamines (glucosamine–galactosamine) were found to be present in a rather constant amount (10% dry weight) and ratio to each other (2:1). Neutral carbohydrate made up about 20% of the dry weight of the material and also appeared in a rather constant amount. There is also a constant relationship between the phosphonate–phosphorus and neutral sugars in the more purified fractions (see Table I). While the structural features of the macromolecule are not yet established, the accumulated evidence (*e.g.*, the resistance to proteolysis (Hilderbrand *et al.*, 1971; Hilderbrand, 1972) and the relative amino acid and carbohydrate content as well as the chromatographic and centrifugal behavior) might suggest that the carbohydrate provides a backbone from which polypeptides project. The phosphonic acid could conceivably provide a linkage between the polypeptide and carbohydrate moieties. This latter point would be consistent with the constant ratio between the neutral sugars and the phosphonic acid as found in a number of fractions (Table I) and the fact that the amino group of the phosphonic acid in unhydrolyzed fraction Gly-2 does not form a N_2ph derivative.

The molecular weight of the phosphonate-rich preparation is not yet known. However, gel filtration studies demonstrated that fraction 10 has an effective Stoke's radius equal to or greater than that of a globular protein of molecular weight 1.5×10^6 daltons. This is in contrast to its ultracentrifugation behavior which yields a sedimentation constant of *ca.* 7 S for the major polydisperse component, a value corresponding in Stoke's radius to a globular protein of *ca.* 1.5×10^5 daltons. Consideration of the composition of the material may be useful in explaining this discrepancy. The high phosphonic acid, aspartic acid, and glutamic acid content, at physiological pH, would provide a high density of negative charge which should maintain a dispersed intramolecular conformation (V. C. Hascall, personal communication). This, together with the hydrated state of the carbohydrate residue could cause the material to be excluded in a gel filtration procedure which would retain a globular material of an equivalent molecular weight. Upon ultracentrifugation the dispersed conformation and the degree of hydration would increase the frictional coefficient of the material and would thus make the sedimentation constant smaller than would be expected for a globular material of the same molecular weight. Assuming that the polydisperse major peak observed on ultracentrifugation is the phosphonate-enriched material, the actual molecular weight of the material probably lies between the molecular weights as indicated by ultracentrifugation and gel filtration.

The glycoprotein preparation obtained by the hot water extraction procedure (fraction 10) and the high molecular weight glycoprotein fraction (Gly-2) prepared by lithium 3,5-diiodosalicylate and phenol extraction have similar composition and properties. Amino acid analysis shows that serine and glycine are present in Gly-2 in quantities about double those in fraction 10, but aminoethylphosphonate is present in equivalent amounts in each fraction (Table II). A significant difference is the presence of phosphate–phosphorus in an amount equal to the phosphonate–phosphorus in fraction Gly-2.

Ultracentrifugation indicates that fraction Gly-2 is as polydisperse as fraction 10 and suggests that both of these phosphonate-rich fractions consists of heterogeneous mixtures of high molecular weight compounds.

Examination of the biology of the amenone might provide a clue to physiological function for macromolecules of this kind. Sea water surrounding the animal contains cations and various materials foreign to the anemone. The stability and the increased buffering capacity (Kittredge and Hughes, 1964) imparted to the biological structure by the phosphonic acid could be of importance in protecting the animal from the environment. A similar protective function could be provided if the phosphonic acid were present in biological structures adjacent to the coelenteron or buccal cavity. Proteolytic enzymes produced by the animal itself may be spilled from the digestive tract (Kittredge and Roberts, 1969) and resistance to these enzymes, a property of phosphonate-containing proteins (*cf.* Rosenberg, 1964; Hilderbrand *et al.*, 1971; Kittredge and Roberts, 1969), could be of importance in maintaining chemical and morphological integrity. Further studies on the structure of these glycoproteins, as well as their location in the animal are currently underway in our laboratories.

Added in Proof

Recent work involving ^{31}P Fourier transform nmr studies on these phosphonoglycoproteins have shown that the relevant relaxation times of the phosphonate–phosphorus atoms are of a magnitude which would account for the line widths of these signals. This suggests that the phosphonic acid residues reside in a relatively ordered environment.

References

Alam, A. U., and Bishop, S. H. (1968), Abstracts, 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept, No. BIOL-276.

Alhadeff, J. A., and Daves, G. D., Jr. (1970), *Biochemistry 9*, 4866.

Brenner, M., Niederwieser, A., and Pataki, H. (1969), *in* Thin Layer Chromatography, Stahl, E., Ed., New York, N. Y., Springer-Verlag, p 764.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.

Glonek, T., Henderson, T. O., Hilderbrand, R. L., and Myers, T. C. (1970), *Science 169*, 192.

Henderson, T. O., Glonek, T., Hilderbrand, R. L., and Myers, T. C. (1972), *Arch. Biochem. Biophys. 149*, 484.

Hilderbrand, R. L. (1972), Ph.D. Thesis, University of Illinois at the Medical Center.

Hilderbrand, R. L., Henderson, T. O., Glonek, T., and Myers, T. C. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1072.

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

Kirkpatrick, D. S., and Bishop, S. H. (1971a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1182.

Kirkpatrick, D. S., and Bishop, S. H. (1971b), *Anal. Chem. 43*, 1701.

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

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

Korn, E. D., Dearborn, D. G., Fales, H. M., and Sokoloski, E. A. (1973), *J. Biol. Chem. 248*, 2257.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marchesi, V. T., and Andrews, E. P. (1971), *Science 174*, 1247.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Biochem. 30*, 1185.
Quin, L. D. (1964), *Science 144*, 1133.
Quin, L. D. (1967), *Top. Phosphorus Chem. 4*, 23.
Rosenberg, H. (1964), *Nature (London) 203*, 229.
Shimizu, H., Kakimoto, Y., Nakajima, T., Kanazawa, A., and Sano, I. (1965), *Nature (London) 207*, 1197.
Snyder, W. R., and Law, J. H. (1970), *Lipids 5*, 800.
Spiro, R. G. (1970), *Annu. Rev. Biochem. 39*, 599.
Winterburn, P. J., and Phelps, C. F. (1972), *Nature (London) 236*, 147.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 210.

Comparison of the Rate Constants for General Base Catalyzed Prototropy and Racemization of the Aldimine Species Formed from 3-Hydroxypyridine-4-carboxaldehyde and Alanine†

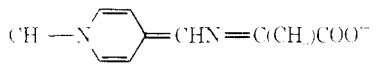
Jack E. Dixon‡ and Thomas C. Bruice*

ABSTRACT: The rate constants for the general base catalyzed racemization of the three aldimine species (SH^-, $S^\pm$, and S; Scheme II) formed from alanine and 3-hydroxypyridine-4-carboxaldehyde have been compared to the general base catalyzed rates of transamination. The two- to threefold difference in the rate constants of racemization and transamination for SH^-, $S^\pm$, and S suggests that the intermediate carbanion is protonated at the carbon originating with the pyridinecarboxaldehyde with about the same ease as protonation takes place at the carbon α to the carboxyl group. These observations are consistent with the large primary deuterium isotope effect noted by Auld and Bruice. The order of ease of proton abstraction from the α carbon by general bases is $SH^- > S^\pm > S$. The conjugated nature of the aldimine $SH^\pm$ with its protonated carboxyl residue and quaternary nitrogen greatly enhances the acidity of the (α)-C–H function of the amino acid moiety but is anti-Hammond in increasing the sensitivity of the rate of proton abstraction to the basicity of the catalyst. These features undoubtedly contribute greatly to the facility of enzymatic transamination.

Studies in this laboratory on model systems for pyridoxal transamination have been carried out in the absence of metal ions in aqueous solution. Our studies have been dictated by the fact that the influence of metal ions upon model transamination reactions (Metzler and Snell, 1952) is not relevant to the enzyme-catalyzed reactions (Jenkins and Sizer, 1957; Matsuo and Greenberg, 1958; Alexander and Greenberg, 1956; Karasek and Greenberg, 1957; Fasella *et al.*, 1962). Bruice and Topping (1963) established that the transamination of pyridoxal with phenylglycine (Scheme I) proceeded *via* imine formation, followed by imidazole general catalysis of the rate-determining prototropic shift. The establishment of proton removal by imidazole marked the first example of the observation of general catalysis in the prototropic shift of an azomethine in aqueous solution. Subsequent investigations have revealed that it is likely that a histidine residue acts as a general base for proton removal in aspartate transaminase (Peterson and Martinez-Carrion, 1970).

Because 3-hydroxypyridine-4-carboxaldehyde meets the minimum requirements for enzymic transamination (Ayling and Snell, 1968) it serves effectively in model studies. The 3-hydroxyl group of 3-hydroxypyridine-4-carboxaldehyde acts as a general catalyst for aldimine formation (French *et al.*, 1965). The rate-determining step in imine formation with 3-hydroxypyridine-4-carboxaldehyde was found to be the formation of carbinolamine, rather than its dehydration as seen with pyridine-4-carboxaldehyde (Scheme I). The overall rate of aldimine formation with 3-hydroxypyridine-4-carboxaldehyde was greater than with pyridine-4-carboxaldehyde, indicating that the 3-hydroxyl group catalyzes, in an intramolecular manner, both carbinolamine formation and dehydration. Since pyridine-4-carboxaldehyde does not undergo transamination and 3-hydroxypyridine-4-carboxaldehyde does, it is evident that the 3-hydroxyl group is involved in the interconversion of aldimine and ketimine species. Thus from considerations of the work of French *et al.* (1965) as well as Thanassi *et al.* (1965), the phenolic hydroxyl group would appear to play an essential role in both imine formation and in prototropy.

The importance of protonation at the pyridine nitrogen to activate the α hydrogen is apparent from the observation of Maley and Bruice (1968) who noted that in the reaction of *N*-methylpyridine-4-carboxaldehyde with alanine I is formed



as an intermediate in the transamination reaction. Recently Abbott and Bobrik (1973) have reported the isolation and characterization of the 1,4-dihydropyridine tautomer formed

† From the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received May 29, 1973*. This work was supported by a grant from the National Science Foundation.

‡ Material submitted by J. E. D. in partial fulfillment of the requirement for the Ph.D. in Chemistry, University of California (1972).