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Phosphonoprotein. Characterization of Aminophosphonic Acid Rich Glycoproteins from Sea Anemones†

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ABSTRACT: Protein from the sea anemones *Metridium senile* and *Anthopleura xanthogrammica* was systematically examined for phosphonic acid compounds. Phosphonic acid compounds were isolated from the acid hydrolysates of fat-free whole body acetone powders of these animals by ion exchange chromatography and identified chromatographically. 2-Aminoethylphosphonate was found in the whole body protein of *M. senile* at a concentration 0.18 μmol/mg. No other phosphonic acid compound was detected in *M. senile* in a concentration greater than 0.3% of the 2-aminoethylphosphonate. 2-Aminoethylphosphate and *N*-methyl-2-aminoethylphosphate were found in the whole body protein of *A. xanthogrammica* at concentrations of 0.02 and 0.15 μmol/mg, respectively. No other phosphonic acid compound was detected in *A. xanthrogrammica* in a concentration greater than 0.3% of the combined 2-aminoethylphosphonic acid and *N*-methyl-2-aminoethylphosphate. Phosphonoprotein, protein rich in aminophosphonic acid, was isolated from the sea anemones *M. senile* and *A. xanthrogammica*. Phosphonoprotein retained its aminophosphonates through dialysis, gel filtration, electrophoresis, trichloroacetic acid precipitation, and solvent extraction. Phosphonoprotein isolated from *M. senile* contained 0.8–0.85 μmol/mg of 2-aminoethylphosphonate, 0.62 μmol/mg of neutral carbohydrate (glucose equivalents), and 0.36 μmol/mg of hexosamine, but no fatty acid or sialic acid. Electrophoresis indicated at least two components in this preparation. Its apparent mol wt by gel filtration was 2.4–3.0 × 10^5. Phosphonoprotein isolated from *A. xanthrogrammica* contained 0.03 μmol/mg of 2-aminoethylphosphonate, 0.37 μmol/mg of *N*-methyl-2-aminoethylphosphonate, 0.44 μmol/mg of carbohydrate (glucose equivalents), and 0.11 μmol/mg of hexosamine, but no fatty acid or sialic acid. The apparent mol wt by gel filtration was 2.5–3.0 × 10^5. Aminophosphonic acids were absent from some soluble proteins and from collagen of *M. senile*. The nonrandom distribution of aminophosphonic acids in protein fractions suggests that they may serve specific functions in a limited number of proteins which comprise 20–40% of the total body protein in these organisms.

Several aminophosphonic acids have been found in nature, *viz.*, 2-amino-3-phosphonopropionic acid, 2-aminoethylphosphonic acid, *N*-methyl-2-aminoethylphosphonic acid, *N*,*N*-dimethyl-2-aminoethylphosphonic acid and *N*,*N*,*N*-trimethyl-2-aminoethylphosphonic acid (see reviews by Quin, 1967; Kittredge and Roberts, 1969). Most of the work with these compounds has centered on their presence in lipid material. However, significant concentrations of 2-aminoethylphosphonic acid have been reported in proteinaceous fractions as well as lipid fractions from some protozoans (Horiguchi and Kandatsu, 1960), lower metazoans including sea anemones

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(Quin, 1965; Kirkpatrick and Bishop, 1971a; Hilderbrand *et al.*, 1971), and from several human tissues (Alhadeff and Davis, 1970, 1971). *N*-Methyl-2-aminoethylphosphonic acid was found in the proteinaceous residue of the sea anemone *Anthopleura xanthogrammica* (Shelburne and Quin, 1967). Recently, 2-aminoethylphosphonic acid and hydroxy-2-aminoethylphosphonic acid have been found in polysaccharide constituents of the plasma membrane of *Acanthamoeba castellonii* (Korn and Wright, 1973; Korn *et al.*, 1973). Although isolation and characterization of the phosphonic acid rich proteinaceous materials from these organisms have not been reported, Hammen (1968) has suggested the presence of "bizarre" proteins containing aminophosphonic acids.

The purpose of this investigation was to isolate and characterize protein(s) containing aminophosphonic acids. The preparation and analysis of phosphonic acid rich glycoprotein from the sea anemones *M. senile* and *A. xanthogrammica* are described in this report.

Materials and Methods

Materials. *Metridium senile* and *Anthopleura xanthogrammica* were collected in the vicinity of Friday Harbor, Washington, or purchased from Pacific Biomarine, Venice, Calif. Reagents and equipment for total phosphorus analysis were previously described (Kirkpatrick and Bishop, 1971b). Aminophosphonic acids, L-2-amino-3-guanidinopropionic acid, and β-2-thienyl-DL-alanine were obtained from Calbiochem, Los Angeles, Calif. Hydroxyproline, hydroxylysine, glucosamine, galactosamine, and pyruvate kinase were obtained from Sigma Chemical Corp., St Louis, Mo. Other amino acid standards were obtained as amino acid calibration mixtures from the Spinco Division of Beckman Instruments, Palo Alto, Calif., and from Bio-Rad, Richmond, Calif. Column chromatographic resins A5, A6, AG-50-2% (200–325), and AG-1-2% (200–400) were obtained from Bio-Rad. Cellulose powder for thin-layer chromatography was Whatman CC41. Cheng Chin polyamide layers were obtained from Gallard-Schlesinger Chemical Corp., Carle Place, N. Y. Dansyl chloride was purchased from Pierce Chemical Corp., Rockford, Ill. Sephadex G-150, Sephadex Blue Dextran 2000, aldolase, and ovalbumin were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Other chemicals and reagents were reagent grade.

Phosphorus Analysis. TOTAL PHOSPHORUS (P_T)[1] ANALYSIS. P_T analysis consisted of wet ashing the sample in a nitric–perchloric–sulfuric acid mixture followed by measuring orthophosphate (1–50 nmol) in the digested residue (Kirkpatrick and Bishop, 1971b). The digestion procedure yielded a uniform amount of acid (3 mequiv of H_2SO_4) in the residue after digestion of samples containing up to 0.040 g of organic material and 0.6 mequiv of inorganic cations. No transfer, dilution, or adjustment of acid concentration was necessary before orthophosphate measurement. The relative standard deviation was 1.7%. Recovery of phosphorus from phosphonic acid compounds was complete.

P_T analyses were performed on the collected fractions from column chromatographies. Up to 1.0-ml sample aliquots from the amino acid analyzer could be analyzed since the eluent contained between 0.0196 and 0.0343 g of sodium citrate–H_2O/ml. After elution with buffer the amino acid analyzer columns were stripped with 0.2 N NaOH. Before P_T analysis the NaOH washings were neutralized with concentrated $HClO_4$ since up to 0.3 mequiv of $NaClO_4$ did not interfere with the P_T analysis.

The sensitivity limit of the P_T analysis was 1–2 nmol of phosphorus. A chromatographic peak with a width at half-height of three fractions contained about 20% of its material in each of the three center fractions. Therefore, the sensitivity limit of the P_T scan was about 0.010 μmol of P_T peak if the entire effluent was collected and analyzed.

DIFFERENCE PHOSPHORUS ANALYSIS FOR C–P PHOSPHORUS (P_{C-P}). Carbon to phosphorus bond phosphorus (P_{C-P}) was calculated by the difference $P_T - P_{i+E}$, where P_{i+E} was the sum of ester plus inorganic phosphorus. P_{i+E} analysis consisted of hydrolyzing the sample in 6 N HCl at 110° for 24 hr, removing HCl by the rotary evaporator, and measuring orthophosphate by the ultramicro method of Bartlett (1959). Hydrolysis of the sample in 6 N HCl frequently resulted in carmelization which interfered with the absorbance at 830 nm. With each P_{i+E} analysis a carmelization blank was included which contained the sample and all reagents except the aminonaphthosulfonate reducing agent. Since the relative standard deviation of the P_T analysis was 1.7%, the relative standard deviation of the P_{C-P} difference phosphorus analysis was about 2.4%.

Chromatography of Aminophosphonic Acids. AMINO ACID ANALYZER. Protein samples for amino acid analysis were hydrolyzed *in vacuo* with 6 N HCl for 24 hr. When homoarginine was added as an internal standard to the sample before acid hydrolysis, 97.6% was recovered. The internal standards L-2-aminoguanidinepropionic acid and β-2-thienyl-DL-alanine were added to the samples after hydrolysis. Amino acid analyses were performed on a Spinco Model 120B amino acid analyzer (Spackman *et al.*, 1958) equipped with Aminex A5 and A6 resins. The buffer system was described by Mashburn and Hoffman (1970) except that the alcohol additive was 4% (v/v) methanol instead of 2% (v/v) propanol. The methanol was added only to the first buffer (pH 3.10). Buffer change from pH 3.28 to 4.25 on the long column occurred at 62.5 min. These conditions allowed resolution of hydroxyproline, glucosamine, and galactosamine as well as the common amino acids at an elution rate of 92 ml/hr at 55.7°. The cochromatographing group, P_i, phosphoserine, and 2-amino-3-phosphonopropionic acid, was completely resolved from the cochromatographing group, 2-aminoethylphosphonic acid, *N*-methyl-2-aminoethylphosphonic acid and *N*,*N*-dimethyl-2-aminoethylphosphonic acid (Table I). Both groups were completely resolved from the common amino acids. The value for the 2-aminoethylphosphonic acid calibration constant (see Table I) was highly dependent upon reaction time in the ninhydrin reaction coil. This dependency may account for the reported variation in 2-aminoethylphosphonic acid calibration constants (deKoning, 1966a,b). This instrument was equipped with a standard Beckman reaction coil (*ca.* 12-ml volume). For tryptophan measurement the protein was hydrolyzed in 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu and Chang, 1971) and chromatographed on a 0.9 × 20 cm column of A5 resin.

For split-stream operation of the A6 column, the column effluent was split by a pump which delivered 0.6 of the stream to the ninhydrin reaction coil; the remainder of the effluent was collected in test tubes by a fraction collector. The decreased flow rate increased the reaction time in the ninhydrin

[1] Abbreviations used are: P_{i+E}, inorganic phosphate plus organic ester phosphorus; P_{C-P}, carbon to phosphorus bond phosphorus; P_T, total phosphorus; dansyl chloride, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

TABLE I: Chromatographic Separation and Identification of Aminophosphonic Acids in the Protein of *M. senile* and *A. xanthogrammica*.[a]

Compound	Aminex A6 Elution Vol (ml)	Aminex A6 Calibration[b]	AG-50-H$^+$ Elution Vol (ml)	AG-1-Formate Elution Vol (ml)	Cellulose R_F Solv 1[c]	Cellulose R_F Solv 2[c]	Dansyl Deriv. Polyamide R_F Solv 3[c]	Dansyl Deriv. Polyamide R_F Solv 4[c]
Cysteic acid	20.7						0.23	0.00
2-Amino-3-phosphonopropionic acid	20.7	6.21	250	399	0.04	0.12	0.64	0.03
P_i	20.7	0	150	718	0.08	0.28		
2-Aminoethylphosphonic acid	29.1	0.57	290	34	0.13	0.18	0.64	0.11
N-Methyl-2-aminoethylphosphonic acid	29.1	0	330	34	0.30	0.41	0.61	0.21
N,N-Dimethyl-2-aminoethylphosphonic acid	29.1	0	350	34	0.53	0.60	0.70	0.40
N,N,N-Trimethyl-2-aminoethylphosphonic acid			350		0.64	0.77		
Valine	137	6.12						
M. senile								
Peak A6(1)	20.7		108–202	607–810	0.08	0.28	N.D.	N.D.
Peak A6(2)	29.1		290	25	0.14	0.19	0.64	0.11
A. xanthogrammica								
Peak A6(1)	20.7		150		0.08	0.28	N.D.	N.D.
Peak A6(2)	29.1		290		0.14	0.18		
			330		0.30	0.41		

[a] Column procedures are described in the text. [b] Calibration constant for 10.0-mm flow cell (Jones and Weiss, 1964); units are absorbance minute per micromole. [c] Solvent system 1, phenol (88%)–ethanol (95%)–NH_4OH (concentrated)–water (12:4:1:3); solvent system 2, phenol (88%)–ethanol (95%)–HCl (concentrated)–water (12:4:1:1.5); solvent system 3, water–90% formic acid (100:1.5); solvent system 4, *N*-heptane–*N*-butyl alcohol–glacial acetic acid (3:3:1).

reaction coil which caused an increase in the 2-aminoethylphosphonic acid calibration constant (see footnote *c* in Table II). After complete elution with the buffers described above, the columns were stripped with 0.2 N NaOH. The collected fractions including the NaOH wash were analyzed for P_T and correlated with the ninhydrin reactive peaks of the amino acid analyzer. Preparative operation of the long column was similar to analytical preparative operation except that there was no stream splitting or ninhydrin analysis and the entire column effluent was collected in test tubes by a fraction collector. Phosphorus-containing material was located by P_T analysis of the collected fractions.

CATION EXCHANGE CHROMATOGRAPHY OF AMINOPHOSPHONIC ACIDS ON AG-50-H$^+$. Aminophosphonic acids were chromatographed at 23° on a 2.5 × 42 cm column of AG-50-H$^+$-2% (200–325) equilibrated and eluted with 1.5 N HCl (Kittredge *et al.*, 1967). The entire effluent was collected in 10-ml fractions. Complete resolution was obtained among P_i, 2-amino-3-phosphonopropionic acid, 2-aminoethylphosphonic acid, and *N*-methyl-2-aminoethylphosphonic acid. The latter was partially separated from the unresolved pair, *N,N*-dimethyl-2-aminoethylphosphonic acid and *N,N,N*-trimethyl-2-aminoethylphosphonic acid (see Table I).

DESALTING 2-AMINOETHYLPHOSPHONIC ACID AND *N*-METHYL-2-AMINOETHYLPHOSPHONIC ACID. Material isolated from the AG-50-H$^+$ column (in 1.5 M HCl) was evaporated to dryness and placed onto a 1 × 20 cm column of AG-1-2% (acetate) in pyridine acetate (0.2 M, pH 9.4) (Hirs, 1967). After washing with water, the phosphonic acid components were eluted with 50% acetic acid and the acetic acid removed by evaporation.

CELLULOSE THIN-LAYER CHROMATOGRAPHY OF AMINOPHOSPHONIC ACIDS. Chromatograms of phosphorus compounds were developed on cellulose thin layers, 0.25 mm × 20 cm × 20 cm, using solvent system 1 (phenol (88%)–ethanol (95%)–ammonia (concentrated)–water (12:4:1:3) (Kittredge *et al.*, 1967)) or 2 (phenol (88%)–ethanol (95%)–HCl (concentrated)–water (12:4:1:1.5)). As shown by the R_F values in Table I both solvent systems completely resolved P_i and the common aminophosphonic acids from each other. Solvent 2 had the advantage of not discoloring. Phosphorus-containing spots were located by spraying with acid molybdate followed by reduced vanadate (Rosenberg, 1959). The limit of detection was less than 0.01 μmol of phosphorus. It should be noted that this spray reagent has produced blue spots characteristic of phosphorus with unidentified compounds whose phosphorus content could not be verified by elution and total phosphorus analysis.

POLYAMIDE THIN LAYER CHROMATOGRAPHY OF DANSYL-AMINOPHOSPHONIC ACIDS. Dansyl-aminophosphonic acids were chromatographed on 7.5 × 7.5 cm polyamide layers (Cheng-Chin). The limit of sensitivity was about 0.05 nmol but normally 0.5–1.0 nmol was chromatographed. In the first dimension the solvent was water–90% formic acid (100:1.5); in the second dimension the solvent was *n*-heptane–*n*-butyl alcohol–glacial acetic acid (3:3:1) (Woods and Wang, 1967). The dansyl derivatives of 2-aminoethylphosphonic acid, *N*-methyl-2-aminoethylphosphonic acid, and 2-amino-3-phosphonopropionic acid were adequately separated from each other (Table I) and from the common dansyl amino acids.

Dansyl derivatives of the aminophosphonic acids were prepared as described by Gray and Smith (1970). Sample aliquots were about 5 nmol. Vacuum drying steps were per-

TABLE II: Quantitative Analysis of Aminophosphonic Acids in Whole Body Protein from *M. senile* and *A. xanthogrammica*.[a]

	μmol/mg of Protein			Ninhydrin Analysis
	P_T	P_{i+E}	P_{C-P}	
M. senile				
Whole body protein	0.33	0.16	0.17	
Phosphorus components of acid hydrolysates[b]				
Peak A6(1)	0.14	0.14	0.00	0.0007[c]
Peak A6(2)	0.18	0.00	0.18	0.22[d]
A. xanthogrammica				
Whole body protein	0.27	0.08	0.19	
Phosphorus components of acid hydrolysates[a]				
Peak A6(1)	0.14	0.14	0.00	0.0004[c]
Peak A6(2)	0.17	0.00	0.17	0.025[d]

[a] Procedures described in the text. [b] These components obtained from chromatography on Aminex A6 in split stream analysis (see text). [c] Component calculated using calibration constant for 2-amino-3-phosphonopropionic acid (see Table I). [d] Component calculated using a calibration constant for aminoethylphosphonic acid of 2.47 absorbance min per μmol determined during split stream analysis. This constant was much higher than the value in Table I due to an increased reaction time in the ninhydrin reaction coil with the decreased flow rate.

formed with the reaction tubes (6 × 50 mm) set in drilled aluminum blocks which were placed on the heated magnesium base plate of a vacuum bell jar (Scientific Glass Apparatus Co., Bloomfield, N. J.).

Protein Preparation. Unless otherwise stated, all protein preparation procedures were performed at 0–2°. Precipitates were collected by centrifugation at 15,000*g* for 15 min in a Sorvall RC-2B refrigerated centrifuge. All protein preparations were prepared according to the procedure of Siekevitz (1952) to ensure removal of adsorbed carbohydrate, nucleic acid, and lipid. This procedure involved hot and cold 5% Cl_3CCOOH precipitation, hot and cold ethanol precipitation, chloroform–methanol extraction, ether–ethanol–chloroform extraction, and ether extractions for each sample prior to analysis for amino acids, carbohydrate, ash, and lipid.

Acetone powders were prepared from freshly chopped *M. senile* or *A. xanthogrammica* by mixing with 4 vol of cold acetone (−20°) and homogenizing in a Waring Blendor at full speed for 1 min. The precipitate was collected by filtration on a Büchner funnel, resuspended in 5 vol of cold acetone, homogenized again, and collected by filtration. This precipitate was washed with 10 vol of cold acetone, suctioned to near dryness, and then spread finely on a sheet of filter paper and dried at room temperature. Preparation of acid-soluble fibrous protein was adapted from the procedure described by Gross (1958).

Protein Chromatography and Electrophoresis. Sephadex G-150 columns (2 × 51 cm) were equilibrated with Tris-citrate, 0.025 M, pH 8.0, and sodium citrate, 0.02 M, pH 6. The columns were calibrated for molecular weight with Blue Dextran (V_0), rabbit muscle pyruvate kinase (235,000), aldolase (156,000), and ovalbumin (45,000) as described by Andrews (1964). Samples of Blue Dextran were passed through the column following elution of protein sample to monitor small changes in V_0 (~56 ml). When phosphorus-containing proteins were chromatographed, the collected fractions were each analyzed for absorbance at 280 nm and for total phosphorus. All chromatography was performed at 2°.

Proteins were electrophoresed on Gelman cellulose acetate membranes at 200 V for 90 min in sodium barbitol, 0.052 M, pH 8.7, 22° (Smith, 1960). The membranes were stained with Ponceau S, destained, cleared, and scanned with a Beckman Labtrol Model R-110 densitometer. After scanning, the membranes were cut transversely and each section analyzed for total phosphorus. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate solutions was performed according to the procedure of Fairbanks *et al.* (1971) for the 11.2% gels and periodic acid Schiff staining and according to the procedure of Weber and Osborn (1969) in Tris-HCl buffer for the 6% gels. Analytical polyacrylamide gel electrophoresis was performed as described by Ornstein and Davis (1964).

Elemental Analysis. Direct current arc emission spectrographic analysis was performed on protein by the Institute for Research, Inc., Houston, Tex. The sample was first ignited at 538° and then analyzed in an Applied Research Laboratories Direct Reading Quantometer, Model 8200.

Carbohydrate Analysis. The carbohydrate content of protein was measured by the phenol–sulfuric acid method (Dubois *et al.*, 1956). With this reaction pentoses and their derivatives produced absorption maxima near 480 nm, hexoses and their derivatives produced absorption maxima near 490 nm, and hexosamines produced negligible color with this reaction. Absorbance of the developed color was measured at 480 and 490 nm on a Gilford Model 240 spectrophotometer.

Sialic Acid Analysis. The sialic acid content of protein was measured by the alkalai–Ehrlich test following 0.12 M HCl hydrolysis at 80° for 30 min to produce free sialic acid (Aminoff, 1961).

Fatty Acid Analysis. The protein was hydrolyzed and methylated by reaction with 10% boron trifluoride in methanol at 100° for 90 min (Morrison and Smith, 1964). The resultant fatty acid methyl esters were quantitated by gas–liquid chromatography as described by Anderson *et al.* (1970). R. E. Anderson kindly made his laboratory available for the performance of this analysis.

Results

Separation and Identification of Aminophosphonic Acids Found in M. senile and A. xanthogrammica Crude Protein. The acid hydrolysates of 118 mg of whole body protein were prepared by Cl_3CCOOH precipitation–solvent extraction of acetone powders of *M. senile* and *A. xanthogrammica* and were each chromatographed on the long A6 column of the amino acid analyzer in 29.6-mg batches. The entire effluent was collected in tubes on a fraction collector for quantitative and qualitative analyses of the phosphorus-containing components. Two phosphorus-containing peaks were eluted from the column in each case. The first peak, designated A6(1), had an elution volume which was the same as P_i and 2-amino-3-phosphonopropionic acid. The second peak, designated A6(2), had an elution volume which was the same as 2-aminoethylphosphonic acid, *N*-methyl-2-aminoethylphosphonic acid, and *N,N*-dimethyl-2-aminoethylphosphonic acid (Table I).

Any compounds not eluted from the A6 column during the routine amino acid analysis were eluted with 0.2 N NaOH.

With protein from both species, the NaOH wash of the column contained no more than 0.032 μmol of P_T which was less than 0.5% of the total P_{C-P} content of the whole body protein. Since the amount of this component varied from batch to batch and in some cases was almost undetectable, it was probably incompletely hydrolyzed material. No components other than peaks A6(1) and A6(2) were found above the detection limit of the P_T analysis for either *M. senile* or *A. xanthogrammica*. The detection limit corresponded to a component containing 0.0008 μmol of P_T/mg of protein or 0.4% of the total P_{C-P} content of the protein. Both P_T and ninhydrin analysis indicated that at sample loads as high as 30 mg, peaks A6(1) and A6(2) were completely resolved from each other and from common amino acids by this chromatographic procedure.

IDENTIFICATION OF PEAK A6(1). When the combined A6(1) peaks from *M. senile* and *A. xanthogrammica* were each chromatographed on AG-50-H^+, no phosphorus-containing peak other than inorganic phosphate was observed for either protein preparation above the detection limits of the P_T analyses of the collected effluent fractions (Table I). The detection limit of both AG-50-H^+ chromatographies corresponded to a component containing 0.00015 μmol of P_T/mg of protein, or 0.09% of the total P_{C-P} of the whole body protein acetone powder. Since 2-amino-3-phosphonopropionic acid was separated from P_i by chromatography on AG-50-H^+, 2-amino-3-phosphonopropionic acid was not present in either *M. senile* or *A. xanthogrammica* in amounts larger than 0.05% of the total P_{C-P} of the whole body protein acetone powder.

The A6(1) peak material of *M. senile* protein contained 0.14 μmol of P_T/mg of protein. The A6(1) peak material from *A. xanthogrammica* contained 0.14 μmol of P_T/mg of protein. Difference phosphorus analysis did not detect any P_{C-P} in the combined A6(1) peaks from either source. Therefore, phosphorus in peak A6(1) from both *M. senile* and *A. xanthogrammica* was at least 97.6% P_{i+E} derived from either inorganic phosphate or acid-hydrolyzed phosphate esters such as phosphoserine originally present in the acetone powders.

Chromatography of the desalted A6(1) peaks isolated from *M. senile* and *A. xanthogrammica* on cellulose thin layers in two solvent systems detected only P_i. When dansyl derivatives of the isolated peaks were prepared and chromatographed on polyamide thin layers, nothing was detected.

The acid hydrolysates of 29.6 mg of whole body acetone powder of *M. senile* and *A. xanthogrammica* were each chromatographed on the long A6 column of the amino acid analyzer and subjected to both phosphorus and ninhydrin analysis on the amino acid analyzer using a split stream (see Methods); the ninhydrin positive material associated with peak A6(1) was calculated in terms of 2-amino-3-phosphonopropionic acid. Thus, *M. senile* contained a maximum of 0.0007 μmol of 2-amino-3-phosphonopropionic acid/mg of protein or 0.4% of the total P_{C-P}. Similarly, *A. xanthogrammica* contained a maximum of 0.0004 μmol of 2-amino-3-phosphonopropionic acid/mg of protein or 0.2% of the total P_{C-P} (Table II). The ninhydrin response reported here may have been caused by small amounts of cysteic acid formed during acid hydrolysis.

IDENTIFICATION OF PEAK A6(2). When the combined A6(2) peak from *M. senile* whole body acetone powder was chromatographed on AG-50-H^+, a phosphorus-containing peak emerged from the column in the retention volume of 2-aminoethylphosphonic acid (Table I). No other phosphorus component was observed above the detection limit of the P_T analyses of the collected effluent fractions. The detection limit corresponded to a component containing 0.00047 μmol of P_T/mg of protein or 0.3% of the total P_{C-P} found in whole body acetone powder. Since 2-aminoethylphosphonic acid was separated from its *N*-methyl derivatives by AG-50-H^+ chromatography, the *N*-methyl derivatives of 2-aminoethylphosphonic acid were not present in *M. senile* in an amount greater than 0.3% of the total P_{C-P} of the whole body protein acetone powder. When combined A6(2) peak material from *A. xanthogrammica* was chromatographed on AG-50-H^+, the major phosphorus component emerged from the column in the retention volume of *N*-methyl-2-aminoethylphosphonic acid, and a minor component emerged in the retention volume of 2-aminoethylphosphonic acid (Table I). No other phosphorus components were observed above the detection limit (0.3% of the total P_{C-P}).

Chromatography of the desalted A6(2) peak isolated from *M. senile* on cellulose thin layers in two solvent systems detected only 2-aminoethylphosphonic acid. Similar chromatography of the A6(2) peak from *A. xanthogrammica* detected mostly *N*-methyl-2-aminoethylphosphonic acid and a faint spot for 2-aminoethylphosphonic acid (Table I). When dansyl derivatives of the isolated peaks were prepared and chromatographed on polyamide thin layers in two solvent systems, only 2-aminoethylphosphonic acid was detected in the *M. senile* material (Table I).

QUANTITATIVE ANALYSIS OF AMINOPHOSPHONIC ACIDS IN WHOLE BODY PROTEIN. The A6(2) peak material from *M. senile* whole body contained 0.18 μmol of P_T/mg of protein. The A6(2) peak material isolated from *A. xanthogrammica* whole body protein contained 0.17 μmol of P_T/mg of protein. Difference phosphorus analysis indicated that the phosphorus in the combined A6(2) peaks from both sources was P_{C-P} (Table II). In four separate acetone powder preparations the micromoles of P_{C-P}/milligram of whole body protein varied from 0.18 to 0.22 for *M. senile* and 0.15 to 0.19 for *A. xanthogrammica*.

The acid hydrolysates of 29.6 mg of whole body acetone powder of *M. senile* and *A. xanthogrammica* were each chromatographed on the long A6 column of the amino acid analyzer in split stream operation (Table II). P_T analysis of peak A6(2) from *M. senile* indicated 0.18 μmol of P_{C-P}/mg of protein; ninhydrin analysis indicated 0.22 μmol of AEP/mg of protein. Since the *N*-methyl derivatives of 2-aminoethylphosphonic acid which cochromatograph with 2-aminoethylphosphonic acid produce negligible ninhydrin response, the phosphorus in peak A6(2) from *M. senile* was primarily 2-aminoethylphosphonic acid. Difference phosphorus analysis of peak A6(2) from *A. xanthogrammica* indicated 0.17 μmol of P_{C-P}/mg of protein; ninhydrin analysis indicated 0.025 μmol of 2-aminoethylphosphonic acid/mg of protein. The *N*-methyl-2-aminoethylphosphonic acid content of whole body protein of *A. xanthogrammica* was calculated by difference to be 0.15 μmol/mg of protein or 85% of the total P_{C-P}. In other analyses of the *A. xanthogrammica* protein from four separate acetone powder preparations in which the split stream operation was not employed, the *N*-methyl-2-aminoethylphosphonic acid comprised 88–92% of the total P_{C-P}.

Phosphonic Acid Content of Protein Fractions. PHOSPHONIC ACID CONTENT OF PROTEIN FRACTIONS PREPARED FROM AN ACETONE POWDER FROM *M. senile*. Because the anemones secreted a great deal of viscous material, proteins were fractionated from an initial acetone powder preparation rather than fresh tissue homogenates (Figure 1). Extraction of the acetone powder with sodium citrate (0.02 M, pH 6) solutions at 4° slowed the action of the powerful tanning enzymes present in this animal sufficiently to prevent formation of the highly

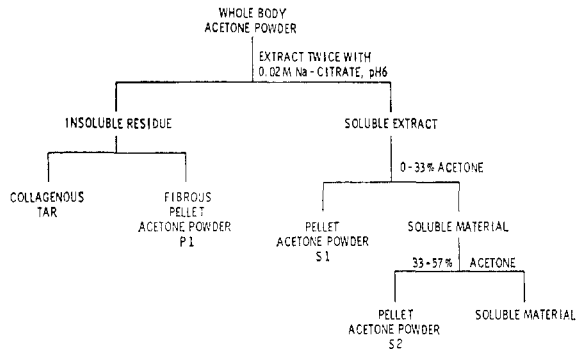

FIGURE 1: Scheme for fractionation of the sea anemone acetone powder extracts. (See text for details.)

cross-linked black tar usually encountered (Fox and Pantin, 1941).

The acetone powder from *M. senile* (about 150 g) was extracted twice by homogenization in 600 ml of 0.02 M sodium citrate, pH 6, and stirring overnight at 2°. Centrifugation separated the polydisperse insoluble material into a hard-packed tar-like collagenous residue and an insoluble fibrous precipitate (P-1) which layered on top of the collagenous residue. The two insoluble layers were carefully scraped apart and the collagenous residue discarded. The combined soluble extracts yielded 1 l. of a viscous soluble protein solution.

Phosphonic acid rich protein could be precipitated from the soluble protein solution by addition of acetone but not by addition of ammonium sulfate. Cold acetone (500 ml) was added dropwise with stirring to 1 l. of the soluble protein solution and the precipitate, fraction S-1, collected by centrifugation. An additional 800 ml of cold acetone was added to the supernatant and the resulting precipitate, fraction S-2, collected in the same manner. Addition of more acetone to the resulting supernatant precipitated no more protein. In separate preparations, the fibrous precipitate (P-1) and fraction S-1 were homogenized in 1 l. of cold acetone, collected by filtration, and dried and stored at −20°.

Fraction S-2 contained only 0.3 g of protein with a P_{C-P} content of 0.15 μmol/mg, which was less than the 0.19 μmol of P_{C-P}/mg found in the whole body protein (Table III). The specific P_{C-P} content of S-1, 0.65 μmol/mg, was threefold greater than that of crude protein but accounted for only 30% of the total protein-bound P_{C-P}. In four separate preparations, the P_{C-P} content of S-1 varied between 0.56 and 0.67 μmol/mg of protein. The specific P_{C-P} content of P-1, 0.31 μmol/mg, was about 1.5 times that of crude protein and accounted for 50% of the total protein-bound P_{C-P}. The P_{C-P} content of the collagenous residue was estimated by difference to be about 20% of the total protein-bound P_{C-P}.

PROTEIN FRACTIONS PREPARED FROM ACID-SOLUBLE FIBROUS PROTEIN OF *M. senile*. Since the collagenous residue contained about 20% of the protein-bound 2-aminoethylphosphonic acid, it was desirable to determine the P_{C-P} content of collagen in spite of the many previous studies of the collagen from this animal (see, for example, Piez and Gross, 1959). Because the collagenous residue prepared from the acetone powder had a tar-like consistency, acid-soluble fibrous protein was prepared separately. Chopped *M. senile* was homogenized at low speed in a Waring Blendor with 10 vol of 0.5 M NaCl. After centrifugation, the pellet was homogenized again in 10 vol of 0.5 M NaCl and centrifuged. The pellet was mixed with 5 vol of 0.1 M acetic acid and stirred slowly overnight. The suspension was centrifuged and the pellet resuspended in 10 vol of 0.1 M acetic acid and stirred slowly for 72 hr. Following centrifugation, NaCl (8 g/100 ml) was added to this supernatant with slow stirring. The heavy fibrous precipitate which formed slowly was collected after standing 12 hr and dissolved in 10 vol of 0.1 M acetic acid. Insoluble material was removed by centrifugation to give an opalescent solution. The sodium chloride–acetic acid precipitating–dissolving cycle was repeated three times. The final sodium chloride precipitate was washed three times with 10 vol of 0.003 M Tris-HCl, pH 7.3. The acid-soluble fibrous protein at this stage contained 0.05 μmol of 2-aminoethylphosphonic acid/mg. The fibrous protein preparation was heated at 121° in 3 vol of the same buffer for 90 min. After cooling and centrifugating, the precipitate was resuspended and autoclaved two more times. The final precipitate was designated the autoclaved protein precipitate (APP). Ethanol was added to the soluble protein solution from the first autoclaving step to give 20% ethanol solution (v/v). The resulting protein precipitate (alcohol fraction 1) was collected and ethanol added to the supernatant to give an 80% ethanol solution. This preparation was held at −20° for 2 days and centrifuged at −15° and the pellet (alcohol fraction 2) dissolved in water. Alcohol fraction 1, alcohol fraction 2 (gelatin), and APP contained 0.14, 0.01, and 0.21 μmol of P_{C-P}/mg of protein, respectively.

PHOSPHONIC ACID CONTENT OF PROTEIN FRACTIONS PREPARED FROM AN ACETONE POWDER FROM *A. xanthogrammica*. Ten grams of a whole body acetone powder from *A. xanthogrammica* containing 0.19 μmol of P_{C-P}/mg (90% *N*-methyl-2-aminoethylphosphonic acid) was subjected to fractionation in the same manner as the acetone powder from *M. senile* (Figure 1). Fraction S-2 contained 400 mg of protein with a P_{C-P} content of 0.09 μmol/mg (Table IV). Fraction S-1

TABLE III: Partial Purification of P_{C-P}-Rich Protein from *M. senile*.

Preparation	Protein Wt (g)	P_{C-P} (mmol)	μmol of P_{C-P}/mg
Whole body protein[a]	150	28.5	0.19
Residue[b]	(90)	(5.6)	(0.06)
P-1	45	14	0.31
S-1	14	9.1	0.65
S-2	0.3	0.05	0.15

[a] Protein was prepared from an acetone powder of the whole animal. [b] Residue P_{C-P} content determined by difference from other fractions (see text).

TABLE IV: Partial Purification of P_{C-P}-Rich Protein from *A. xanthogrammica*.

Preparation	Protein Wt (g)	P_{C-P} (mmol)	μmol of P_{C-P}/mg
Whole body protein[a]	10	1.9	0.19
P-1	7.6	1.04	0.15
S-1	1.7	0.56	0.33
S-2	0.4	0.036	0.09

[a] Protein was prepared from an acetone powder of the whole animal.

TABLE V: Amino Acid Analysis of Protein Preparations from *M. senile* and *A. xanthogrammica*.[a]

Amino Acid	*M. senile*						*A. xantho-grammica*
	Whole Body Protein	S-1[b]	S1-DS[b]	P-1[c]	Gelatin	APP[d]	S1-DS
Ala	77.8	57.1	47.9	71.4	67.4	78.1	52.5
Gly	127.9	62.8	51.7	84.3	337.3	83.6	61.6
Val	53.1	59.7	54.8	60.8	26.8	65.6	68.2
Leu	60.9	58.1	48.1	75.3	31.2	80.5	67.6
Ileu	42.8	43.3	34.1	47.8	17.5	56.2	36.8
Pro	55.5	43.4	47.0	51.5	62.4	29.7	47.1
Phe	28.8	31.1	26.5	36.2	6.0	43.7	33.0
Tyr	21.8	28.9	23.3	33.3	3.3	34.4	25.4
Ser	57.2	73.4	101.2	71.7	38.9	56.2	56.7
Thr	50.2	57.5	60.6	50.6	37.2	50.8	62.2
Half-Cys	12.3	17.2	13.4	19.2	ND	21.9	21.7
Met	19.7	17.4	16.1	22.9	9.9	26.6	19.9
Arg	47.7	35.0	26.7	50.2	66.3	58.6	44.1
His	16.4	21.2	14.8	26.4	2.2	11.7	19.9
Lys	62.1	70.1	54.9	75.0	20.3	64.8	79.1
2-Aminoethylphosphonic acid	31.3	134.0	171.2	32.1	1.6	39.1	5.7
N-Methyl-2-aminoethylphosphonic acid	ND	ND	ND	ND	ND	ND	70.8
Asp	96.7	69.8	57.5	73.6	75.0	99.2	100.8
Glu	104.5	84.8	67.2	103.0	88.7	93.7	114.7
OH-Pro	17.7	ND	ND	ND	77.8	ND	ND
OH-Lys	6.6	ND	ND	ND	29.0	ND	ND
Glu-NH_2	5.7	29.6	72.8	71.0	1.0	5.5	9.7
Gal-NH_2	3.3	3.5	10.3	3.6	ND	ND	9.0
Amide-N	(125.5)	(167)	(166)	(122.6)	(87.1)	(125.8)	(160.6)
% recovery by weight[e]	77	57	57	61	71	59	70
% C–P recovery[f]	108	98.5	86	82	*g*	84	N.A.

[a] Values are expressed as residues/1000 residues; abbreviations for preparations are described in the text. [b] Tryptophan content was 3.08 and 3.00 residues/1000 residues in separate analyses; S1-DS in this analysis represents P_{C-P}-rich protein from combined fractions after passage through the G-150 column. [c] P-1 insoluble protein after exhaustive washing. [d] Autoclaved precipitated protein from acid-soluble fibrous protein preparation. [e] Per cent recovery represents per cent weight of amino acid residues using 107.5 and 121.1 g/mol as residue weights for 2-aminoethylphosphonic acid and *N*-methyl-2-aminoethylphosphonic acid, respectively. [f] Phosphorus recovery was the ninhydrin response to 2-aminoethylphosphonic acid as the percentage by weight of the P_{C-P} analysis. [g] Ninhydrin response to 2-aminoethylphosphonic acid near the detectable limit.

contained 1.7 g of protein with a P_{C-P} content of 0.33 μmol/mg (90% *N*-methyl-2-aminoethylphosphonic acid). The fibrous residue (P-1), which did not separate into two distinct layers, contained 7.6 g of protein with a P_{C-P} content of 0.15 μmol/mg (89% *N*-methyl-2-aminoethylphosphonic acid).

Characterization of Aminophosphonic Acid Rich Protein from M. senile and A. xanthogrammica. AMINOETHYLPHOSPHONIC ACID RICH PROTEIN IN *M. senile*. The P_{C-P}-rich protein fraction S-1 from *M. senile* (Table III) contained 0.655 μmol of P_{C-P}/mg by difference phosphorus analysis which compared closely with 0.645 μmol of 2-aminoethylphosphonic acid/mg by amino acid analysis (Table V). Amino acid analysis indicated that 2-aminoethylphosphonic acid was present in much greater amounts (134 residues/1000 residues) than any other amino acid. Recovery of amino acids and amino sugars by amino acid analysis accounted for 57% of the weight of S-1.

Neutral carbohydrate content of fraction S-1, measured by the phenol–sulfuric acid method, produced an absorption spectrum virtually identical with that produced by glucose (absorption maximum near 490 nm). By this criterion the protein-bound neutral carbohydrate content of S-1 was mostly hexose (Dubois *et al.*, 1956). Expressed as glucose, the neutral carbohydrate content of S-1 was 10% of the weight or 0.56 μmol/mg of protein. The sialic acid content of S-1 was less than the limit of detection of 0.02 μmol/mg of sample. The fatty acid content of S-1 was less than the limit of detection of 0.003 μmol/mg of sample. Moisture was less than 1% by weight.

The residue, after ashing at 538°, was 14% by weight. The 2-aminoethylphosphonic acid content of S-1 all converted to orthophosphate combined with the sulfur amino acids all converted to inorganic sulfate would account for 9% by weight of the protein. The total of the nonvolatile metals detected by emission spectroscopy accounted for less than 1% by weight of the protein. Thus, 4% of the weight of the protein was unaccounted for in the ash. The total weight of S-1 which could be accounted for as amino acids, carbohydrates, and ash was 71%. Recovery of 2-aminoethylphosphonic acid by amino acid analysis, relative to P_{C-P} analysis, was between 82 and 100%. The low recovery of the protein weight (71%) may be

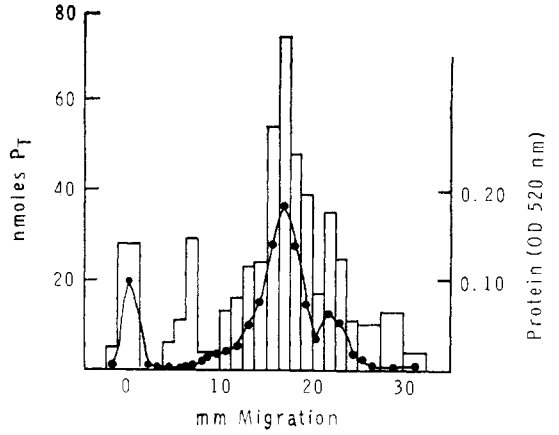

FIGURE 2: Separation of the phosphonoprotein fraction S1-DS from *M. senile* after electrophoresis on cellulose acetate strips. A 20-μl sample was applied to wetted, cellulose acetate membrane. After electrophoresis (see text) the strip was stained with Ponceau S, destained, cleared, and scanned in a densitometer. The strip was then cut into cross-sectional pieces and analyzed for P_T.

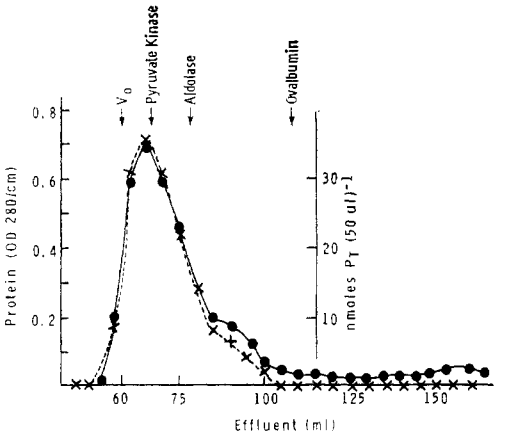

FIGURE 3: Elution pattern of the phosphonic acid enriched protein fraction (S1-DS) from *M. senile* after chromatography on Sephadex G-150. A 4-ml sample of phosphonoprotein fraction S1-DS from *M. senile* was dialyzed against 0.02 M Tris-citrate (pH 8) and applied to a calibrated Sephadex G-150 column (see text). The column was eluted with the same buffer at a flow rate of 9 ml/hr. The eluent was collected in test tubes and samples analyzed for P_T (×) and absorbancy at 280 nm (●).

due to destruction of amino acids during acid hydrolysis in the presence of carbohydrate or to the presence of an as yet unidentified constituent in the protein.

Fraction S-1 was homogenized in 0.02 M Tris-citrate, pH 8.7, and dialyzed twice against 6.7 vol of the same buffer. Of the total P_T, only 1.3% was dialyzable. After dialysis and centrifugation, 85% of the total 2-aminoethylphosphonic acid containing material was solubilized and was designated S1-DS. In four separate S1-DS preparations, the 2-aminoethylphosphonic acid content by P_{C-P} analysis varied between 0.81 and 0.86 μmol/mg of protein and the neutral carbohydrate content increased to 0.63 μmol/mg of protein. Except for these increases in neutral carbohydrate, 2-aminoethylphosphonic acid, and glycosamine, the amino acid composition and the results from other analyses were not significantly changed from S1.

Electrophoresis of S1-DS was performed on cellulose acetate membranes (Figure 2). The A_{520} and P_T analyses indicated all protein to be associated with phosphorus and the specific 2-aminoethylphosphonic acid content of the protein to be nearly constant and independent of migration rate. Samples of S1-DS (20–50 μg) were subjected to electrophoresis in 7% polyacrylamide gels (Ornstein and Davis, 1964) in Tris-HCl buffer at pH 8.3. After electrophoresis the periodic acid Schiff's reagent positive and Coomassie Blue staining regions were coordinate and confined to the first 3 mm at the top (origin) of the gel columns indicating little or no penetration into the gel. Electrophoresis of samples of S1-DS through both 6 and 11.2% polyacrylamide gels in 1% sodium dodecyl sulfate with and without 8 M urea using the procedures of Korn and Wright (1973) gave similar results; some of the Coomassie Blue and all of the periodic acid Schiff's reagent staining material remained in the first 5 mm at the top of the gel. Faint Coomassie Blue staining bands were evident between 0.2 (R_m) and 0.6 (R_m). The 6 and 11.2% urea-sodium dodecyl sulfate gels were sliced in 3-mm cross-sections and each cross-section analyzed for P_T. No interference with the ashing and phosphorus recovery was noted when 0.04 μmol of 2-aminoethylphosphonic acid was included with control gel sections. Only the strongly periodic acid Schiff's reagent positive and faintly Coomassie Blue staining region in the first 3 mm at the top of the gels contained measurable amounts of P_T. Bovine serum albumin (25–50 μg) was included with each electrophoresis experiment on a separate gel column to ensure proper functioning of the gel electrophoresis experiment.

When S1-DS of *M. senile* was chromatographed on a calibrated Sephadex G-150 column, the A_{280} and P_T analyses of the collected fractions were nearly congruent (Figure 3). The material eluted in one main peak behind the V_0 of the column and just ahead of pyruvate kinase with an apparent mol wt of 2.5–3.0 × 10^5. The specific 2-aminoethylphosphonic acid content of the protein was constant throughout the peak. The 2-aminoethylphosphonic acid content, carbohydrate content, and amino acid composition of the protein in the combined protein in the S1-DS G-150 peak (Table V) remained the same as that of S1-DS.

Fraction S1-DS (20 ml) from *M. senile* was dialyzed against 500 ml of 0.02 M sodium citrate, pH 6. After dialysis, the final protein concentration was about 5 mg/ml. To a 10-ml portion of this dialyzed fraction powdered ammonium sulfate was added to saturation at 4°. After standing overnight at 4°, no precipitate formed indicating that this protein was soluble in saturated ammonium sulfate under these conditions. No precipitate formed when another portion was heated to 90° for 10 min.

The fibrous precipitate (P-1), which contained 50% of the total P_{C-P} in the whole body protein, was investigated to evaluate the possible existence of other phosphonic acid rich proteins (Table III). To exhaustively extract the soluble proteins, P-1 (200 mg) was homogenized three times with 10 ml of 0.02 M citrate buffer at pH 6.0 and one time in 20 ml of 0.02 M Tris-citrate at pH 8.7. The insoluble residue after each homogenization was collected by centrifugation. The final residue of insoluble protein (washed P-1) had a specific 2-aminoethylphosphonic acid content (0.22–0.25 μmol/mg) about equal to whole body protein and somewhat less than P-1. The amino acid composition was significantly different from that of the 2-aminoethylphosphonic acid rich protein, S1-DS (Table V). Additionally, P-1 contained no hydroxyproline or hydroxylysine, had a low glycine content, and had a moderate amount of glycosamine. In spite of exhaustive extraction in dilute citrate buffer, some soluble 2-aminoethylphosphonic acid rich proteinaceous material may have remained in the matrix of this insoluble protein with a myosin-like consistency.

FIBROUS PROTEIN FROM *M. senile*. After autoclaving and alcohol fractionation of the acid soluble fibrous protein, alcohol fraction 2 had an amino acid composition characteristic of gelatin or collagen (Table V). The 2-aminoethylphosphonic acid content of the gelatin was no more than 1.6 residues/1000. The autoclaved protein precipitate (APP) had an amino acid composition (Table V) which differed from that of the gelatin; for instance, APP had no hydroxyproline or hydroxylysine, a low glycine content, and 2-aminoethylphosphonic acid in concentrations approximating whole body and P-1 protein. Alcohol fraction 1 of the material made soluble by autoclaving the acid-extracted fibrous protein was intermediate in amino acid composition and 2-aminoethylphosphonic acid content. Apparently the small amount of 2-aminoethylphosphonic acid found in the gelatin was associated with other proteins which fractionate with collagen.

N-METHYL-2-AMINOETHYLPHOSPHONIC ACID RICH PROTEIN FROM *A. xanthogrammica*. A sample (200 mg) of the P_{C-P}-rich protein fraction (S-1 in Table IV) from *A. xanthogrammica* was homogenized in 20 ml of 0.02 M sodium citrate, pH 6.0. The precipitate was collected and homogenized again with 0.02 M Tris-citrate, pH 8, in the manner described previously for the same protein fraction isolated from *M. senile*. After dialysis of the combined soluble protein against 0.02 M sodium citrate, a sample of this S1-DS from *A. xanthogrammica* with a P_{C-P} content of 0.40 μmol/mg of protein was applied to a calibrated Sephadex G-150 column. The elution pattern (Figure 4) indicated that the P_{C-P}-rich protein was associated with proteins with a mol wt of 2.5–3.0 × 10^5. The ratio of P_{C-P} to absorbancy at 280 nm for the material eluting from the column was the same as that applied to the column (about 0.5 μmol of P_{C-P}/A_{280}). The fractions containing the P_{C-P} were combined (25 ml), and the protein precipitated by addition of 50 ml of cold ethanol (−20°) at 0°. The alcohol-precipitated protein, which contained all of the protein and phosphorus, was dissolved in the barbital buffer and a sample subjected to electrophoresis on cellulose acetate strips as described above (Figure 2). The two Ponceau S staining regions contained phosphorus and had about the same electrophoretic migration as described for the *M. senile* P_{C-P}-rich protein. Experiments with polyacrylamide gel electrophoresis of samples from this fraction, performed as described for the *M. senile* S1-DS, indicated the same meager penetration of the periodic acid Schiff's reagent positive Coomassie Blue staining material.

The amino acid analysis of the P_{C-P}-rich protein (S1-DS from *A. xanthogrammica* (Table V) indicated a glycoprotein containing *N*-methyl-2-aminoethylphosphonic acid and 2-aminoethylphosphonic acid in a ratio of about 12:1. The hexose content was 0.44 glucose equiv/mg of protein. Sialic acid was less than 0.02 μmol/mg and the fatty acid content was 0.01 μmol/mg of protein. The P_{C-P}-rich protein in S1-DS fractions of *A. xanthogrammica* did not precipitate in saturated ammonium sulfate solutions at pH 5.5 and did not precipitate after heating at 90° for 10 min at pH 6 in 0.02 M sodium citrate.

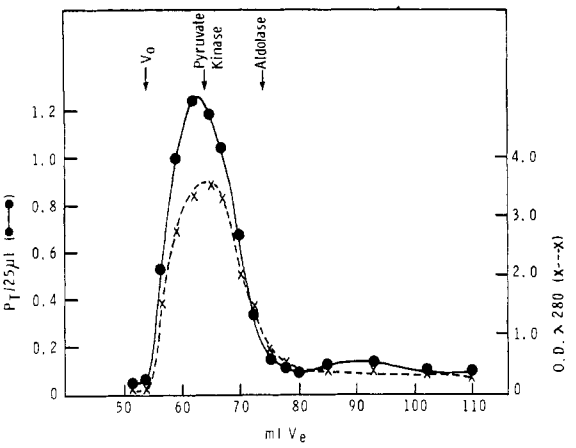

FIGURE 4: Elution pattern of the phosphonic acid rich protein fraction (S1-DS) from *A. xanthogrammica* after chromatography on Sephadex G-150. A 5-ml sample of S1-DS containing 40 μmol of P_{C-P} was applied to a calibrated Sephadex G-150 column (see text) in 0.2 M sodium citrate, pH 6. The column was eluted with the same buffer at a flow rate of 9 ml/hr. The eluent was collected in test tubes and analyzed for P_T and absorbancy at 280 nm.

Discussion

In this investigation of the occurrence of phosphonic acid rich proteins in lower metazoans, the P_{C-P} content in whole body protein is 0.18–0.22 μmol/mg in *M. senile* and 0.15–0.19 μmol/mg in *A. xanthogrammica*. In *M. senile* 99.7% of the protein-bound aminophosphonic acid is 2-aminoethylphosphonic acid with less than 0.3% as *N*-methyl derivatives of 2-aminoethylphosphonic acid, 2-amino3-phosphonopropionate, or other aminophosphonic acids. In *A. xanthogrammica* 99% of the protein-bound aminophosphonic acid is accounted for as *N*-methyl-2-aminoethylphosphonic acid (90%) and 2-aminoethylphosphonic acid (10%). In addition to data presented here, the identity of the aminophosphonic acids in these organisms has been established by infrared and nuclear magnetic resonance (nmr) spectroscopy (Kittredge *et al.*, 1967; Shelburne and Quin, 1967; Henderson *et al.*, 1972). Quin (1965) reports protein-bound P_{C-P} in whole body protein of 0.086 μmol/mg and 0.052 μmol/mg in the sea anemones *Metridium dianthus* and *Telia felina* and the mollusc *Buscycon canaleculatum*, respectively. Two other species of mollusc, *Mytilus edulis* and *Venus mercenaria*, and the starfish *Asterias forbesi* had less than 0.016 μmol of P_{C-P}/mg of crude protein. Shelburne and Quin (1967) report the isolation of 0.0043 μmol of 2-aminoethylphosphonic acid/mg and 0.014 of *N*-methyl-2-aminoethylphosphonic acid/mg from a proteinaceous material of *A. xanthogrammica*. This *N*-methyl-2-aminoethylphosphonic acid to 2-aminoethylphosphonic acid ratio of 4:1 reported by Shelburne and Quin (1967) differs somewhat from the ratio reported here. The absolute levels of P_{C-P}/mg of crude protein in Quin's reports are lower than the levels reported here using the amino acid analyzer in combination with the differential phosphorus analysis. The level of 0.008 μmol of 2-aminoethylphosphonic acid/mg of proteinaceous residue of human brain and liver deserves reinvestigation (Alhadeff and Davis, 1971).

In both *M. senile* and *A. xanthogrammica* enrichment of the phosphonic acid content of these proteins is obtained by acetone fractionation, dialysis, and Sephadex gel filtration. The isolation procedure (Figure 1) isolates a group of proteins whose solubility is greatly influenced by their phosphonic acid and carbohydrate content. Although the mode of bonding of aminophosphonic acids to this protein has not been described, the bonding is strong enough to resist severe extraction conditions and, accordingly, the complex is designated phosphonoprotein. The P_{C-P}-rich protein contains neutral sugars, amino sugars, and most amino acids, has no lipid, sialic acid, hydroxyproline, hydroxylysine, nor large amount of glycine, has a mol wt between 2.5 and 3.0 × 10^5 by gel filtration, is relatively heat stable, is soluble in saturated ammonium sulfate solutions, and migrates as protein when subjected to electrophoresis on cellulose acetate strips. The

preparations show coordinate positive staining with the periodic acid Schiff's reagent and Coomassie Blue with gel electrophoresis in various systems used by Korn and Wright (1973) to separate the phosphonoglycan from membrane protein. The lack of penetration of these phosphonoproteins into the sodium dodecyl sulfate–urea gels suggests that the molecular weight estimation by gel filtration may be low, that the phosphorus and glycan moieties are closely associated, and that this treatment does not completely resolve the glycan from the protein. In *M. senile* all of the protein-bound aminophosphonic acid exists as 2-aminoethylphosphonic acid while the phosphonoprotein from *A. xanthogrammica* contains both *N*-methyl-2-aminoethylphosphonic acid and 2-aminoethylphosphonic acid. In *A. xanthogrammica*, the *N*-methyl-2-aminoethylphosphonic acid to 2-aminoethylphosphonic acid ratio of 10 or 12:1 in all protein fractions isolated during purification suggests a uniformity of insertion in a group of similar proteins or the presence of a single phosphonoprotein. The purified phosphonoprotein fraction from *M. senile* contains a remarkable 171 residues/1000 residues and a fourfold enrichment of protein-bound P_{C-P} from the whole body protein. The same protein fraction from *A. xanthogrammica* contains 76 residues of aminophosphonic acid/1000. Since the P_{C-P}-rich protein fraction (S-1) in both organisms accounts for only 30% of the total protein-bound aminophosphonic acid, other distinctly different P_{C-P}-rich proteins may exist in the P-1 protein fraction.

2-Aminoethylphosphonic acid was absent from collagen *M. senile* which comprises 23% of the total protein of this animal as calculated from the hydroxyproline content. The retention volume of 2-aminoethylphosphonic acid on the long column of the amino acid analyzer (Table I) corresponds closely with the retention volume shown for the ninhydrin positive compound "X" in some invertebrate collagens (Piez and Gross, 1959). The substance X may be 2-aminoethylphosphonic acid since there is a correspondence between its relative concentrations and values for ash among the various collagens studied. Although the collagen from *M. senile* may contain a trace of X, Piez and Gross (1959) report that the collagens of spongin A and spongin B definitely contain substance X. Some 2-aminoethylphosphonic acid containing protein associated with the collagen from *M. senile* dissociated from collagen by autoclaving. The amino acid composition of this collagen-associated protein resembles the P-1 protein to some extent but has no resemblance to the phosphonoprotein or proteins reported to be associated with collagen (*i.e.*, elastoidin (Gross, 1963)).

Aminophosphonic acids may also be absent from most of the proteins of the S-2 fraction, which contains many of the soluble enzymes. An enzyme, adenosine deaminase, purified from the same S-2 protein fraction of *M. senile* contains no 2-aminoethylphosphonic acid (Bishop *et al.*, 1972). A proteolytic enzyme isolated from a similar fraction of *M. senile* may contain a small amount of 2-aminoethylphosphonic acid (Gibson and Dixon, 1969; Kittredge and Roberts, 1969). Aminophosphonic acids have not been reported as constituent amino acids in the limited number of proteins isolated from other coelenterates. These proteins include proteases from *Renilla reniformis* (Coan and Travis, 1970), luminescent protein from *Aqueora* (Shimomura and Johnson, 1969), structural coral protein from a variety of coral species (Young, 1971; Silberberg *et al.*, 1972), collagen (Katzman and Kang, 1972; Nordwig and Hayduk, 1969; Pikkarainen *et al.*, 1968; Piez and Gross, 1959), and nematocyst protein (Blanquet and Lenhoff, 1966).

These data indicate that aminophosphonic acids are distributed in some but not all proteins of these sea anemones, suggesting a specific physiological role for the protein. For instance, the distribution of aminophosphonic acids in lipid is nonrandom and some specific functions have been ascribed to these phosphonolipids. In lower metazoans which apparently possess the ability for *de novo* biosynthesis of the aminophosphonic acids, the aminophosphonic acids are primarily found in sphingolipids (Hori *et al.*, 1964, 1966, 1967; Hori and Arakawa, 1969; Higashi and Hori, 1968; Hayashi *et al.*, 1969; Hayashi and Matsuura, 1971; deKoning, 1966b; Sampugna *et al.*, 1972; Rouser *et al.*, 1963; Simon and Rouser, 1967). Although a small amount of 2-aminoethylphosphonic acid ceramide has been found in lipids from *Tetrahymena* (Carter and Gaver, 1967), most lipid-bound aminophosphonic acid is found in phosphodiglycerides (Rosenberg, 1964; Liang and Rosenberg, 1966; Thompson, 1967; Dawson and Kemp, 1967; Kennedy and Thompson, 1970; Smith *et al.*, 1970; Sugita and Hori, 1971; Jonah and Erwin, 1971; Nozawa and Thompson, 1971). In *Tetrahymena*, 2-aminoethylphosphonic acid diglycerides are heavily concentrated in the glyceryl ether fraction and the greatest portions of the phospholipids associated with the ciliary membranes are phosphonolipids (Thompson, 1967; Kennedy and Thompson, 1970; Nozawa and Thompson, 1971; Smith *et al.*, 1970; Jonah and Erwin, 1971).

Speculations about the function of P_{C-P}-containing proteins must be very general. Considering the several minor nonphosphorus-containing proteins evident after the sodium dodecyl sulfate–urea treatment and gel electrophoresis and the small degree of enrichment during purification, phosphonoproteins isolated from these two anemones probably represent major fractions of the total protein of the organisms. If the phosphonic acid group is not completely derivatized, the phosphonoprotein may act as an ion exchanger in the maintenance of salt balance within the organism such as with algenic acid (Eppley, 1958; Smidsrød and Haug, 1965). It may serve some structural role as with the phosphopolysaccharide "onuphic acid" of some parchment worm tubes (Pautard and Zola, 1967a,b) or the membrane-bound phosphonoglycan of the amoeba membrane preparations (Korn and Wright, 1973). As a highly hydrated polyanion, it may provide viscoelastic properties similar to hyaluronic acid (Balazs, 1966). Although the tissue distribution within these organisms has not been determined, the distribution becomes increasingly interesting in view of recent attempts to account for the viscoelastic properties of the mesogleal tissue from *M. senile* (Gosline, 1971a,b).

The bonding of aminophosphonic acids in protein has not been investigated thoroughly. Aminophosphonic acids may be attached to the phosphonoprotein directly as constituents within the peptide backbone, directly as side-chain additions to the peptide backbone, or indirectly as constituents of a lipid or polysaccharide side-chain addition to the peptide backbone as phosphoesters, phosphoamidates, or amides. Acid hydrolysis of the phosphonoprotein produces a free aminophosphonic acid indicating bonding through an acid-labile linkage such as an amidate, amide, or ester. Protein-bound 2-aminoethylphosphonic acid from *M. dianthus* cannot be derivitized with 2,4-dinitrofluorobenzene without prior hydrolysis of the protein in acid (Quin, 1965). Dansyl-aminophosphonic acid derivatives could not be prepared without prior acid hydrolysis of the phosphonoprotein isolated from *M. senile* (Kirkpatrick and Bishop, unpublished). These experiments suggest that the amino group may be involved in

bonding to the protein or N-acylated as reported by Hori and Arakawa (1969) for some 2-aminoethylphosphonic acid ceramides. Our fatty acid analysis would not have detected short-chain fatty acids such as acetic acid.

It is unlikely that aminophosphonic acids occur as constituents within the peptide backbone because of the lability of the phosphoamidate bond. No amino acid in *M. senile* phosphonoprotein occurs in amounts equimolar with 2-aminoethylphosphonic acid (Table V) suggesting that bonding directly as a side-chain addition to the peptide backbone is unlikely unless several species of amino acids serve as "anchor" sites. For instance, the combined serine and threonine content (162 residues/1000 residues) could only account for the 2-aminoethylphosphonic acid content if virtually all were derivatized as phosphomonoesters; the combined aspartate and glutamate content (125 residues/1000 residues) cannot account for all of the 2-aminoethylphosphonic acid as ω-carboxyamides. If the aminophosphonic acids are polymerized as polyphosphate chains, then a single serine, threonine, aspartate, or glutamate residue could account for bonding of several aminophosphonic acid residues.

The possibility of indirect bonding to the peptide backbone through a lipid or polysaccharide side chain seems most probable. The absence of fatty acids eliminates the lipid side-chain possibility. The carbohydrate concentration, on the other hand, is approximately equal to the aminophosphonic acid concentrations in preparations of phosphonoprotein from both species. The experiments with the sodium dodecyl sulfate–urea gel electrophoresis suggest a close similarity to the phosphonoglycan reported by Korn *et al.* (1973). Carbohydrate in most glycoproteins is linked glycosidically through a hexosamine moiety to a hydroxyamino acid or to asparagine (Montgomery, 1970). In *M. senile*, the glucosamine concentration (73 residues/1000 residues) suggests that the carbohydrate chain could consist of one glucosamine and two other saccharides. Serine, threonine, asparagine, or glutamine could serve as the "anchor" point for the heteropolysaccharide side chain. Again, the possibility of a polymeric polyphosphate chain of aminophosphonic acid residues linked as an ester to hydroxyl group on a sugar residue is open.

Considering the relative abundance of aminophosphonic acids, carbohydrates, hydroxyamino acids, and dicarboxylic amino acids, phosphoesterification of the aminophosphonic acid to the carbohydrate moiety with N-acylation of the amino group by a short-chain fatty acid or other acid-labile linkage seems the most probable mode of bonding. The recent demonstration of a separate phosphonoglycan in amoeba membranes (Korn *et al.*, 1973) indicates a precedence for this type of linkage. The lability of the bonds involving aminophosphonic acids during hydrolysis in 6 N HCl is consistent with this model.

Experiments to determine whether the aminophosphonic acids are associated with the protein backbone or the glycan and whether or not the glycan is covalently linked to the protein are in progress.

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