An Examination of Marine Animals for the Presence of Carbon-bound Phosphorus[1]

Louis D. Quin and Frank A. Shelburne

Department of Chemistry
Duke University
Durham, North Carolina 27706

ABSTRACT

Thirty-one marine animals from nine different phyla were analyzed for the presence of carbon-bound phosphorus. Representatives of the Phyla Coelenterata, Mollusca, and Echinodermata had previously been shown to contain this type of phosphorus (C-P); the present study has added a number of new animals from these phyla to the positive list and has shown that members of the Phyla Annelida, Arthropoda, and Porifera may also contain carbon-bound phosphorus. C-P phosphorus has been found in material extracted with 70% ethanol as well as in the largely proteinaceous residues from these extractions. While animals from all the phyla mentioned contain C-P phosphorus in extractable material, it appears that Coelenterates, especially sea anemones, are unique in containing large amounts in the proteinaceous residues.

Introduction. Prior to 1959, phosphorus in organisms had been considered to be combined chemically only in the form of derivatives (esters, amides or anhydrides) of phosphoric acid. The discovery in that year (2) of a natural compound containing the carbon-phosphorus (C-P) bond has necessitated a new attitude toward the biological involvement of phosphorus. The compound isolated was 2-aminoethylphosphonic acid (AEPA), $NH_2CH_2CH_2PO(OH)_2$; it was first found in members of the Phylum Protozoa growing in sheep rumen. It was not long before animals of the marine environment were implicated in this new development; in 1962 a sea anemone was reported (5) to contain AEPA, and it was announced in 1965 (7) that a few other common marine invertebrates also possessed significant amounts of the substance. The amount of C-P phosphorus may be large; one sea anemone (*Tealia felina*) was found to have as much as 50% of its phosphorus in this form (7). Table I provides a summary of the marine animals previously reported to contain compounds with the C-P bond.

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The C-P bond is very stable; it is resistant to cleavage by oxidizing agents and is not hydrolyzed by either acidic or basic media. Phosphonic acids, therefore, cannot serve biologically in the same manner as the well-known phosphoric acid derivatives, which readily release or transfer phosphate; such acids would appear to have their own unique role in life processes. At present, there is no information revealing what the role might be, and no enzyme system has yet been identified that is capable of either making or breaking the C-P bond. As noted, the amount of carbon-bound phosphorus is appreciable in some animals and must be of real significance to them in some way. This long-overlooked aspect of the biological involvement of phosphorus raises a number of fascinating questions and offers a fertile field for investigation.

The earliest studies revealed that AEPA is incorporated in structures extractable along with lipids; later work showed more specifically that AEPA occurred in a form resembling a cephalin (6) and occurred in association with sphingosine (9). However, it was shown early that insoluble proteinaceous residues from extractions of marine animals contain large amounts of bound AEPA (7). The exact nature of the chemical association of AEPA in proteins remains to be established. While AEPA stands as that C-P compound most commonly encountered in living systems, other aminophosphonic acids have also been detected. These include α-amino-β-phosphonopropionic acid (3), 2-(N-methylamino)ethylphosphonic acid (4, 10), 2-(trimethylammonio)ethylphosphonic acid (4), and possibly 2-(dimethylamino)ethylphosphonic acid (4). A survey of this new and rapidly developing field was published in 1967 (8).

Table I. Marine animals previously known to contain carbon-bound phosphorus.

Animal	Fraction containing C-P: Extractable	Fraction containing C-P: Insoluble residue	Reference
Sea Anemones			
Anthopleura elegantissima	x	x	(3, 5)
Metridium dianthus	x	x	(7)
Tealia felina	x	x	(7)
Anthopleura xanthogrammica	x	x	(4, 10)
Coral			
Zoanthus sociatus	x		(3)
Mollusks			
Mytilus edulis	x	absent	(7)
Venus mercenaria	x	absent	(7)
Busycon canaliculatum	x	x*	(7)
Haliotis midae	x		(1)
Starfish			
Asterias forbesi	x	absent	(7)

* The present study has not confirmed this observation.

The purpose of the present study has been to explore in greater detail than heretofore the extent of the occurrence of carbon-bound phosphorus in marine members of the animal kingdom. Particular emphasis has been given to the extent of incorporation of C-P compounds in insoluble proteinaceous material. Earlier studies had suggested that sea anemones may be unusual in containing large amounts of C-P in protein association (7).

In the absence of a direct method for the determination of the amount of phosphorus bonded to carbon, a difference method, developed earlier (7), was employed. This method takes advantage of the stability of the C-P bond to hydrolysis; only a few organophosphorus compounds, notably those with functionality on the carbon *alpha* to phosphorus, lack good stability to vigorous acid hydrolysis. Therefore, a determination of total phosphorus by a combustion method and a determination of phosphate phosphorus released on acid hydrolysis will, by difference, provide a measure of C-P phosphorus. On several occasions, specific analysis for AEPA, which has been found to respond well on automatic aminoacid analyzers, was used to confirm an indication from the phosphorus analyses that C-P phosphorus was present.

Experimental Methods. Animal Fractionation. The animals included in this study are listed in Table II; they were collected in the Beaufort, North Carolina, area. The exoskeleton of members of the Phyla Mollusca and Arthropoda was removed and was not processed further unless otherwise indicated. The whole animal, or a dissected part thereof, was chopped and then homogenized in a Waring Blendor with three to four times its volume of 70% ethanol. The homogenate was centrifuged and the ethanolic extract was decanted. This process was repeated three or four times until the supernatant liquid was colorless. The combined alcohol extracts were stripped *in vacuo* on a rotary evaporator, and the residue was further dried in a vacuum desiccator. The residue from the centrifugation was dried similarly, ground to a fine powder in a Wiley Mill, and then analyzed as described below for C-P phosphorus. When positive results were obtained, the residue was subjected to chloroform extraction in a Soxhlet apparatus for 24 hours to insure complete removal of the lipid-bound phosphorus. The amount of chloroform solubles was small, and this material was not generally analyzed. Data from the fractionation of various animals are also given in Table II.

Phosphorus Analyses. Samples were analyzed for total phosphorus by a combustion method and for phosphorus released as phosphate on vigorous hydrolysis.

The combustion was performed by the Schöniger oxygen-flask method, using the apparatus supplied by A. H. Thomas Co., Philadelphia, Pa. Combustion was performed on a 10–30-mg sample in a 500-ml flask containing 10 ml of nitric acid water (1:5). After a 15–20-minute period, or until all "fog" had condensed, the platinum sample carrier was allowed to drop into

Table II. Animal fractionation data (in grams).

	Wet weight	Alcohol solubles	Insoluble residue
PHYLUM PORIFERA			
Sponge			
Hymeniacidon heliophila	80.0	1.9	8.9
PHYLUM COELENTERATA			
Class Hydrozoa			
Hydroid			
Tubularia crocea	10.0	–	0.2
Man-of-war			
Physalia physalius			
Float	–	–	0.8
Tentacles	–	–	0.2
Class Scyphozoa			
Jellyfish			
Dactylometra quinquecirrha	–	4.9	0.7
Class Anthozoa			
Sea anemone			
Aiptasia pallida	30.0	0.4	0.8
Bunadosoma cavernata	202	9.2	50.0
Paractis rapiformis	3.6	–	0.2
Cerianthus americanus	23.0	–	1.6
Soft coral			
Leptogorgia virgulata			
Rind	24.0	–	18.0
Axial rod	11.0	–	7.2
Sea pansy			
Renilla reniformis	43.0	0.9	6.0
PHYLUM CTENOPHORA			
Mnemiopsis leidy	–	9.0*	2.9
PHYLUM ANNELIDA			
Segmented worm			
Chaetopterus variopedatus ...	62.0	3.6	3.1
PHYLUM BRYOZOA			
Bugula neritina	18.0	0.9	2.7
PHYLUM MOLLUSCA			
Class Gastropoda			
Knobbed whelk			
Busycon carica	218.0	–	52.0
Channeled whelk			
Busycon canaliculatum	74.0	–	15.3
Snail			
Fasciolaria hunteria	22.0	–	5.0
Nudibranch			
Archidoris sp.....	–	–	0.1
Class Pelecypoda			
Oyster			
Crassostrea virginica	95.0	2.2	11.0

(Table II continued)	Wet weight	Alcohol solubles	Insoluble residue
Scallop			
Lucina radians	76.0	3.7	6.3
Class Cephalopoda			
Squid			
Loligo pealei	31.0	2.7	4.4
PHYLUM ARTHROPODA			
Class Crustacea			
Shrimp			
Peneus duorarum			
Muscle	13.0	0.7	2.1
Organs	7.8	0.9	1.0
Shell	4.7	–	1.4
Stone crab			
Minippe mercenaria			
Muscle and organs	73.7	5.7	27.0
Shell	75.0	–	33.0
Blue crab			
Callinectes sapidus			
Muscle and organs	50.0	3.1	8.1
PHYLUM ECHINODERMATA			
Class Asteroidea			
Starfish			
Asterias forbesi	37.0	1.8	2.8
Astropecten articulatus	21.0	–	8.6
Class Ophiuroidea			
Brittle star			
Ophiomusium lymani†	–	0.76	5.9
Class Echinoidea			
Sea urchin			
Arbacia punctulata	35.5	–	13.8
Class Holothuroidea			
Sea cucumber			
Euphronides sp.	107.0	–	3.5
PHYLUM CHORDATA			
Class Ascidiacea			
Tunicate			
Styela plicata	38.0	1.4	1.5
Class Osteichthyes			
Pinfish			
Lagadon rhomboides			
Organs**	20.0	1.1	3.7
Muscle	88.0	5.1	21.4

* The alcohol solubles contained considerable inorganic material. The recorded weight is that portion extractable with chloroform.

† Obtained from Blake Plateau at 1500 m.

** Includes eyes, brain, intestinal system.

the solvent. The top was disconnected and washed with a small amount of water. After boiling for 2 minutes, the contents of the flask were washed through Whatman No. 41 filter paper into a 100-ml volumetric flask and diluted to the mark. An aliquot of 50 ml was transferred to a 100-ml volumetric flask; 13.0 ml of the 1:5 nitric acid solution was added, giving a final acid concentration of 0.5 N.

The hydrolysis was performed on a sample of 20–60 mg with 30 ml of 6 N hydrochloric acid in a 4-oz teflon bottle. The bottle was capped and heated in an oil bath at 105° ± 2°C for 48 hours. A small portion of activated charcoal (Nuchar CN) was added to the bottle while hot. If color remained, the charcoal treatment was repeated. The hydrolysate was filtered through Whatman No. 41 paper into a 100-ml volumetric flask and diluted to the mark. A 25-ml aliquot was transferred to a 100-ml volumetric flask and 1.2 ml of the 1:5 nitric acid was added, making a final acid concentration of 0.5 N.

The analysis for phosphate in the solutions from both sources was performed as follows. Reagents were: (i) Vanadate solution. A 2.5-g portion of ammonium vanadate, NH_4VO_3, was dissolved in about 500 ml of hot water; 20 ml of conc. nitric acid was added, and the solution was diluted to 1000 ml, and (ii) Molybdate solution. A 50.0-g portion of ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, was dissolved in warm water. The solution was filtered into a 1000-ml volumetric flask and diluted to the mark.

Ten ml of each reagent solution was added to the volumetric flask containing the sample to be analyzed and water was added to 100 ml. A blank was also prepared from 18.0 ml of 1:5 nitric acid and 10 ml of each reagent solution, with dilution to 100 ml. After 30 minutes, the absorbance of the sample versus the reagent blank was read at 360 mμ in a 1-cm cell using a Beckman DU Spectrophotometer. The absorbance was converted to percent phosphorus by use of a calibration chart prepared from solutions of known phosphate content. For consistent results, it has been found necessary to conduct the color development at constant temperature. Duplicate analyses were performed on several samples; values generally differed by no more than 0.03%.

2-Aminoethylphosphonic Acid Analysis. A Beckman Spinco Model 120B automatic aminoacid analyzer was used; elution was performed with a sodium citrate buffer of pH 3.25. Synthetic AEPA was eluted in about 26 minutes, before common acidic aminoacids and close to taurine. (A mixture of AEPA and taurine could not be resolved but gave a broadened peak.) In a typical analysis, a 5–10-mg sample of defatted insoluble residue was hydrolyzed with 1 ml of 6 N hydrochloric acid in an evacuated sealed tube for 22 hours. The acid was removed on a rotary evaporator, and the residue was diluted with deionized water so as to obtain an aminoacid concentration of about 0.1 micromole per ml. A 1.0-ml aliquot was applied to the analyzer column. The results are given in Table IV.

Table III. Phosphorus analyses on animal fractions (percent phosphorus).

	Alcohol solubles			Insoluble residue		
	Total	Hydrolyzable	C-P	Total	Hydrolyzable	C-P
PHYLUM PORIFERA						
H. heliophila	0.53	0.47	0.06	0.17	0.10	0.07
PHYLUM COELENTERATA						
A. pallida	0.24	0.22	0.02	0.80	0.50	0.30
B. cavernata	0.65	0.41	0.24	0.90	0.53	0.37
P. rapiformis				0.80	0.59	0.21
C. americanus				0.64	1.04*	−0.40
Actinia equina†				1.10	0.60	0.50
L. virgulata						
Rind	0.48	0.22	0.26	0.42	0.32	0.10
Axial rod				7.62	7.47	0.15
T. crocea	0.22	0.18	0.04	0.74	0.68	0.06
P. physalius						
Float				0.64	0.43	0.21
Tentacles				0.65	0.45	0.20
D. quinquecirrha	0.16	0.06	0.10	0.85	0.85	0.00
R. reniformis	0.48	0.26	0.22	0.29	0.12	0.17
PHYLUM CTENOPHORA						
*M. leidy***	0.11	0.11	0.00	1.17	1.17	0.00
PHYLUM ANNELIDA						
C. variopedatus	0.60	0.50	0.10	1.02	1.03	−0.01
PHYLUM BRYOZOA						
B. neritina	0.29	0.33	−0.04	0.53	0.56	−0.03
PHYLUM MOLLUSCA						
C. virginica	0.88	0.56	0.32	0.88	0.83	0.05
L. radians	1.03	0.96	0.07	1.47	1.49	−0.02
L. pealei	1.92	1.75	0.17	0.73	0.77	−0.04
B. carica				0.35	0.35	0.00
B. canaliculatum				0.54	0.58	−0.04
F. hunteria				0.62	0.59	0.03
Archidoris sp.				0.64	0.50	0.14
PHYLUM ARTHROPODA						
P. duorarum						
Muscle	2.09	1.95	0.14	0.52	0.49	0.03
Organs	0.38	0.21	0.17	1.38	1.37	0.01
Shell				1.36	1.29	0.07
M. mercenaria						
Muscle and organs	0.81	0.70	0.11	0.85	0.90	0.05
Shell				0.77	0.82	−0.05
C. sapidus††				2.04	2.03	0.01

(Table III continued)

	Alcohol solubles			Insoluble residue		
	Total	Hydrolyzable	C-P	Total	Hydrolyzable	C-P
PHYLUM ECHINODERMATA						
A. forbesi	0.69	0.54	0.15	0.13	0.15	−0.02
A. punctulata	0.31	0.23	0.08	0.10	0.11	−0.01
Euphronides sp.**	1.11	1.14	−0.03	0.61	0.71	−0.10
O. lymani	0.27	0.30	−0.03	0.12	0.21	−0.09
A. articulatus				0.24	0.24	0.00
PHYLUM CHORDATA						
L. rhomboides						
Muscle	0.83	0.83	0.00	1.44	1.41	0.03
Organs	2.62	2.60	0.02	1.25	1.25	0.00
S. plicata	0.24	0.24	0.00	0.53	0.56	−0.03

* This value is high due to considerable background color of the hydrolysate, which resisted decolorization with charcoal.

† Fractions were contributed by R. C. Denney from another study.

** The alcohol extract was of high salt content; the analysis was performed on chloroform extractables from this fraction.

†† The chloroform extractables (0.6 g) from the alcohol-insoluble fraction contained 0.16% total phosphorus and 0.06% hydrolyzable phosphorus.

Table IV. Specific AEPA analysis on some insoluble residues (in percent).

	AEPA	Phosphorus as AEPA	% of C-P found as AEPA
A. pallida	1.07	0.27	90.0
B. cavernata	1.80	0.45	>100.0
C. americanus	0.56	0.18	–
L. virgulata			
Rind*	1.18	0.29	52.0
Archidoris sp.	0.28	0.07	50.0

* After removal of calcium carbonate by washing with 5% hydrochloric acid. The residue then contained 0.82% total phosphorus and 0.26% hydrolyzable phosphorus.

Results. A total of 31 animals from nine phyla were examined. Fractionation provided two main samples for most of the animals: (i) material extractable with 70% ethanol and (ii) material insoluble in this solvent. A chloroform extraction was performed on the second sample to insure complete removal of lipids; the small amount of chloroform-soluble material was not generally included in the analytical work. The animals examined in this survey, as well as relative weights of the pertinent fractions, are listed in Table II.

The indirect method described earlier (7) for C-P determination was employed in this study; values for phosphorus found as phosphate after combustion and after hydrolysis are given in Table III. When the difference in total and hydrolyzable phosphorus was less than about 0.05%, it was not considered that a reliable indication of C-P was at hand, since experimental error could

account for a difference of this magnitude. Very small amounts of C-P would be overlooked in this approach. However, as can be seen in Table III, the content of C-P in many animals well exceeds this limit.

While little difficulty was experienced in applying the combustion method to the analysis of either soluble or insoluble material, the hydrolyzable phosphorus determination was occasionally complicated by the presence of colored material formed in the hydrolysis; this material interfered with the spectroscopic method. Repeated treatment of such hydrolysates with decolorizing charcoal was occasionally necessary to obtain a colorless solution. One sample (from *C. americanus*), however, resisted even this treatment, and, as can be seen in Table III, the value for hydrolyzable phosphorus greatly exceeds that for total phosphorus. A recourse in this situation is to perform a direct analysis (see below) for AEPA; this procedure was applied to this fraction and AEPA was indeed found to be present. It remains a possibility that AEPA analysis of other fractions where the C-P value is very low or even negative would be similarly revealing.

No attempt was made to determine the exact chemical nature of the compounds containing carbon-bound phosphorus in every sample giving a positive result; much of it may be assumed to be in the form of AEPA, although one sea anemone is known from previous work to contain larger amounts of 2-(N-methylamino)ethylphosphonic acid than of AEPA (10). A few samples were specifically checked for the presence of AEPA by use of conventional automatic amino acid analysis. AEPA responds well to this method; with an acidic eluant, it emerges early, even before the common acidic aminoacids. It has a low ninhydrin color yield (16.7% of the average color yield of twelve common α-aminoacids). Results of these analyses appear in Table IV. In not every case was the amount of C-P associated with AEPA in good agreement with the amount of C-P found by the phosphorus analyses. Thus, for the soft coral, *L. virgulata*, and the nudibranch, *Archidoris*, only about 50% of the C-P material could be accounted for on the basis of the AEPA value. The possible presence of other C-P compounds is indicated, though hardly established, by these results.[2]

The usual precaution of employing fresh or properly preserved animals in the study was followed. The need for this was revealed in an examination of the sea anemone, *B. cavernata*. The first analysis on this animal was performed on a specimen that had died of starvation in a sea-water tank. The insoluble residue from this animal had 0.20% C-P by the phosphorus analyses, but aminoacid analysis revealed only a trace of AEPA. The analysis was then repeated on a specimen processed immediately after collection. By phosphorus analyses, the C-P content was found to be 0.37%; analysis for AEPA provided

2. The C-P analyses were run before chloroform extraction, while AEPA analyses were made after extraction. This slight difference in the nature of the samples is very unlikely to account for the observed discrepancy.

a value of 1.80%, equivalent to 0.45% phosphorus as C-P. These results suggest that some of the carbon-bound phosphorus may be degraded by processes occurring during the starvation period or even after death of the animal, in part to deaminated forms.

Discussion. Of the 31 animals included in this screen, 21 gave positive results for the presence of carbon-bound phosphorus in one or both of the two fractions examined. Three phyla – Porifera, Annelida, and Arthropoda – have been added to the list of those whose members may contain this type of phosphorus. Previously, the Phyla Coelenterata, Mollusca, and Echinodermata had been recognized in this sense, and it has been found that several new examples of animals in these phyla give positive results. It is now clear that compounds with carbon-bound phosphorus are of common occurrence among the marine invertebrates, and indeed they stand as an important type of constituent in many animals. It is also clear, however, that animals of the lower phyla, especially Coelenterata, contain appreciably more C-P than do those of the higher phyla. On the other hand, the occurrence of C-P in marine vertebrates remains to be demonstrated; while only one vertebrate[3] was analyzed in this study, it gave totally negative results.

Although carbon-bound phosphorus has thus been shown to have a wide occurrence among marine invertebrates, the chemical nature of the forms in which the C-P is incorporated in these animals is by no means uniform. Some animals contained C-P in alcohol-extractable material as well as in the proteinaceous residues; others contained C-P only in extractable forms. In only one instance (*A. pallida*) has C-P been detected in residues without the simultaneous discovery in alcohol-extractable material.

In this study, particular emphasis has been placed on the proteinaceous residues. It is now obvious that most members of the Phylum Coelenterata contain significant amounts of C-P phosphorus in such material. Indeed, every one of nine sea anemones so far studied – five in this program and four in earlier studies (Table I) – are positive in this respect. Those of the present study contained 25–45% of the total phosphorus as C-P phosphorus. Only three animals other than members of Coelenterata contained phosphorus in these fractions. One of these is a nudibranch (*Archidoris*), which is known to feed on sea anemones and to use their nematocysts for defensive purposes. The other exceptions are a sponge (*H. heliophila*) and the shell of a shrimp (*P. duorarum*). The C-P value of 0.07% for both is just beyond the range ascribable to experimental error and should not be considered significant until confirmed by more rigorous studies.

Two points relative to earlier published work need mention. It had been noted (7) that analysis of *A. forbesi* gave contradictory results; one specimen

3. During this study, a lipid extract of the liver from a basking shark became available. Analysis revealed no phosphorus to be present.

showed C-P in extractable form while another specimen was negative in this respect. As may be seen in Table III, the present study has confirmed the presence of C-P in the extractable material of this animal. The second point is the failure to reproduce the observation (7) that the whelk, *B. canaliculatum*, contains a significant amount of C-P in its insoluble residue. This is an important matter, for this earlier finding would constitute a major exception to the generality arrived at in this study that animals other than Coelenterates do not contain appreciable, if any, C-P in this material. The present study disposes of this exception; this animal, as well as the related *B. carica*, gave negative results. The earlier finding may have been due to imcomplete defatting of the residue.

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