The Presence of Compounds with a Carbon-Phosphorus Bond in Some Marine Invertebrates*

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ABSTRACT: By a difference in total and phosphate phosphorus, the presence of compounds containing the carbon-phosphorus bond was detected in certain fractions derived from six marine invertebrates. The phyla *Coelenterata*, *Mollusca*, and *Echinodermata* are represented. 2-Aminoethylphosphonic acid was isolated from hydrolysates of several of these fractions. In one case (sea anemone *Metridium dianthus*) it was demonstrated that this compound was present in lipid as well as insoluble protein. Peptic hydrolysis of this protein gave a polypeptide containing 4.4% of this acid. Dinitrophenylation studies revealed its amino group not to be free in the polypeptide, nor in smaller peptides from further hydrolyzed material.

Phosphorus was detected in nonsaponifiable lipid from *M. dianthus*, and some concentration of this was achieved by solvent extraction and chromatography.

Natural compounds with a carbon-phosphorus bond have only recently been observed. 2-Aminoethylphosphonic acid has been isolated from ciliate *Protozoa* (Horiguchi and Kandatsu, 1959, 1960; Kandatsu and Horiguchi, 1962) and the sea anemone *Anthopleura elegantissima* (Kittredge *et al.*, 1962). Evidence for a glyceride (Kittredge *et al.*, 1962) and a sphingolipid (Rouser *et al.*, 1963) involvement for this compound has been reported. A related compound, α-amino-β-phosphonopropionic acid, was detected in *Tetrahymena* and a zoanthid (Kittredge and Hughes, 1964). We now describe some results of a search among marine invertebrates for substances containing the carbon-phosphorus bond. Such compounds have been detected in diverse animals, and evidence for their presence in the biochemical classes of proteins and nonsaponifiable lipids is recorded for the first time.[1]

Screening Methods and Results

No direct method is presently available for detection of the C-P bond. In principle, ^{31}P nuclear magnetic resonance spectroscopy might be useful for this, as the chemical shift of ^{31}P attached to one or more carbons is different (negative) from that of ^{31}P attached only to oxygen or nitrogen in phosphates. However, the present high concentration requirement of the method prevents its utilization in the screening of extracts. A semiquantitative measure of the amount of phosphorus present in such a bond was obtained by comparing phosphate formed on combustion against phosphates formed on hydrolysis. The former value represents total phosphorus; the latter represents only phosphorus present as derivatives of phosphoric acid, and does not include carbon-bonded phosphorus, which is generally hydrolytically stable. In many cases, the difference was appreciable (0.2–0.3%), well beyond a differential due only to experimental error. In several cases where this method suggested C-P material to be present, isolation studies did indeed provide substantiation of its presence.

Three major fractions were generally derived from each animal and subjected to the analysis: (A) material soluble in 70% ethanol, (B) chloroform-solubles from the insoluble residue in preparing A, (C) insoluble material from the chloroform extraction. Results of analysis of six marine animals appear in Table I.

Most of the fractions indicated by analysis to have C-P compounds were examined for the presence of 2-aminoethylphosphonic acid. This was accomplished by acid hydrolysis and removal of amino acids from the solution on Dowex 50 (H^+). The acids were eluted with ammonia and fractionated by ion-exchange chromatography, employing a modification of a published procedure (Kittredge *et al.*, 1962). 2-Aminoethylphosphonic acid was recrystallized from aqueous alcohol and was identified by comparison of several of its properties with a known specimen. From every fraction studied (Table I), 2-aminoethylphosphonic acid was isolated. No evidence of significant amounts of other amino

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[1] Some of this work was described in a preliminary communication (Quin, 1964). The insoluble lipid-free residue of *Tetrahymena* was later reported to contain 2-aminoethylphosphonic acid (Rosenberg, 1964).

TABLE I: Phosphorus Analyses of Marine Animals.

Fraction	Weight (g)	Per Cent Phosphorus: Total	Phosphate	As C-P[a]
	(A) *Metridium dianthus*, 467 g[b]			
A	16.6	0.68	0.47	0.21[c]
B	4.5	2.28	1.53	0.75[c]
C	40.7	0.95	0.66[d]	0.29[c]
	(B) *Tealia felina*, 230 g[b]			
A	8.9	1.33	0.60	0.77
B	4.5	0.15	0.11	[e]
C	35.2	0.77	0.41	0.37[c]
	(C) *Busycon canaliculatum*, 122 g[b]			
A	6.7	0.67	0.45	0.22
B	0.15	0.98	0.55	0.43
C	18.7	0.72	0.56	0.16
	(D) *Mytilus edulis*, 53 g[b]			
A	1.83	1.34	1.13	0.21[c]
B, C[f]	3.21	0.61	0.59	[e]
	(E) *Venus mercenaria*, 64 g[b]			
A	1.96	0.97	0.80	0.17[c]
B, C[f]	10.1	0.37	0.38	
	(F) *Asterias forbesi*, 63 g[b]			
A	2.44	0.57[g]	0.35[g]	0.22[g]
B, C[f]	14.4	0.19	0.15	[e]

[a] By difference. [b] Wet weight. [c] Aminoethylphosphonic acid was found in this fraction. [d] As judged by later work, this value is probably high. [e] Values less than 0.05% are not presently considered as reliable evidence for C-P compounds. [f] Insoluble residue from extraction of fraction A. [g] Analysis of a second animal gave 0.86% total and 0.85% phosphate P.

acids containing phosphorus was obtained. However, small amounts of phosphorus were indicated in several fractions from the ion-exchange chromatographic procedure, and it is not known if new compounds are represented by these values.

In two cases, insoluble oil remaining after extended hydrolysis still contained phosphorus. Since only carbon-bonded phosphorus should survive the hydrolysis, assignment of C-P material to the nonsaponifiable lipid fraction could be tentatively made. The material eluted with water from Dowex 50, before elution of the amino acids, in no case contained C-P compounds.

The foregoing program permits verification and partial description of any C-P material indicated to be present by the differential phosphorus analysis. These methods lack sensitivity, however, and very small amounts of C-P compounds may easily go undetected. Indeed, it is only because the animals were so heavily endowed with such compounds that the approach was as satisfactory as it was. It is not unlikely that other C-P compounds are present in fractions passed by in this work.

From the sea anemone *Metridium dianthus* was isolated a considerable amount of 2-aminoethylphosphonic acid. Fractions A, B, and C from 467 g of wet animals (62 g dry) provided 107, 66, and 440 mg, respectively. This represents a remarkable 0.99% of the dry weight. This value checks reasonably well with the value 1.21% derived from the data in Table I with all C-P calculated as the amino acid. An amount equivalent to 4 mg P of apparently organic phosphorus was also detected in nonsaponifiable lipid of fraction B. The manner in which 2-aminoethylphosphonic acid is present in fractions A and B has not been explored. In fraction A, it may be free or lipid-bound (Kittredge *et al.*, 1962; Rouser *et al.*, 1963); fraction B is clearly lipid and contains only bound 2-aminoethylphosphonic acid. The appearance of considerable 2-aminoethylphosphonic acid in fraction C was surprising. The extraction process can leave little, if any, lipid in this insoluble material, and a new form for bound 2-aminoethylphosphonic acid had to be considered. Evidence will be presented in the next section that 2-aminoethylphosphonic acid is associated in some fashion with protein structure.

The sea anemone *Tealia felina* is even a richer source of C-P compounds than *M. dianthus*. Chromatographic evidence for the presence of 2-aminoethylphosphonic acid in fraction C was obtained, but the compound has not yet been isolated. If all C-P is considered present as 2-aminoethylphosphonic acid, there is some 1.8% of this compound present, on the dry basis.

In addition to the phylum *Coelenterata*, we found C-P compounds in *Mollusca* and possibly in *Echinodermata*. The bivalve mollusks *Mytilus edulis* (blue mussel) and *Venus mercenaria* (quahog or littleneck clam) were suggested by analysis to have C-P compounds in fraction A only, and in each case 2-aminoethylphosphonic acid was isolated from hydrolysates of these fractions. The amount was quite small, however, relative to that from *M. dianthus*. The gastropod mollusk *Busycon canaliculatum* (channeled whelk), however, had by analysis C-P compounds in all three fractions, but no isolation work has been performed. The representative of *Echinodermata* studied, *Asterias forbesi* (starfish), has not yet given clear-cut results. Two animals were analyzed separately; as seen in Table I, only one gave indication of C-P material.

In Table II is given a comparison of relative amounts

TABLE II: Relative Amounts of C-P Material in Marine Animals.[a]

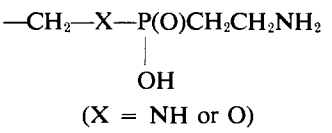
T. felina	410
M. dianthus	302
B. canaliculatum	215
M. edulis	76
A. forbesi	32
V. mercenaria	28

[a] In mg P/100 g of dry tissue.

of C-P compounds for these animals. The bivalves deserve a special comment in that they are edible. While they are not the richest in C-P compounds, they contain enough so that one might ingest as much as 20–30 mg of 2-aminoethylphosphonic acid on eating four clams or eight mussels. The disposition made of this by the human body remains an interesting question for study.

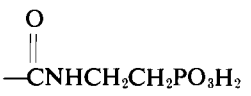
2-Aminoethylphosphonic Acid as a Constituent of Protein. The insoluble residue from alcohol and chloroform extraction of the anemone *M. dianthus* was found to be largely protein. After complete hydrolysis, about 80–85% of its weight of amino acids was obtained. The protein dissolved on standing in 1 N sodium hydroxide at room temperature for 1 hour; since it was not reprecipitated on neutralization, partial hydrolysis must have occurred. The first indication that 2-aminoethylphosphonic acid may be incorporated in the protein, and is not merely present as incompletely extracted lipid or some other form, came with the finding that a precipitate with trichloroacetic acid from this neutralized solution contained C-P material by analysis, and on hydrolysis gave a mixture of amino acids, including the phosphonic acid. Enzymatic hydrolysis with pepsin also gave soluble polypeptides, and a precipitate with trichloroacetic acid contained 2-aminoethylphosphonic acid. More importantly, the pepsin-solubilized material (about 80% of the protein) could be easily separated on Sephadex G-25 into a fast-moving polypeptide fraction and a slower-moving mixture of apparently smaller peptides. The polypeptide had, by the differential phosphorus analysis, 4.4% of 2-aminoethylphosphonic acid, which accounted for 90% of the amount of the acid present in the protein. From the content of 2-aminoethylphosphonic acid, the minimum molecular weight of the polypeptide is 2840.

The aminoethylphosphonic acid may be bonded in the polypeptide in three general ways: (1) through one or both acidic functions of the phosphonic group:

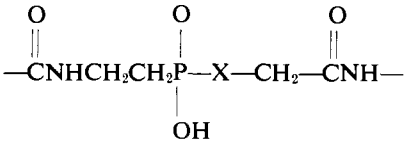


(X = NH or O)

(2) through the amino group:



(3) through both amino and phosphonic groups:



(X = NH or O)

The present work has not provided a complete solution to this important problem. The first possibility, however, has been eliminated by dinitrophenylation studies. The DNP-polypeptide on acid hydrolysis did not provide DNP-2-aminoethylphosphonic acid, which was prepared independently and found to be stable to acid hydrolysis. The implication is that some function, probably acyl, is protecting the amino group. In discriminating between the second and third structural types, smaller peptides seemed necessary for future study, and partial degradation of the polypeptide by refluxing for 1 hour in 1 N hydrochloric acid was performed. Separation on Sephadex G-25 revealed little of the polypeptide to have survived, and several small peptides were recovered in admixture. On dinitrophenylation, followed by hydrolysis, no DNP-2-aminoethylphosphonic acid was found, and once again it is evident that none of this acid with amino group free is present in the peptide mixture. The mixture of peptides should provide a suitable subject for continued studies permitting discrimination between the second and third structural types.

In view of the above work, it is felt that adequate reason exists for assigning a role in protein structure to 2-aminoethylphosphonic acid, even though the exact role has not been determined. It is likely but not yet established that C-P material detected in fraction C of other animals is similarly protein bound.

Nonsaponifiable Lipid Containing Phosphorus. Acid hydrolysis of fractions B of *M. dianthus* and A of *B. canaliculatum* left an oil containing a small amount of phosphorus (0.1–0.2%). To eliminate the possibility that this was caused by incompletely hydrolyzed phospholipid, each oil was again hydrolyzed with acid and then with base. The oils still contained phosphorus, and it is suggested that this is bound to carbon. Column chromatography on silica gel provided fractions showing enrichment of phosphorus (0.44% P with carbon tetrachloride; 0.48% P with chloroform). More polar solvents also eluted small amounts of phosphorus. The carbon tetrachloride and chloroform eluates were combined and rechromatographed to provide four fractions of different ease of elution but all of significant phosphorus content, in the range 0.4–0.8%. Behavior on thin-layer chromatography further suggested a different identity for the phosphorus compounds of these fractions. For example, on cellulose one fraction moved near the solvent front without resolution, and phosphorus was found there. Another fraction in the same solvent moved at about R_F 0.50, where the phosphorus was located.

It is strongly suggested that neutral phosphorus compounds are present in nonsaponifiable lipids from *M. dianthus*. Although it is essential that individual compounds be obtained and identified, there appears to be little possiblity that phosphorus compounds not containing a phosphorus-to-carbon bond could have survived the hydrolytic procedure, or that inorganic material could account for the observations.

Conclusions

It is apparent from this work that phosphorus bonded to carbon can account for a significant fraction of the total phosphorus of some lower marine invertebrates. In *T. felina*, as much as 50% of the phosphorus is indicated to be in this form. It is also made apparent that 2-aminoethylphosphonic acid has a widespread distribution among low marine invertebrates, and in some cases is an important constituent. This compound seems to find a place in several biochemical structures. The full biochemical significance of these and related observations remains to be established. It seems clear that new attitudes toward the metabolism of phosphorus, at least in the lower animals, are required. This new aspect of phosphorus chemistry may also have an impact on studies of phosphorus in the marine environment, including sedimentary material and dissolved compounds in sea water.

Experimental

Specimens. Tealia felina, preserved in methanol, was kindly supplied by Dr. H. Barnes of the Millport Marine Station, Scotland. All other animals were collected from Vineyard Sound, Massachusetts, and were either held alive in sea-water tanks or frozen.

Analytical Methods. Total phosphorus was determined by the Schöniger oxygen flask method, using the F & M Scientific Corp. Model 141-A safety ignition unit. Paper-wrapped samples of about 20–50 mg were ignited in a 500-ml flask containing 10 ml of 1:5 nitric acid (Barney *et al.*, 1959). The platinum head was then boiled for 2 minutes in the nitric acid, and the mixture was diluted to 100 ml. Aliquots were adjusted to the proper acidity and the phosphomolybdovanadate color developed. Absorbance was determined at 391 mμ in 5-cm cells with a Cary Model 14 spectrophotometer. Phosphate phosphorus was determined by heating a 40- to 50-mg sample in 30 ml of 6 N hydrochloric acid in a 4-oz Teflon bottle for 48 hours at 105 ± 2°, decolorizing the solution with a pinch of Norit, adjusting to 100 ml, and developing the phosphomolybdovanadate color on an aliquot. In some of the earlier work, hydrolysis was accomplished by refluxing in 6 N hydrochloric acid in glass flasks for 24 hours. A small blank was introduced from the glass, which could be troublesome at low phosphate values.

Animal Processing. The entire tissue of each animal, exclusive of shell, was chopped and homogenized in a Waring Blendor with 70% ethanol for 5 minutes. The mixture was centrifuged and the solid was returned to the Blendor for another treatment with alcohol. The blending was performed 3–4 times. The combined extracts were stripped on a rotary evaporator to a brittle or gummy, occasionally hygroscopic, solid (fraction A). The insoluble residue was dried in a vacuum desiccator over silica gel to constant weight. The residue was either powdered for analysis or broken into small chunks, and placed in a Soxhlet extractor with chloroform for 24 hours. Stripping of the chloroform left fraction B, generally a yellow to orange oil. Insolubles from the extraction constituted fraction C, which was powdered before any further use was made of it. Weights and analysis for the various fractions appear in Table I.

Synthesis and Properties of 2-Aminoethylphosphonic Acid. The procedure of Kosolapoff (1947) was used to prepare this compound. It was crystallized by adding ethanol to a concentrated water solution; so prepared, it had uncorrected mp 282–283° decomp. It had R_F 0.23 in freshly prepared 1-butanol–acetic acid–water (4:1:2)[2] and 0.54 in pyridine-water (65:55). These solvents were used extensively in this work and will be referred to as the butanol and the pyridine systems. Whatman No. 1 paper was used. For identification of the natural compound, the synthetic sample was always run simultaneously; the R_F values so obtained are included in parentheses.

The dinitrophenyl derivative was prepared by adding a solution of 40 mg of 2,4-dinitrofluorobenzene in 2 ml of ethanol to a solution adjusted to *p*H 9 of 20 mg of the amino acid in 3 ml of water. After 1 hour at 40°, the mixture was extracted with ether and then adjusted to *p*H 1 with hydrochloric acid. Ethyl acetate was used to extract the product; ether, benzene, and chloroform were ineffective as extractants. The yellow powder was recrystallized from ethanol-ether. This product, mp 193–194° decomp, proved to be the hydrochloride of the DNP derivative. It gave a positive test for chloride ion.

Anal. Calcd. for $C_8H_{11}ClN_3O_7P$: N, 12.83. Found: N, 12.57.

If in the DNP preparation the *p*H was adjusted to 5 before solvent extraction, no product was removed with ethyl acetate. Apparently the DNP hydrochloride is more soluble than the neutral form, whose behavior suggests dipolar ion character. A similar peculiarity showed up on paper chromatography. When a water solution of the hydrochloride was applied to paper, a low R_F (0.12) was obtained in butanol saturated with water (Mellon *et al.*, 1953). The R_F increased as the *p*H of the application solution was decreased: *p*H 4, R_F 0.16; *p*H 2.5, R_F 0.21; *p*H 1, R_F 0.41; 6 N hydrochloric acid, R_F 0.60. This behavior requires that, in comparative paper chromatography, the *p*H of the media of application be the same. Solutions in 6 N hydrochloric acid were used consistently, as this medium was encountered in DNP-peptide hydrolysis studies.

DNP-2-aminoethylphosphonic acid was tested for stability to refluxing 6 N hydrochloric acid for 6 hours. No change in its chromatographic behavior was observed, and no amino acid was detected on paper chromatography. With concd ammonia, little displacement of the amino acid (Lowther, 1959) occurred after 3 hours in a sealed capillary tube at 107°, but the displacement was extensive after 24 hours.

Isolation and Identification of 2-Aminoethylphosphonic

[2] R_F 0.14 has been reported for this system (Horiguchi and Kandatsu, 1960). This value is approached on aging of the solution.

Acid. Ten g of fraction C from *M. dianthus* was refluxed for 24 hours in 200 ml of 4 N hydrochloric acid, and the solution then was stripped to dryness. The residue was taken up in water and the solution again was evaporated. It was again dissolved in water and filtered onto a 20 × 2.8–cm column of Dowex 50 (H^+). The column was eluted with 1 liter of water, and the eluate was stripped to dryness. The residue had 2.21% total P and 2.22% phosphate P.[3] The column was then eluted with 1 liter of 1:9 concd ammonia–water, and then with water until the washings were neutral. The combined eluates were stripped, leaving 8.1 g of amino acids. A 3.0-g portion in 10 ml of 0.5 N acetic acid was applied to a 28 × 2.8–cm column of Dowex 1-X8 (200–400 mesh, acetate form, in 0.5 N acetic acid) and eluted with 0.5 N acetic acid (Kittredge *et al.*, 1962). Almost all of the phosphorus was eluted in the 50- to 110-ml fraction, which contained 2.4 g of solid having 0.63% P. The 110- to 260-ml fraction contained 0.3 g of solid having 0.085% P, and elution finally with 3 N acetic acid gave 0.5 g of solid having 0.094% P. The latter two fractions were not examined further. The column was then thoroughly washed with water, and the solid from the 50- to 110-ml fraction in 12 ml of water was chromatographed on it, in two separate, identical operations. On elution with water, the bulk of the sample (2.25 g) was eluted rapidly (50–130 ml) but contained only 0.088% P. The 130- to 170-ml fraction gave 0.05 g of solid (0.54% P) in which paper chromatography showed the presence of some 2-aminoethylphosphonic acid along with several other amino acids. The 170- to 570-ml fraction was rich in phosphorus and was seen to be predominantly 2-aminoethylphosphonic acid on paper chromatography. This material was purified by a second passage through the Dowex 1 (acetate) column with water. Some contaminating amino acids were removed in the 50- to 130- and 130- to 170-ml fractions; the latter also contained some 2-aminoethylphosphonic acid. The 170- to 570-ml fraction (40 mg) was almost pure 2-aminoethylphosphonic acid, and showed only two very weak amino acid spots in a large sample application. It was freed of a slight coloration with a pinch of Norit and then twice recrystallized by adding ethanol to a concentrated water solution. The total yield from 10 g of fraction C was 108 mg, mp 282–283° decomp, R_F in the butanol system, 0.24 (0.23); R_F in the pyridine system, 0.54 (0.54).

Anal. Calcd. for $C_2H_8NO_3P$: P, 24.8. Found: P, 25.1.

Identical proton magnetic resonance spectra for the natural and synthetic compounds, in D_2O, were obtained with a Varian A-60 spectrometer. The spectra showed equal-area methylene multiplets centered at 2.47 and 3.72 ppm, relative to tetramethylsilane as 0.

In a similar manner, water-soluble C-P material released on hydrolysis of several fractions (Table I) was traced into the 2-aminoethylphosphonic acid fraction. In no case was C-P indicated by analysis in the material eluted from Dowex 50 (H^+) with water. Some pertinent data are: *M. dianthus:* 5.4 g of fraction A gave 35 mg of 2-aminoethylphosphonic acid; R_F in butanol system, 0.21 (0.21); in pyridine system, 0.54 (0.55); mp 280–283° decomp; P, 24.5%. *M. dianthus:* 2.5 g of fraction B gave 37 mg of 2-aminoethylphosphonic acid; R_F in butanol system, 0.13 (0.13); in pyridine system, 0.55 (0.54); mp 280–283° decomp; P, 24.3%. The residue from the 50- to 100-ml eluate from Dowex 1 (acetate) with water had 0.25% P and may contain another phosphorus compound. No 2-aminoethylphosphonic acid was detected in this fraction by paper chromatography. *M. edulis:* 3.0 g of fraction A gave about 5 mg of 2-aminoethylphosphonic acid; R_F in butanol system 0.19 (0.19); in pyridine system, 0.43 (0.43); mp 280–283° decomp. *V. mercenaria:* 1.86 g of fraction A gave about 5 mg of 2-aminoethylphosphonic acid, R_F in butanol system, 0.17 (0.17); in pyridine system, 0.57 (0.56); R_F of DNP derivative on thin-layer chromatography (silica gel) in 7:3 2-propanol-ammonia (Brenner, *et al.*, 1961), 0.17 (0.18). No phosphorus analyses were performed on any ion-exchange fractions.

The presence of 2-aminoethylphosphonic acid in a hydrolysate of fraction C of *T. felina* was detected through the DNP derivative. A mixture of 0.166 g of this fraction and 50 ml of 6 N hydrochloric acid was refluxed 24 hours and then stripped to dryness. The residue was reacted with 2,4-dinitrofluorobenzene in the same manner used for the amino acid itself. The DNP mixture was separated into several bands on silica gel plates in the 2-propanol-ammonia system. The slowest band (R_F 0.17) was removed from the plate and eluted with water. The eluate was stripped to dryness, the residue was dissolved in concd ammonia, and the solution was sealed in a capillary tube. After 20 hours at 107°, the tube contents was applied to paper and run in the butanol system. Ninhydrin-positive spots appeared at R_F 0.06, 0.12, and 0.23; reference 2-aminoethylphosphonic acid was located at 0.11.

Partial Hydrolysis of Fraction C. (A) WITH SODIUM HYDROXIDE. A mixture of 2.0 g of fraction C from *M. dianthus* and 25 ml of 1 N sodium hydroxide was shaken at room temperature for 30 minutes. Centrifugation showed that the bulk of the solid had dissolved. The supernatant liquid was adjusted to *p*H 7, and a slight precipitate was removed by centrifugation. The supernatant liquid was then treated with 30 ml of 30% trichloroacetic acid. A precipitate formed immediately and was removed by filtration (0.39 g). It had 0.69% total P and 0.53% phosphate P. It was hydrolyzed by refluxing for 24 hours in 6 N hydrochloric acid; paper chromatography showed the hydrolysate to have several amino acids, and it was then placed through the Dowex 50 and Dowex 1 separation procedures. The presence of 2-aminoethylphosphonic acid was detected in the proper fraction by two-dimensional paper chromatography in the butanol system and then the pyridine system; a spot coinciding with 2-aminoethylphosphonic acid was observed.

[3] The appearance of different values in the preliminary report (Quin, 1964) is owing to an error of data transcription.

(B) WITH PEPSIN. A mixture of 4.0 g of fraction C of *M. dianthus* and 40 mg of pepsin in 100 ml of water was adjusted to *p*H 1.5 with hydrochloric acid, and allowed to stand for 6 hours at 40°. It was boiled briefly to destroy the pepsin, and insolubles (0.77 g) were removed by centrifugation. One-fourth of the supernatant liquid was treated with 40 ml of 30% trichloroacetic acid. No precipitate formed until enough solid trichloroacetic acid was added to make the entire solution about 30% in this acid, whereupon 0.44 g of a curdy precipitate was obtained (0.24% total P, 0.13% phosphate P). The remainder of the supernatant liquid was concentrated to 23 ml and applied to a 18 × 2.8-cm column of Sephadex G-25 (medium). The column was eluted with water; 10-ml fractions were collected from the first appearance of color in the eluate. The first three fractions (cloudy brown) had a *p*H of about 6–7, were immobile in the butanol system but ninhydrin-positive at the origin, and combined left 0.79 g of cream solid on freeze-drying. The *p*H of the eluate began to drop with fraction 4, which had some mobility in the butanol system, and reached about 1–2 in fractions 8–9, which were completely mobile in the butanol system to give poorly differentiated streaks. Analytical values for total and phosphate P on combined, freeze-dried fractions were: fractions 1–3, 1.49 and 0.38; fractions 4–5, 0.49 and 0.36; fractions 6–9, 0.57 and 0.56.

Dinitrophenylation of the Polypeptide from Peptic Hydrolysis. A solution of 25 mg of the polypeptide (fractions 1–3 above) in 1.2 ml of 0.8 N sodium bicarbonate was treated with a solution of 20 mg of 2,4-dinitrofluorobenzene in 0.3 ml of ethanol; the mixture was shaken at 25° for 2 hours, and then the *p*H was adjusted to about 1. The precipitate was removed and washed several times with acetone and once with ether. A portion was hydrolyzed for 6 hours in refluxing 6 N hydrochloric acid. A chromatogram developed in the butanol system had several ninhydrin-positive spots, including one for 2-aminoethylphosphonic acid. A chromatogram in water-saturated 1-butanol had yellow spots at R_F 0.15, 0.39, and 0.59 (darkest). The last was coincident with DNP-2-aminoethylphosphonic acid (R_F 0.60), but this compound had R_F 0.20 in benzene–acetic acid–water (5:10:1), whereas the hydrolysate had a single spot at 0.09. Lack of identity was also indicated by eluting the R_F 0.60 area from a large sample of the hydrolysate and displacing the DNP-bound amino acid with concd ammonia in a sealed capillary tube for 24 hours at 107°. A chromatogram in the butanol–acetic acid–water system had a major spot at R_F 0.60, with weak spots at 0.39, 0.30, and 0.19. Authentic 2-aminoethylphosphonic acid was located at 0.13. A similar check was made with thin-layer chromatography. The hydrolysate was applied as a band to silica gel and developed with 2-propanol–concd ammonia (7:3). Bands were located at R_F 0.14, 0.26, and 0.34 (darkest). The first was coincident with DNP-2-aminoethylphosphonic, but the amino acid released on ammonia displacement as above had R_F 0.22 (darkest) and 0.28. The authentic phosphonic acid appeared at R_F 0.15.

Partial Hydrolysis of the Polypeptide. Trypsin at *p*H 8 for 24 hours at 40° had no effect on the polypeptide. However, after 1 hour's refluxing of 128 mg with 50 ml of 1 N hydrochloric acid, considerable change in chromatographic behavior was observed. The solution was stripped and the residue applied to a 20 × 2.8-cm Sephadex G-25 column. About 12 mg of unchanged polypeptide was recovered in fractions 1–3 (10 ml each). The *p*H dropped at fraction 5, which on paper showed considerable streaking. Fractions 6 and 7 had a *p*H of about 1.5; each had a complex chromatogram, with resemblances and differences to each other and to fraction 5. Later fractions had negligible residues and were discarded. Fractions 5–7, combined and freeze-dried, gave 100 mg of hygroscopic solid. This contained 1.19% total and 0.28% phosphate phosphorus, and after complete hydrolysis gave a spot for 2-aminoethylphosphonic acid in the butanol system. A similar preparation (100 mg) in 8 ml of 0.8 M sodium bicarbonate was treated with a solution of 200 mg of 2,4-dinitrofluorobenzene in 20 ml of ethanol. After 4 hours at 25° the mixture was acidified and the precipitate washed with ethyl acetate. Neither this precipitate nor the water solution itself gave an indication of the presence of DNP-2-aminoethylphosphonic acid on silica gel plates, either before or after 6 N hydrochloric acid hydrolysis for 8 hours at reflux. The hydrolysate from the precipitate had a spot for 2-aminoethylphosphonic acid in the butanol system.

Nonsaponifiable Lipid. After refluxing for 24 hours in 150 ml of 4 N hydrochloric acid, 1.86 g of fraction A of *V. mercenaria* left 0.17 g of a brown oil (total P, 0.26%), extracted with chloroform from the mixture. The oil was refluxed 24 hours in 30 ml of 6 N hydrochloric acid and then 24 hours in 1 N sodium hydroxide. The mixture was acidified and extracted with chloroform. After filtration, the extract was evaporated to dryness. The brown tar remaining contained 0.10% P.

Fraction B (5.5 g) of *M. dianthus* was refluxed 48 hours with 150 ml of 6 N hydrochloric acid. The initially yellow oil was transformed to a voluminous semisolid, and finally to a black oil. This was removed with chloroform and refluxed for 20 hours in 0.5 N sodium hydroxide. The oil was partly soluble in this medium while hot, but insoluble cold. The solution was saturated with salt and extracted with ether. Removal of ether left 5.1 g of gummy brown solid (total P, 0.10%). A solvent extraction procedure was performed prior to chromatography. A portion (4.1 g) was mixed with 100 ml of ethanol, leaving 1.31 g of tan powder (0.23% P). This was placed in 30 ml of chloroform; a thin gel was formed, but dilution with 30 ml of benzene gave a mixture amenable to centrifugation. After benzene and ether washes, the residue weighed 0.37 g and contained 0.07% P. The extracts were evaporated, leaving a brown powder (0.29% P). This was dissolved in chloroform and applied to a 19 × 1.6-cm column of silica gel (as received from J. T. Baker) packed in pentane. Results of elution were: (a) pentane, trace of yellow oil; (b) carbon tetrachloride, tan powder (0.44% P); (c) chloroform, brown wax (0.48% P); (d) acetone, brown wax (0.11% P);

(e) 75% methanol and then glacial acetic acid, tan powder (0.12% P). Fractions (b) and (c) were placed in benzene, mixed with silica gel, and stripped to dryness. The residue was applied to a 20 × 1.6-cm column of silica gel (in pentane). Distinct, colored fractions were eluted with benzene in pentane: (a) 100 ml each of 40%, 45%, and 50% mixture, brown solid, 0.47% P; (b) 100 ml of 60% mixture, brown solid, 0.64% P. Elution with chloroform in benzene followed: (c) 200 ml of 25% mixture, brown wax, 0.81% P; (d) 100 ml of 50% mixture, brown wax, 0.56% P. All color was finally removed from the column with 2-propanol, giving a brown wax (0.21% P). Thin-layer chromatography on cellulose (without binder) in 2-propanol-benzene (1:19) moved the phosphorus in fraction (b) with the colored material, near the solvent front. For fraction (d), the bulk of the colored material moved to R_F 0.5, and phosphorus was concentrated there. Location of phosphorus was accomplished by analysis of sections of each plate. Each section was eluted with 2-propanol-benzene (1:1), and the eluate was directly evaporated onto the paper for the Schöniger analysis.

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Catalysis of the Oxidation of Norethynodrel by Horseradish Peroxidase*

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ABSTRACT: Autoxidation of 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one (norethynodrel) occurs slowly in aqueous solution. The initial products of the reaction are 17α-ethynyl-10β-hydroxy-19-nortestosterone and 17α-ethynyl-10β-hydroperoxy-19-nortestosterone. The oxidation is rapidly catalyzed by horseradish peroxidase in the presence of hydrogen peroxide and manganese ion.

Hemoglobin also catalyzes the reaction, although the ratio of the 10β-hydroperoxy- to the 10β-hydroxy-metabolites is lower when hemoglobin is substituted for peroxidase in the incubation medium.

In studies on the metabolism of orally administered 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one (norethynodrel) in the rabbit (Arai *et al.*, (1962) and in the human (Layne *et al.*, 1963), we have shown that hydroxylation at position 10 plays a major part in the metabolism of this steroid. Incubation of norethynodrel with blood (Arai *et al.*, 1962) led to the formation of 17α-ethynyl-10β-hydroxy-19-nortestosterone and of another unidentified ketone. The latter has since been found to be 17α-ethynyl-10β-hydroperoxy-19-nortestosterone, which strongly suggested that a peroxidative reaction was involved in the *in vitro* conversion of norethynodrel by blood, and possibly also in the *in vivo* metabolism of this steroid. The present paper reports the finding that norethynodrel and related $\Delta^{5(10)}$ steroids are rapidly oxidized by horseradish peroxidase at *p*H 7.4 in the presence of hydrogen peroxide and manganese ion. The effect of several factors on the rate of oxidation

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