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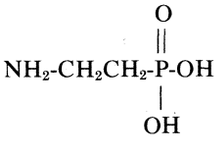
2-Aminoethylphosphonic Acid Metabolism During Embryonic Development of the Planorbid Snail *Helisoma*

Abstract. *In freshly laid egg masses of* Helisoma *sp., more than 95 percent of the phosphorus is found in alkylphosphonic acids, as determined by phosphorus-31 nuclear magnetic resonance spectroscopy. These compounds are metabolized during embryonic development, as shown by differential acid hydrolysis and experiments with phosphorus-33–labeled phosphoric acid. Further, nuclear magnetic resonance spectroscopy indicates phosphonic acid involvement in related snail families, including the schistosomal vector* Biomphalaria glabrata.

Alkylphosphonates are compounds containing a direct carbon-to-phosphorus bond. Compounds of this class were first observed in nature in 1959, when Horiguchi and Kandatsu (*1*) isolated 2-aminoethylphosphonic acid [(AEP), structure **I**] from hydrolyzates of sheep rumen protozoa. Since then, AEP and related alkylphosphonates have been detected in many invertebrate and some vertebrate species as components of lipids (*2*) and as components of high-molecular-weight proteoglycans (*3*). Although the unique properties of these molecules have led to hypotheses about their functions, much still needs to be learned about their biology (*4*). One useful characteristic of these compounds is their resistance to hydrolytic conditions that break most ester phosphorus bonds. Alkylphosphonic acids can thus be determined in biological samples as "nonhydrolyzable" phosphorus. Another technique that has proved useful is the direct determination of these compounds by phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P NMR); this is due to the large differences in chemical shift between ester phosphorus and phosphonate phosphorus.



2-Aminoethylphosphonic acid, structure **I**

This report presents evidence for a novel role for AEP as a phosphorus source in the embryonic development of the freshwater pulmonate snail *Helisoma* sp., of the family Planorbidae.

Spectrum A of Fig. 1 shows the ^{31}P NMR spectrum of freshly laid intact egg masses of *Helisoma* sp. Of major significance in this spectrum is the great abundance of phosphonate phosphorus, as indicated by the large downfield signal at +21.5 ppm. Of equal significance is the absence of detectable free or esterified orthophosphate in the egg masses, as indicated by the absence of any resonances in the region of the spectrum near 0 ppm. These initial results with the intact eggs were confirmed by running samples of acid-hydrolyzed egg masses and hydrolyzed adult snails (Fig. 1, spectra B and C). It is notable that the egg masses contain resonances from two phosphonates. We had previously determined that the compound giving rise to the major peak (85 percent of total phosphorus) was 2-AEP by using thin-layer chromatography, ^{31}P NMR, and ^{1}H NMR to compare the isolated compound and authentic 2-AEP (*5*). The compound giving rise to the minor peak has not yet been identified. We have also determined that the phosphonates in the intact eggs are components of high-molecular-weight molecules consisting mainly of carbohydrate (a phosphonoglycan) and are not found free or as components of lipids. The adult snail also contains appreciable phosphonate phosphorus (10 to 15 percent of the total phosphorus) as 2-AEP. These ^{31}P NMR results were confirmed by colorimetric measurements of total and hydrolyzable phosphorus by the procedures of Snyder and Law (*6*) and Kirkpatrick and Bishop (*7*) (data not shown).

Because the phosphorus composition of *Helisoma* sp. may be different at different developmental stages, we measured the changes over time in total phosphorus and hydrolyzable phosphorus in developing egg masses. The results (Fig. 1D) demonstrated that as the embryonic snails developed there was a marked increase in the amount of hydrolyzable phosphorus per milligram of dry weight, with no change in the total phosphorus content. Whereas hydrolyzable phosphorus represented only 3 to 5 nmole per milligram of dry weight in freshly deposited egg masses, it was 110 nmole per milligram of dry weight in 12-day-old egg masses (just prior to hatching).

We also exposed egg-laying adult *Helisoma* sp. to $H_3{}^{33}PO_4$ (carrier-free), and monitored the aqueous medium. We determined that the snails took up 92 percent of the [^{33}P]orthophosphate from the medium after 24 hours (average of two experiments). After 48 hours of exposure to the phosphorus-33, we moved the snails to nonradioactive medium and collected the egg masses laid by the radioactive adults. Portions of the egg masses were used for determining the initial levels and distribution pattern of phosphorus-33 incorporation. Other portions were allowed to develop in phosphorus-free medium so that changes in the phosphorus-33 labeling pattern as a function of time of embryonic development could be monitored. This experiment showed a progressive diminution of phosphorus-33 in AEP, with a concomitant increase in phosphorus-33 in orthophosphate released by acid hydrolysis (Fig. 2). The results demonstrate that the carbon-to-phosphorus bond in AEP was being broken and acid-labile phosphorus was being produced as the embryos developed.

The bottom two radiochromatographic strip scans in Fig. 2 show that, in the infant snail, most of the phosphorus-33 was in the form of acid-labile phosphorus, whereas in the rigid structural material left behind after the infant snails hatched, approximately 87 percent of the remaining phosphorus-33 was in the form of AEP. Indeed, there was very little hydrolyzable phosphorus in the empty cases, since the ^{33}P-labeled material that ran toward the front was the unidentified phosphonate compound (compound X) rather than orthophosphate. These results indicate that alkylphosphonic acids, particularly AEP, may play a role in the development of the planorbid snail *Helisoma* sp.

Based on the efficient uptake of

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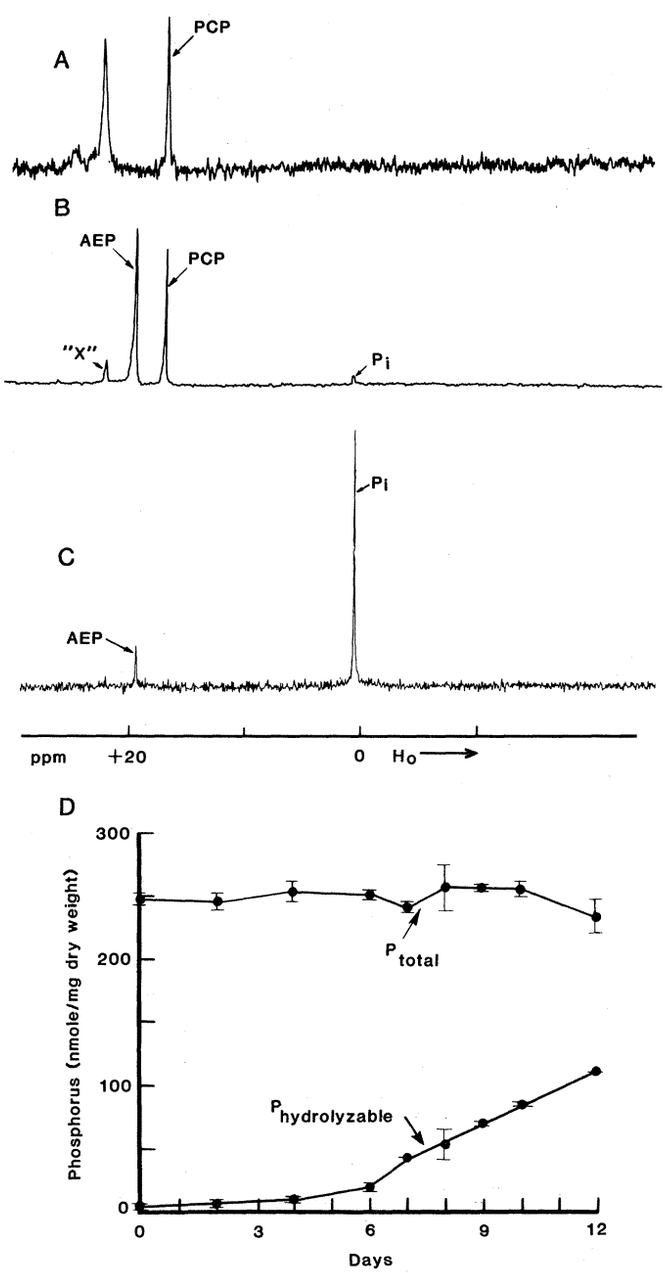

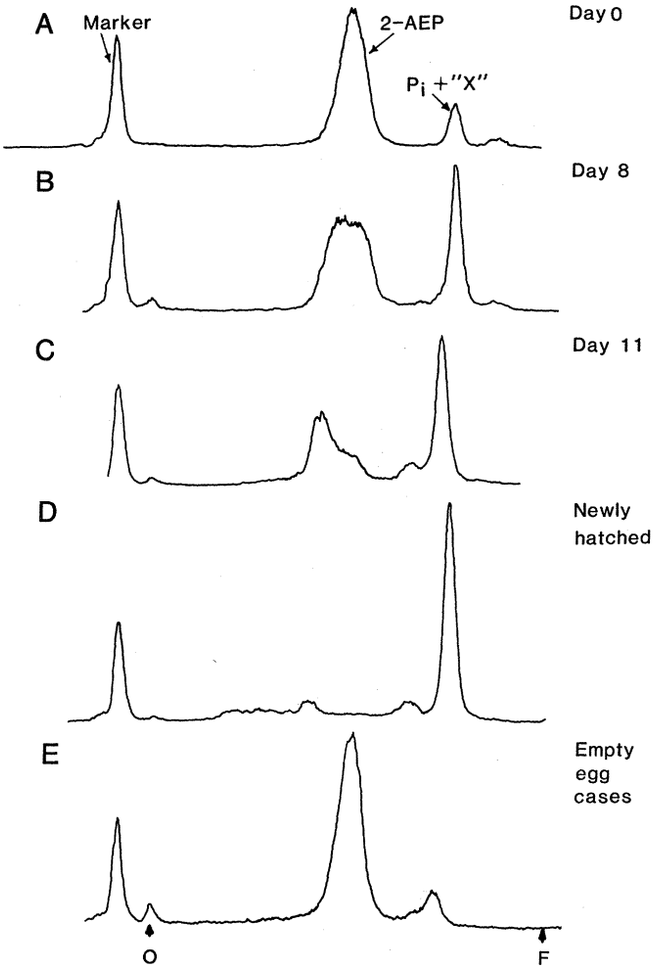

Fig. 1 (left). Proton decoupled ^{31}P NMR spectra of (A) intact egg masses, (B) acid-hydrolyzed egg masses, and (C) snails. The spectrometer was a Bruker HFX-5 operating at 36.43 MHz for phosphorus, with facilities for deuterium stabilization and Fourier transform signal averaging. Detailed description of the instrumental conditions and procedures are given in (*8*). Spectrum A was obtained from freshly laid *Helisoma* egg masses (0 to 48 hours after laying) to which 0.2 ml of 99.8 percent D_2O had been added. The marker compound at +16.1 ppm was an external capillary of methylenediphosphonic acid (*PCP*). Zero was assigned to 85 percent H_3PO_4, with positive shifts associated with decreasing field strength. The number of pulses was 10,000. The probe temperature was 22°C for all spectra. The sample used for spectrum B was similar to that used for spectrum A, but had been treated to hydrolysis with 6*N* HCl at 120°C for 20 hours; the sample was treated with Norite and passed through Chelex 100 (Bio-Rad) (Na^+ form) to remove metal ion contaminants. The sample was assayed in 10 percent D_2O at *p*H 4.35 and required 16,000 pulses. The symbols *AEP*, P_i, and "*X*" represent the signals from 2-aminoethylphosphonate, inorganic phosphate, and the unidentified phosphonate, respectively. Spectrum C was obtained from adult snails treated in a manner similar to that described for B, except the shell was first removed and the adults were hydrolyzed in 6*N* HCl at 110°C for 40 hours. The number of pulses was 5300, and the sample was tested at ~ *p*H 4. (D) Time course of the change in relative amounts of total and hydrolyzable phosphorus during development. Egg masses laid in the previous 24 hours (0 day) were removed from the adults and placed in a separate pan containing distilled water and added salts (*9*). Egg masses were removed at intervals and examined microscopically for viability and stage of development. The dry weight was determined in tared tubes (typically 10 to 12 mg), and hydrolysis in 6*N* HCl and alkaline phosphatase treatment were carried out as in (*6*). After enzymatic hydrolysis, the samples were acidified with 1*N* HCl to at least 0.5*N* acid to prevent precipitation of the calcium phosphate salts. The orthophosphate released by the digestion was determined by the microphosphate method (*10*) and total phosphorus was determined by the wet ash procedure (*7*). The data shown represent duplicate determinations on two different experiments and error bars indicate the standard deviation of the four values. Hatching typically took place between days 11 and 13 after laying. Fig. 2 (right). Radiochromatographic scans of thin-layer chromatograms of acid hydrolyzates of egg masses during embryonic development. Egg masses from snails labeled with $H_3{}^{33}PO_4$ were treated essentially as described in Fig. 1D, except that these samples were hydrolyzed for 20 hours at 120°C and the enzymatic step was omitted. Adult snails were labeled by incubating with 1 mCi of carrier-free [^{33}P]orthophosphoric acid (New England Nuclear) for 48 hours in 1 liter of tap water. After 24 hours the snails had taken up 91 percent of the labeled phosphorus, as indicated by scintillation counting of the snails' water. After 48 hours the snails were moved to fresh water. Eggs laid during 24- or 48-hour periods were removed for determination of radioactivity levels or for developmental experiments. In this experiment, egg masses exhibited the highest radioactive incorporation 3 to 5 days after the start of labeling (440,000 cpm/mg, dry weight). The egg masses deposited during this time (0 to 48 hours old) were used for the above series of thin-layer chromatograms. After hydrolysis, the samples were treated with Norite for 10 minutes on a steam bath, checked for radioactivity and total phosphorus content, and dried under N_2 prior to chromatography. One hundred nanomoles of total phosphorus was streaked on a 5 by 20 cm cellulose plate (Eastman). The solvent used was acetone, acetic acid, and water (5:3:2 by volume). The scans were obtained on a radiochromatogram scanner (Packard model 7201).

$H_3{}^{33}PO_4$ from the medium by adult snails and the subsequent incorporation of the phosphorus-33 into AEP, as well as the breakdown of AEP during embryonic development, *Helisoma* appears to be an excellent animal for use in fundamental studies on the metabolism of the naturally occurring alkylphosphonates such as AEP.

We have also obtained ^{31}P NMR spectra from the egg masses of other species of the family Planorbidae, as well as representatives of the families Physidae and Lymnaeidae. In general, these gave rise to ^{31}P NMR spectra similar to that shown in Fig. 1A. Included in these preliminary studies was the planorbid snail *Biomphalaria glabrata*, an obligate intermediate host for the human pathogenic trematode *Schistosoma mansoni*. As is the case for the related *Helisoma* sp., alkylphosphonates represent more than 95 percent of the total phosphorus in freshly laid egg masses of *B. glabrata*.

These studies are of particular interest, especially in *B. glabrata*, because of the potential for control of snail proliferation. Further work may lead to the discovery of specific metabolic sites in which these biochemical processes can be interdicted without harming other life forms, since most life forms, particularly higher vertebrates, have no significant metabolic involvement with alkylphosphonates.

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1α,25-Dihydroxyvitamin D_3 Nuclear Receptors in the Pituitary

Abstract. *Specific binding of 1α,25-dihydroxyvitamin D_3 was found in nuclear and cytosol fractions of the bovine pituitary. For nuclear binding, the dissociation constant was 0.1 nanomole per liter, and maximum binding was 104 femtomoles per milligram of protein. In competition studies, 25-hydroxyvitamin D_3 was 300 times weaker than 1α,25-dihydroxyvitamin D_3. The existence of high-affinity sites supports a physiologic role for 1α,25-dihydroxyvitamin D_3 in the pituitary.*

Growth hormone (GH) and prolactin influence the synthesis of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] (*1*). Conversely, vitamin D metabolites may affect the production of hormones in the pituitary. It is moreover established that other steroid hormones have pituitary target sites (*2*). Receptors for 1α,25-$(OH)_2D_3$ have been characterized in small intestine, kidney, parathyroid, pancreas, and bone (*3*–*7*). To assess pituitary vitamin D interactions, we examined the specific binding of tritiated 1α,25-$(OH)_2D_3$ to subcellular fractions of bovine pituitary. Specific binding was observed in both cytosol and nuclear fractions. Binding to crude nuclear fractions is rapid in onset, saturable, and has properties strikingly similar to those observed with cytosolic 1α,25-$(OH)_2D_3$ receptors in a variety of recognized target tissues.

Intact bovine pituitaries and brain areas were excised immediately after the animals were killed. Subsequent tissue preparation was performed at 0° to 5°C. Connective tissue, white matter, and blood were removed, and tissues were minced in a medium containing 0.01*M* tris-HCl (*p*H 7.4 at 25°C), 0.25*M* sucrose, 1.5 m*M* disodium EDTA, and 0.5 m*M* dithiothreitol. Homogenates (1:10, weight to volume) centrifuged at 1200*g* for 10 minutes produced a crude nuclear (P_1) pellet; the mitochondrial pellet (P_2) was prepared by centrifuging the supernatant at 8000*g* for 20 minutes. Further centrifugation at 105,000*g* for 60 minutes yielded a microsomal pellet (P_3) and a supernatant (cytosol) fraction.

For membrane studies, subcellular fractions were resuspended at 1:10 or 1:20 (weight to volume) in 0.05*M* tris-HCl (*p*H 7.4 at 25°C), 1.5 m*M* EDTA, and 0.5 m*M* dithiothreitol, and incubated for 30 minutes at 0°C. The suspensions were then incubated with 1α,25-$(OH)_2$-$[^3H]D_3$ (0.5 to 0.7 n*M*) in the presence or absence of unlabeled steroids (in a total volume of 0.23 ml), for 20 minutes at 25°C, and rapidly filtered under vacuum through Whatman GF/B glass fiber filters. The filters were washed with 12 ml of ice-cold buffer containing 0.01*M* tris-HCl, *p*H 7.8, and 0.1 percent Triton X-100. For studies of binding to cytosol, 1.3 to 4.3 n*M* 1α,25-$(OH)_2$-$[^3H]D_3$ and unlabeled steroids were distributed into borosilicate tubes and dried under nitrogen. The steroids were redissolved in 15 to 20 μl of ethanol before the addition of 150 to 200 μl of cytosol. Samples were incubated for 120 to 180 minutes at 0°C; 50 μl of the total incubation volume was filtered under vacuum through DE81 cellulose filters, which were then rinsed with 5 ml of ice-cold 0.02*M* tris-HCl, *p*H 7.9 to 8.2. Radioactivity on the filters (fraction bound to membranes or cytosolic proteins) was assayed by liquid

Table 1. Specific binding of 1α,25-dihydroxyvitamin-$[^3H]D_3$ in subcellular fractions of bovine pituitary. Subcellular fractions were prepared as described in the text. Data are from two representative experiments. In experiment a, the 1α,25-$(OH)_2$-$[^3H]D_3$ concentration was 0.62 n*M* (110 Ci/mmole; Amersham). In experiment b, the 1α,25-$(OH)_2$-$[^3H]D_3$ concentrations were 0.49 nm (P_1 to P_3 fractions) and 1.0 n*M* (cytosol fraction) (160 Ci/mmole, New England Nuclear). The specific binding of 1α,25-$(OH)_2$-$[^3H]D_3$ in each subcellular fraction in experiment b was expressed as a percentage of the specific binding obtained in all four fractions; thus P_1 percent = $[P_1/(P_1 + P_2 + P_3 + \text{cytosol})] \times 100$ with each value in the equation expressed as femtomoles bound per fraction.

Experiment	Tissue fraction	Amount of 1α,25-$(OH)_2$-$[^3H]D_3$ bound (count/min)				Percent of total tissue binding
		Total	Blank	Specific	Femtomoles per milligram of protein	
a	P_1	1298	271	1027	21.90	
b		705	216	489	7.09	47
a	P_2	176	129	47	0.51	
b		107	84	23	0.18	1
a	P_3	121	86	35	0.64	
b		69	77			
b	Cytosol	2697	2008	689	8.54	52

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