flasks are used, but more frequent medium renewals are required and infection is less stable without periodic subcultures. A similar infection can be established in this macrophage culture by use of amastigotes derived from infected animals.

Amastigotes were separated from the host cells and their cellular components in the material collected from the roller bottles. The recovery rate was more than 80 percent (Table 1), and isolated parasites were devoid of any visible host cell debris (Fig. 1B). Amastigotes were first released from heavily infected cells that were suspended in PBS plus 2 m*M* EDTA by needle passage as described above or by three cycles of vigorous mixing in a vortex mixer followed by centrifugation at 3500*g* for 5 minutes. The material was then suspended in 45 percent Percoll (Pharmacia) in PBS, layered over a cushion of 1 ml of 100 percent Percoll, and centrifuged at 3500*g* for 30 minutes. After centrifugation, amastigotes formed a sharp band at the interface between the 45 and 100 percent Percoll solutions while cells and their debris floated at the top near the meniscus. This method can also be used for isolating amastigotes from cutaneous lesions of mice infected with *L. mexicana amazonensis*. The amastigotes isolated from the in vitro culture system proved to be as infectious to laboratory animals as lesion-derived amastigotes. Parasites from both sources were injected at 10^7 per animal into the nose tips of Syrian golden hamsters (in groups of four for each parasite source). Two months later, the lesion sizes were 1.64 ± 0.3 and 1.61 ± 0.4 cm in diameter for culture- and lesion-derived amastigotes, respectively.

The in vitro system for the *L. mexicana amazonensis*-mouse macrophage line compares favorably with the in vivo animal model for this parasite in routine maintenance and production of amastigotes. The roller bottle cultures, with little attention, provide an essentially perpetual host-parasite system from which infective amastigotes can be isolated every week in quantities adequate for investigations. Flexibility is perhaps the greatest advantage of such an in vitro system, which can provide parasites on short notice and infected cells at any time for various types of assays. These cultured materials have been used to test for potential antileishmanial drugs and to study host-parasite interactions.

K.-P. CHANG

Laboratory of Parasitology, Rockefeller University, New York 10021

References and Notes

1. L. Lamy, A. Samso, H. Lamy, *Bull. Soc. Pathol. Exot.* **57**, 16 (1964); H. C. Miller and D. W. Twohy, *J. Protozool.* **13** (Suppl.), 19 (1966); T. E. Frothingham and E. Lehtimaki, *J. Parasitol.* **55**, 196 (1969); R. L. Berens and J. J. Marr, *J. Protozool.* **26**, 453 (1979); K.-P. Chang and W. Fish, in *Cultivation of Pathogenic Protozoa of Domestic Animals and Man*, J. B. Jensen, Ed. (CRC Press, Boca Raton, Fla., in press).
2. P. Ralph, J. Prichard, M. Cohn, *J. Immunol.* **114**, 898 (1975). Cells for the present study were cultured as suspensions in spinner bottles with minimal essential medium (MEM) plus 10 percent HIFBS or as monolayers in plastic tissue culture flasks with Hepes-buffered RPMI 1640 plus 20 percent HIFBS at 35° or 37°C.
3. K.-P. Chang, *Exp. Parasitol.* **48**, 175 (1979).
4. It is important to note that the counting method is made possible by certain properties of the parasite species and the macrophage line used. The fact that J774G8 cells under the present culture conditions are loosely adherent and can be washed from the culture flasks by vigorous aspiration facilitates their counting. The relatively large sized amastigotes of the leishmania species and the huge parasitophorous vacuoles produced by them in the macrophages render their visualization and enumeration rather easy by phase-contrast microscopy of fresh material with an oil immersion lens. Previously, infected cultures were fixed and stained before observations, and it was very difficult to estimate the total cell numbers.
5. Fetal bovine serum from commercial sources must be screened for its ability to support the growth of the macrophage and the parasite before use.
6. Under the culture conditions described, macrophages double their numbers twice during the first 2 weeks, after which the cell population frequently decreases in heavily infected cultures. When macrophages used for infection experiments were previously cultured in MEM plus 10 percent HIFBS, they normally did not grow during the first 2 weeks.
7. Large numbers of free amastigotes may appear in heavily infected cultures when the medium is not changed for more than 3 days. Such cultures usually deteriorate. Heavily infected cells do not burst, but trap large numbers of amastigotes until they deteriorate. They are thus not made available to infect other macrophages in the same culture. This would explain the fact that uniform infection cannot be maintained in the stationary culture unless amastigotes are purposely released to initiate a new infection. There is no evidence for extensive destruction of macrophages by leishmania in natural infections. Such an event may not be advantageous for intracellular parasites that use these cells exclusively as their host cells.
8. Repeated collections of the floating cell population may sometimes thin out the cell layer and necessitate the addition of infected cells.
9. I thank W. Trager for suggestions in reviewing this report; J. Michl and R. S. Bray for supplying the macrophage cell line and the parasite, respectively; and J. Stanorski and C. Klein for technical help. Supported by the U.N. Development Programme-World Bank-World Health Organization Special Program for Research and Training in Tropical Diseases contract 780040, by research grant AI-15183 from the National Institute of Allergy and Infectious Diseases, and by an Irma T. Hirschl career scientist award.

2 April 1980; revised 17 June 1980

Cerebral Arteriolar Damage by Arachidonic Acid and Prostaglandin G_2

Abstract. *Application of arachidonic acid or prostaglandin G_2 to the brain surface of anesthetized cats induced cerebral arteriolar damage. Scavengers of free oxygen radicals inhibited this damage. Prostaglandin H_2, prostaglandin E_2, and 11,14,17-eicosatrienoic acid did not produce arteriolar damage. It appears that increased prostaglandin synthesis produces cerebral vascular damage by generating free oxygen radicals.*

Severe arterial hypertension causes arteriolar necrosis by mechanisms that are not well understood (*1*). In cats, acute hypertension, induced by experimental brain injury or by intravenous administration of vasoconstrictor agents causes discrete destructive lesions in the endothelial lining of brain pial arterioles (*2–4*). After the hypertensive episode, these arterioles display sustained vasodilation and reduced responsiveness to change in the partial pressure of CO_2 in arterial blood (P_aCO_2) and to changes in arterial blood pressure. All of these abnormalities are minimized or completely inhibited by treatment with cyclooxygenase inhibitors or by topical application of free radical scavengers to the brain surface (*3, 4*). These findings suggest that the cerebral arteriolar abnormalities in acute hypertension are caused by free oxygen radicals generated as a result of increased prostaglandin synthesis. It is known that intermediate steps in the biosynthesis of prostaglandins from their precursor, arachidonic acid, produce free oxygen radicals (*5–9*). These highly reactive forms of oxygen are injurious to tissues and may cause lysis of red cells (*10*), destruction of endothelial cells (*11*), and peroxidation of lysosomal (*12*) and mitochondrial membranes (*13*).

If this hypothesis for the pathogenesis of the cerebral arteriolar damage in acute hypertension is correct, the cerebral arteriolar abnormalities seen in hypertension would be produced by application of exogenous prostaglandins, and this effect of the prostaglandins would be inhibited by administration of free radical scavengers. We tested this hypothesis in cats anesthetized with pentobarbital (30 mg/kg, given intravenously) and ventilated artificially with a positive-pressure respirator, after skeletal muscles were paralyzed with decamethonium bromide (0.5 mg/kg, intravenously). Arterial blood pressure and CO_2 concentration in expired air were monitored continuously. The pial microcirculation of the parietal cortex was

0036-8075/80/0912-1242$00.50/0

viewed through a cranial window (*14*).

After control measurements were made of resting arteriolar caliber and responsiveness to arterial hypocapnia induced by hyperventilation, the space under the cranial window was filled with artificial cerebrospinal fluid (CSF) containing one of the agents listed below. Each solution was prepared fresh immediately prior to the experiment from stock solutions of these agents and was allowed to remain in contact with the brain for 15 minutes. Then, the space under the window was flushed with fresh CSF. Observations were repeated 1 hour later to verify the degree of residual arteriolar dilation. Subsequently, the animal's head was perfused at the animal's own blood pressure, first with 0.9 percent NaCl solution, and then with a fixative solution consisting of 2.5 percent glutaraldehyde and 2 percent paraformaldehyde in 0.1*M* phosphate buffer (*15*). The pial vessels, which were studied in vivo, were examined by scanning and transmission electron microscopy with a technique that permits precise correlations of morphology and function (*15*). The density of any endothelial lesions seen by scanning electron microscopy was quantified by counting the number of lesions in five random microscopic fields, each measuring 2100 μm^2. The examination of pial vessels and counting of the lesions was done by a morphologist who was not aware of the nature of the agents used in each animal.

We used 40 cats, randomly assigned to eight groups. Animals of each of these groups received one of the following agents under the cranial window.

1) Arachidonic acid (200 μg/ml).

2) Arachidonic acid (200 μg/ml) administered 30 minutes after treatment of the animal with the cyclooxygenase inhibitor indomethacin (3 mg/kg, intravenously); this dose of indomethacin causes significant inhibition of the pial arteriolar dilation produced by topical application of arachidonic acid on the brain surface (*16*).

3) Prostaglandin E_2 (PGE_2), 10 μg/ml.

4) Prostaglandin G_2 (PGG_2), 10 μg/ml.

5) Prostaglandin H_2 (PGH_2), 10 μg/ml.

Note that because their molecular weights are similar, PGE_2, PGH_2, and PGG_2 at 10 μg/ml provide similar molar concentrations.

6) 11,14,17-Eicosatrienoic acid (200 μg/ml). This unsaturated fatty acid is not a substrate for cyclooxygenase and therefore does not stimulate prostaglandin synthesis (*17*). It was used to determine whether or not 200 μg/ml of a fatty acid produces nonspecific effects.

7) Arachidonic acid (200 μg/ml) plus 20 m*M* mannitol, a scavenger of the hydroxyl free radical (*18, 19*); application of 20 m*M* mannitol by itself produced 6 percent arteriolar dilation and did not affect the vasoconstrictor response to arterial hypocapnia.

8) Prostaglandin G_2 (10 μg/ml) plus superoxide dismutase (Biotics Research, Houston, Texas) (10 μg/ml); this enzyme catalytically scavenges the superoxide anion radical (*20*). Application of superoxide dismutase (10 μg/ml) by itself produced no significant change in either resting arteriolar diameter or in the response to arterial hypocapnia.

The prostaglandin cyclic endoperoxides PGG_2 and PGH_2 were biosynthesized from radiolabeled arachidonic acid by using the cyclooxygenase from sheep

Table 1. Cerebral arteriolar abnormalities induced by topical application of prostaglandins and fatty acids. Values are means ± standard error. Control diameters and P_aCO_2 during normocapnia and hypocapnia were comparable in all groups. Decreases in P_aCO_2 during hypocapnia averaged 14 to 17 mm-Hg. Arteriolar dilation reported here represents the residual dilation seen 1 hour after agents were washed away from brain surface.

Treatment	Number of vessels	Number of endothelial lesions per 2100 μm^2	Residual arteriolar dilation (percent of control)	Decrease in arteriolar diameter with arterial hypocapnia (percent of control)	
				Before	After
Arachidonic acid	28	18.9 ± 2.2	35 ± 6.3	14 ± 0.8	9 ± 1.9†
Arachidonic acid after indomethacin	31	3.6 ± 1.6*	5 ± 7.0*	15 ± 0.9	13 ± 1.1
11,14,17-Eicosatrienoic acid	22	4 ± 1.9*	4 ± 2.5*	17 ± 0.9	16 ± 1.0
PGG_2	20	13.3 ± 1.8	30 ± 6.3	12 ± 0.8	8 ± 1.3†
PGH_2	31	0 ± 0*	3 ± 2.3*	14 ± 0.8	13 ± 1.2
PGE_2	29	0 ± 0*	1 ± 4.0*	15 ± 0.9	14 ± 1.1
PGG_2 plus superoxide dismutase	28	8.8 ± 1.0*	17 ± 2.6*	13 ± 0.8	12 ± 0.8
Arachidonic acid plus mannitol	24	3.5 ± 0.6*	14 ± 4.5*	15 ± 0.9	15 ± 1.2

*Significantly ($P < .05$) different from either arachidonic acid or PGG_2 means [Tukey's t (*27*) after analysis of variance]. †Significantly ($P < .05$) different from corresponding value before treatment (t-test).

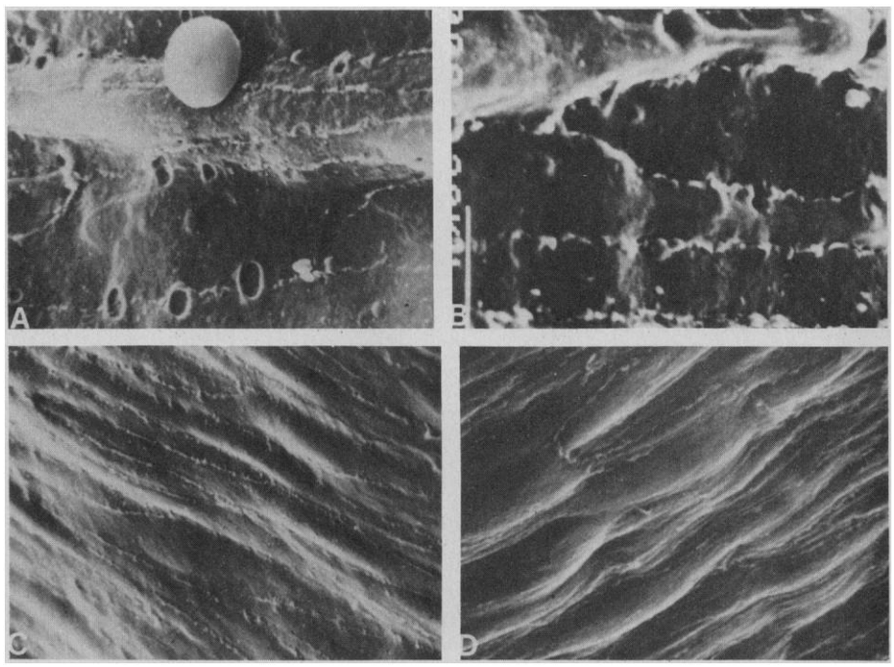


Fig. 1. Representative scanning electron micrographs of the luminal surface of pial arterioles from animals treated with (A) topical PGG_2, (B) arachidonic acid, (C) arachidonic acid after intravenous administration of indomethacin, and (D) PGE_2. Note the presence of endothelial lesions in the vessels treated with PGG_2 and arachidonic acid and the absence of endothelial lesions in the vessels treated with arachidonic acid after indomethacin or PGE_2. Magnification, ×5000.

seminal vesicle microsomes. After biosynthesis and isolation, PGG_2 and PGH_2 were dissolved in acetone and stored at −40°C until experimental use. The endoperoxides were quantified from the specific activity of the radioactive arachidonic acid substrate. Confirmation of the purity of the endoperoxides was obtained by the method of Hamber *et al.* (*21*), whereby endoperoxides were reduced to $PGE_{2\alpha}$ by triphenylphosphine. Analysis of the reduced endoperoxides by high-pressure liquid chromatography yielded only $PGF_{2\alpha}$, evidence that the endoperoxides were pure. Additional confirmation of the authenticity of the endoperoxides was obtained by showing that they induced platelet aggregation in vitro. A stock solution of PGE_2 was prepared by dissolving PGE_2 in 100 percent ethanol. Stock solutions (10 mg/ml) of sodium arachidonate and of 11,14,17-eicosatrienoic acid were prepared by dissolving three parts of the fatty acid (Nuchek Preparations, Elysia, Minnesota) and one part of sodium carbonate (by weight) in distilled water. A stock solution (10 mg/ml) of the sodium salt of indomethacin (Sigma, St. Louis, Missouri) was prepared by dissolving three parts of indomethacin and one part of sodium carbonate (by weight) in distilled water. From these stock solutions, appropriate solutions were prepared in artificial CSF having the same composition of CSF as for cats. The CSF was equilibrated with 6 percent oxygen, 6.5 percent carbon dioxide, and 87.5 percent nitrogen, to give the same *p*H and gas tensions as natural feline CSF (*14*). When necessary, the *p*H of the final drug-containing solutions was adjusted to 7.35. The ethanol or acetone vehicles, in the same concentration as in the various solutions, had no effect on pial arteriolar caliber (*22*).

Arachidonic acid and PGG_2 produced sustained vasodilation, reduced responsiveness to arterial hypocapnia, and a large number of endothelial lesions (Table 1 and Fig. 1). These lesions were similar to those that followed acute hypertension resulting from experimental brain injury or administration of vasoconstrictor agents (*2–4*). There were two types of lesions. When seen with scanning electron microscopy, one consisted of a domelike protrusion into the lumen of the vessels, and the other had the appearance of a crater. With transmission electron microscopy, the domelike lesions appeared as large vacuoles, whereas the craters appeared as localized defects at which the superficial layer of the cell membrane had been ruptured or completely eliminated. The administration of arachidonic acid after treatment with indomethacin, and the administration of 11,14,17-eicosatrienoic acid, PGE_2, or PGH_2 produced significantly less, or no, sustained vasodilation, significantly fewer lesions, and no change in the responsiveness to arterial hypocapnia. Similarly, the administration of superoxide dismutase with PGG_2 or of mannitol with arachidonic acid resulted in fewer endothelial lesions, less vasodilation, and no change in the responsiveness to hypocapnia compared to that seen in the absence of the free radical scavengers. There was no significant change in mean arterial blood pressure or in P_aCO_2 as a result of the application of any of the agents used.

Reference to the normal biosynthetic pathway for prostaglandins in vascular tissue (*23*), which proceeds from arachidonic acid to PGG_2 to PGH_2 and then to PGI_2 or to stable prostaglandins such as PGE_2, shows that the key compound for the induction of vascular damage is PGG_2. This is evidenced by the production of vascular damage by PGG_2 itself, but not by PGH_2 or PGE_2. Similarly, arachidonic acid, which leads to increased synthesis of PGG_2, produced damage. Inhibition of this increased synthesis by indomethacin inhibited the vascular damage. 11,14,17-Eicosatrienoic acid, which does not stimulate prostaglandin synthesis, did not cause vascular damage.

The demonstration that PGG_2 is the important agent implicated in the production of vascular damage points to the probable mechanism by which this is brought about. It is known that the conversion of PGG_2 to PGH_2 releases free oxygen radicals (*5*). The exact nature of these radicals is not known. It is likely that several types of oxygen radicals are involved, including the superoxide anion radical, hydrogen peroxide, singlet oxygen, and the hydroxyl radical (*5–9*). The sequential formation of new radicals can be accomplished by reactions initiated by radicals formed earlier (*24*). The fact that mannitol inhibited the vascular damage produced by arachidonic acid and that superoxide dismutase inhibited the vascular damage produced by PGG_2 suggests the involvement of the hydroxyl free radical and of the superoxide anion radical. In both instances inhibition of vascular damage was incomplete, possibly because the concentration of the radical scavengers was suboptimal or because their access to the vascular wall was limited.

The demonstration that increased prostaglandin synthesis induced by exogenous arachidonic acid is capable of producing vascular damage in cerebral arterioles and that this damage seems to be the result of free oxygen radicals generated by the conversion of PGG_2 to PGH_2 is consistent with the hypothesis that a burst of prostaglandin synthesis initiated by acute hypertension leads to generation of free oxygen radicals which are immediately responsible for cerebral vascular damage. Whether this mechanism has relevance to vascular damage caused by sustained human hypertension is not known. Pronounced transient elevations in arterial blood pressure occur frequently in both normal individuals and in patients with hypertension (*25*). The cerebral vessels of spontaneously hypertensive rats have endothelial lesions similar to those which we have observed after acute hypertension (*26*).

HERMES A. KONTOS
ENOCH P. WEI
Department of Medicine, Medical College of Virginia, Richmond 23298
JOHN T. POVLISHOCK
W. DALTON DIETRICH
CHRISTOPHER J. MAGIERA
Department of Anatomy, Medical College of Virginia
EARL F. ELLIS
Department of Pharmacology, Medical College of Virginia

References and Notes

1. G. W. Pickering, *High Blood Pressure* (Grune & Stratton, New York, 1968), pp. 148–153.
2. E. P. Wei, W. D. Dietrich, J. T. Povlishock, R. M. Navari, H. A. Kontos, *Circ. Res.* **46**, 37 (1980).
3. H. A. Kontos, E. P. Wei, W. D. Dietrich, R. M. Navari, J. T Povlishock, N. R. Ghatak, E. F. Ellis, J. L. Patterson, Jr., in preparation.
4. H. A. Kontos, in *Proceedings of the Symposium on Cerebral Microvasculature—Investigation of the Blood-Brain Barrier* (Plenum, New York, in press).
5. R. W. Egan, J. Paxton, F. A. Kuehl, Jr., *J. Biol. Chem.* **251**, 7329 (1976).
6. L. J. Marnett, P. Wlodawer, B. Samuelsson, *ibid.* **250**, 8510 (1975).
7. R. V. Panganamala, N. R. Brownlee, H. Sprecher, D. G. Cornwell, *Prostaglandins* **7**, 21 (1974).
8. R. V. Panganamala, H. M. Sharma, R. E. Heikkila, J. C. Geer, D. G. Cornwell, *ibid.* **11**, 599 (1976).
9. R. V. Panganamala, H. M. Sharma, H. Sprecher, J. C. Geer, D. G. Cornwell, *ibid.* **8**, 3 (1974).
10. E. W. Kellogg III and I. Fridovich, *J. Biol. Chem.* **252**, 6721 (1977).
11. T. Sacks, C. F. Moldow, P. R. Craddock, T. K. Bowers, H. S. Jacob, *J. Clin. Invest.* **61**, 1161 (1978).
12. K. L. Fong, P. B. McCay, J. L. Poyer, *J. Biol. Chem.* **248**, 7792 (1973).
13. K. Goda, J. W. Chu, T. Kimura, A. P. Schaap, *Biochem. Biophys. Res. Commun.* **52**, 1300 (1973).
14. J. E. Levasseur, E. P. Wei, A. J. Raper, H. A. Kontos, J. L. Patterson, Jr., *Stroke* **6**, 309 (1975).
15. W. D. Dietrich, E. P. Wei, J. T. Povlishock, H. A. Kontos, *Am. J. Physiol.* **238**, H172 (1980).
16. E. P. Wei, E. F. Ellis, H. A. Kontos, *ibid.*, p. H226.
17. S. Bergstrom, L. A. Carlson, J. R. Weeks, *Pharmacol. Rev.* **20**, 1 (1968).
18. C. Beauchamp and I. Fridovich, *J. Biol. Chem.* **245**, 4641 (1970).

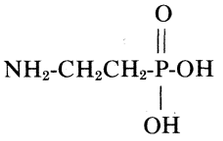
19. P. Neta and L. M. Dorfman, *Adv. Chem. Ser.* **81**, 222 (1968).
20. I. Fridovich, *Annu. Rev. Biochem.* **44**, 147 (1975).
21. M. Hamberg, T. Svensson, B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 345 (1974).
22. E. F. Ellis, E. P. Wei, H. A. Kontos, *Am. J. Physiol.* **237**, H381 (1979).
23. W. E.-M. Lands, *Annu. Rev. Physiol.* **41**, 633 (1979).
24. E. W. Kellogg and I. Fridovich, *J. Biol. Chem.* **250**, 8812 (1975).
25. D. W. Richardson, A. J. Honour, G. W. Fenton, F. H. Scott, G. W. Pickering, *Clin. Sci.* **26**, 445 (1964).
26. F. Hazama, T. Ozaki, S. Amano, *Stroke* **10**, 245 (1979).
27. C. C. Li, *Introduction to Experimental Statistics* (McGraw-Hill, New York, 1964), pp. 418–428.
28. Supported by grants HL-21851 and NS-12587 from the NIH. The PGE_2 was a gift from the Upjohn Company, Kalamazoo, Mich.

2 May 1980; revised 24 June 1980

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

0036-8075/80/0912-1245$00.50/0