

Fig. 2. (A) Protoconch of *Buccitriton sagenum* shown by the scanning electron microscope. (B) A detail of the fifth whorl reveals the formation of axial ribs before spiral threads and adult cancellate sculpture.

unmetamorphosed larval shells are not studied. They are small (usually from 60 to around 300  $\mu\text{m}$  in diameter), and macroinvertebrate workers generally deal with animals that are at least 1 mm in diameter. Microinvertebrate workers, on the other hand, may not have the taxonomic knowledge required to successfully identify species from several phyla. Under an optical microscope larval shells of different species tend to look similar, mainly because of relatively poor resolution of these microscopes. In the scanning electron microscope apparently featureless protoconch whorls can reveal an enormous amount of sculptural detail. The ornament is species-specific, so that even closely related species can be differentiated by their larval shells alone. Scanning electron micrographs of the protoconchs of *Buccitriton sagenum* and *B. texanum* (Figs. 2 and 3) from upper middle Eocene deposits of the Gulf Coast show some distinguishing characteristics. *Buccitriton texanum* has a spiral thread on the shoulder of the fourth and fifth whorl that starts before or at the same time as the first axial rib (Fig. 3B). The number of spiral threads increases as the individual grows, and the ribs and spiral threads gradually become more pronounced until they form a cancellate pattern in the adult stage. In

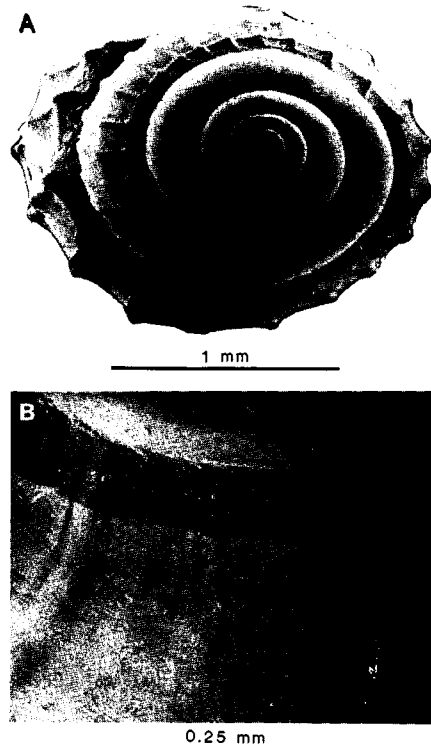


Fig. 3. (A) Protoconch of *Buccitriton texanum* shown by the scanning electron microscope. (B) A detail of the shoulder of the fourth whorl reveals the formation of a spiral thread before the first axial rib.

*B. sagenum*, spiral threads and axial ribs also form a cancellate pattern in the adult, but the axial ribs start well in advance of the spiral threads in the protoconch stage (Fig. 2B). It is not necessary to use the scanning electron microscope for each shell since many structures seen originally with the scanning

scope may also be seen with light optics. Well-preserved adult or juvenile specimens commonly have the larval stages represented by the protoconch at the apex or umbo of the shell. By documenting the protoconch morphology of well-preserved adult specimens, a catalog of larval forms can be compiled. Then the species of a free larval shell may be identified from the catalog.

In summary, the free larval shells of molluscs are common in the fossil record and with proper documentation they can be reliably identified to species. The study of fossil molluscan larvae can considerably enhance the biostratigraphic potential of molluscs by increasing their known geographic range and facies independence.

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6. Molluscan larval shells are more delicate than planktic foraminifera and may not be as abundantly preserved in the rock record. They are robust enough, however, to be found in well cuttings from unconsolidated sediments and are probably present whenever aragonitic shell material is preserved.
7. I thank J. Sprinkle and P. Chambers for critical review of the manuscript. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the Geology Foundation of the University of Texas at Austin for support of this research.

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## Cloned Poliovirus Complementary DNA Is Infectious in Mammalian Cells

**Abstract.** A complete, cloned complementary DNA copy of the RNA genome of poliovirus was constructed in the *Pst* I site of the bacterial plasmid pBR322. Cultured mammalian cells transfected with this hybrid plasmid produced infectious poliovirus. Cells transfected with a plasmid which lacked the first 115 bases of the poliovirus genome did not produce virus.

Poliovirus is a positive-strand RNA virus with a genome of one molecule of single-stranded RNA containing the information for synthesis of poliovirus proteins (1-5). During infection of mammalian cells, the viral RNA is translated into a single continuous polypeptide of 250,000 molecular weight, which is subsequently cleaved by proteases to form the functional viral proteins. To better understand the structure and functions of the viral genome, complementary

DNA (cDNA) copies of the viral RNA were cloned into a bacterial plasmid and three clones which together represent the entire RNA were used to determine the complete 7440-nucleotide sequence of the poliovirus genome (3).

An important question is whether these cDNA clones could generate infectious virus in mammalian cells as was previously shown in bacteria for the cDNA clones of a bacterial RNA virus (6). The availability of infectious cloned

poliovirus cDNA would make it possible to perform genetic manipulations that are not possible with RNA and would open a variety of new approaches to the study of similar RNA viruses. To determine whether poliovirus cDNA is infectious, the three cDNA clones described previously (3) were joined to produce a single, full-length cDNA copy of the poliovirus genome in the Pst I site of plasmid pBR322. When this hybrid plasmid was transfected into CV-1 or HeLa cells, infectious poliovirus was produced.

Construction of the partial poliovirus cDNA clones has been described (3). Poliovirus RNA was used as a template for the synthesis of double-stranded cDNA and the cDNA's were cloned in pBR322 at the Pst I site with the use of oligo(dG) · oligo(dC) (2) homopolymer tails. Plasmid pVR101 contained an insert representing bases 4865 through the 3' end of the viral RNA (base 7440) followed by 37 A residues in the "sense" strand. The insert in plasmid pVR103 represented bases 116 to 6526, and plasmid pVR105 represented bases 1 to 220 of the viral genome. Plasmid pVR105 was constructed from primer-extended material (3).

The manipulations used for joining these three plasmids into a single, full-length clone are shown in Fig. 1. Plasmids pVR101 and pVR103 were joined at the unique, common Bgl II site, producing plasmid pVR104 which represents bases 116 through the 3' poly(A) stretch of the viral RNA. This plasmid was then joined with plasmid pVR105 at the Bam HI site at nucleotide 220 by means of partial digestion products (see legend to Fig. 1). The structure of the full-length poliovirus cDNA clone pVR106 is shown in Fig. 1. The poliovirus-specific DNA is linked to pBR322 at the Pst I site with oligo(dG) · oligo(dC) tails. Beginning at the Pst site and moving clockwise around the plasmid (Fig. 1), the "sense" strand consists of 50 to 60 G's, bases 1 to 7440 of the viral RNA, 37 A's, 17 C's, and finally another Pst I site.

The infectivity of the full-length clone (pVR106) was tested by transfection into mammalian cells. Semiconfluent monolayers of CV-1 cells were transfected, in duplicate, with closed circular forms of pVR106 or unsubstituted pBR322. The modified calcium phosphate method of transfection was used (7). After treatment with glycerol, one plate of cells was covered with fluid medium, and the other plate was covered with an agar overlay. After a 4- to 5-day incubation period under liquid medium, cells transfected with pVR106 had completely detached

from the plate. Cells transfected with pBR322 remained as an intact monolayer. The medium from cells transfected with pVR106 or pBR322 was examined for the presence of virus by plaque assay on HeLa cell monolayers (Table 1). A high virus titer was found in the medium from cells transfected with pVR106, but no virus was released from pBR322-transfected cells. Cells transfected with pVR106 and incubated under agar displayed, on being stained with crystal violet, plaques similar to those induced by poliovirus (Table 1). No plaques were observed on cells transfected with pBR322 DNA. Usually from 10 to 70 plaques were observed per 100-mm plate

of cells transfected with 10 µg of pVR106. If approximately 10 percent of the cells receive DNA (7), then infectious foci arose at an efficiency of about  $2 \times 10^{-5}$  to  $8 \times 10^{-5}$ . An independently derived, full-length poliovirus cDNA clone, designated pVR106a, also yielded infectious virus after transfection into cells (data not shown).

Other experiments (Table 1) show that virus production in transfected cells is directed by plasmid pVR106 DNA. No virus was detected in cells transfected with pVR106 DNA cleaved by Hinf I, an enzyme that cuts the plasmid at 45 sites and does not reduce the infectivity of viral RNA. The infectivity of pVR106

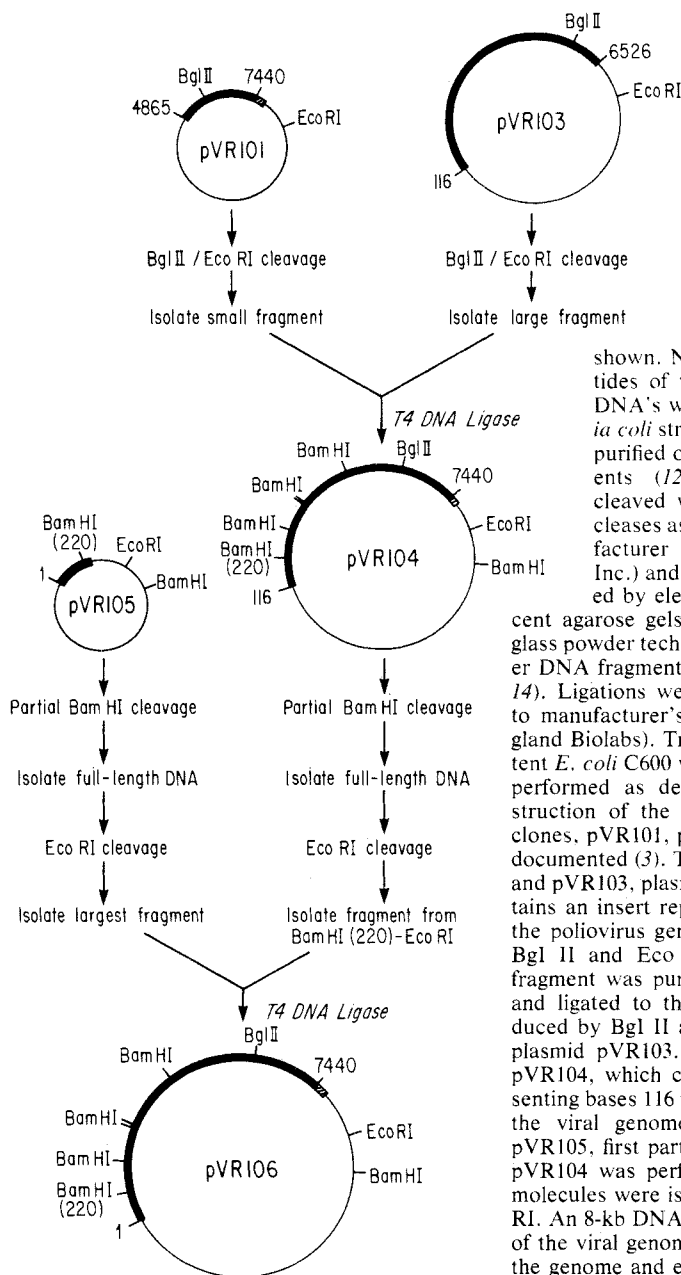


Fig. 1. Construction of full-length poliovirus cDNA clone. Single lines, pBR322 DNA; double lines, poliovirus-specific cDNA. Light area in the poliovirus-specific region represents oligo(A) · oligo(T); the oligo(G) · oligo(C) tails on both ends of the insert are not

shown. Numbers refer to nucleotides of the viral RNA. Plasmid DNA's were grown in *Escherichia coli* strain C600, extracted, and purified on cesium chloride gradients (12). The DNA's were cleaved with restriction endonucleases as described by the manufacturer (New England Biolabs, Inc.) and fragments were separated by electrophoresis on 0.6 percent agarose gels. A modification of the glass powder technique was used to recover DNA fragments from agarose gels (13, 14). Ligations were performed according to manufacturer's instructions (New England Biolabs). Transformation of competent *E. coli* C600 with various DNA's was performed as described (15). The construction of the three poliovirus cDNA clones, pVR101, pVR103, and pVR105, is documented (3). To join plasmids pVR101 and pVR103, plasmid pVR101, which contains an insert representing the 3' end of the poliovirus genome, was cleaved with Bgl II and Eco RI. The smaller DNA fragment was purified by electrophoresis and ligated to the largest fragment produced by Bgl II and Eco RI digestion of plasmid pVR103. This ligation produced pVR104, which contains an insert representing bases 116 through the 3' poly(A) of the viral genome. To join pVR104 to pVR105, first partial Bam HI digestion of pVR104 was performed, and then linear molecules were isolated and cut with Eco RI. An 8-kb DNA fragment from base 220 of the viral genome through the 3' end of the genome and extending to the pBR322 Eco RI site was purified by electrophoresis.

This fragment was ligated to an isolated fragment of pVR105 consisting of nucleotides 1 to 3607 of pBR322 linked to DNA representing bases 1 to 220 of the poliovirus genome. The resulting plasmid pVR106 contained a full-length cDNA copy of the poliovirus genome at the Pst I site of pBR322.

Table 1. Transfection of cultured mammalian cells with various plasmid DNA's. CV-1 or HeLa cells were grown to 80 percent confluence in 10-cm plastic dishes. Cells were then transfected by the modified calcium-phosphate technique (7), in duplicate, with 10  $\mu$ g of plasmid DNA per dish or 2  $\mu$ g of viral RNA prepared as described (3). Briefly, medium was removed from the cells, and DNA was added as a calcium-phosphate precipitate in Hepes-buffered saline. After 20 minutes at room temperature, cells were covered with warm medium (Dulbecco's modified Eagle medium plus 10 percent calf serum) and incubated for 4 hours at 37°C. After this time, medium was removed, cells were washed once with warm medium, and 2.5 ml of 15 percent glycerol in Hepes-buffered saline was added to each. After 3.5 minutes at 37°C, the glycerol was removed and cells were washed once with warm medium. One of the duplicate dishes was then covered with warm medium, and the other was covered with medium containing 1 percent agarose (Sigma). Plates were incubated at 37°C for 4 to 5 days. To count plaques, the agar overlay was removed, and cells were stained with 0.1 percent crystal violet in 50 percent ethanol. Medium from cells incubated under liquid was assayed for infectious poliovirus on HeLa cell monolayers. Virus titers shown are values for a typical experiment. Usually between 5 and 70 plaques were observed on monolayers transfected with pVR106 DNA. For ribonuclease treatment, 5  $\mu$ g of boiled pancreatic ribonuclease (Worthington) were used for 10  $\mu$ g of DNA or 2  $\mu$ g of RNA.

Nucleic acid	Plaque-forming units per milliliter in medium	Number of plaques on transfected cell monolayer
CV-1 cells		
pVR106	$1.2 \times 10^9$	22
pVR106 + Hinf I	0	0
pVR106 + ribonuclease	$1.3 \times 10^9$	10
pVR106, phenol extracted	$1 \times 10^9$	22
pVR106, phenol extracted, then ribonuclease	$1.4 \times 10^9$	26
pVR104	0	0
pBR322	0	0
Viral RNA	$1.5 \times 10^9$	71
Viral RNA + ribonuclease	0	0
Viral RNA + Hinf I	$1.4 \times 10^9$	20
HeLa cells		
pVR106	$3.7 \times 10^8$	69
pBR322	0	0

DNA was not significantly reduced by treatment with ribonuclease under conditions that abolish the infectivity of viral RNA. Therefore, infectivity was not due to viral RNA contaminating the pVR106 DNA. Phenol extraction of pVR106 DNA alone or followed by ribonuclease treatment did not lower the infectivity of the plasmid. Thus, virions did not appear to be present in the pVR106 preparation. When pVR106 DNA was assayed directly for contaminating virions on HeLa cell monolayers, no infectivity was detected (data not shown). These results indicate that the infectivity of pVR106 is inherent in the plasmid DNA.

To examine the identity of the virus produced in pVR106-transfected cells, an antibody neutralization test was performed. About 100 plaque-forming units of the virus released into the culture medium of pVR106-transfected cells was mixed with various dilutions of rabbit antiserum to poliovirus and assayed for infectivity. In parallel, 100 plaque-forming units of authentic poliovirus was assayed for neutralization by the serum. Both viruses were neutralized 50 percent by a 1/50,000 dilution of the serum, indicating that the pVR106-derived virus was authentic poliovirus.

Plasmid pVR104, which was an inter-

mediate in the construction of the full-length clone, lacked the first 115 bases of the poliovirus genome (Fig. 1). There are no AUG codons in this region (3, 4) and therefore bases 1 to 115 of the viral RNA are probably not translated into protein. Transfection of plasmid pVR104 into CV-1 cells did not result in virus production (Table 1), an indication that the 5' end of poliovirus RNA is required for infectivity even though it does not encode protein. This requirement for the 5' end is not surprising, because it is known that at least the first ten bases are conserved among all three poliovirus types and Cocksackie virus B1 (8, 9). Although the AUG that initiates the poliovirus polypeptide appears to be located at nucleotide 743, there are also eight other AUG codons in the preceding region (3). It is possible that some of this region is translated into protein, and it will be of interest to create deletions in this area and determine how much of it is necessary for viral replication.

It is surprising that a cDNA copy of an RNA virus can initiate the infection process. The mechanism by which this event proceeds will be important to understand. It is possible that the plasmid enters the nucleus and RNA synthesis initiates at one or more of the areas in

pBR322 which may function as a promoter in vitro (10). Alternatively, the plasmid DNA may integrate next to a promoter in cellular DNA. The first sense strands of RNA made in this way would probably have extra, nonviral sequences at either end. Precisely how these molecules could replicate is not clear; the extra sequences might be randomly cleaved off, removed by a specific nuclease, or perhaps not removed at all. In the latter case, replication might occur because of initiation and termination specificity of the viral replicase. To help clarify this process, the termini of RNA's in virions liberated from pVR106-transfected cells are being examined.

This report is the first that demonstrates the infectivity of cloned DNA derived from the RNA genome of a lytic animal virus. Cloned DNA copies of RNA tumor virus genomes are infectious in mammalian cells; retroviruses, however, normally pass through a DNA phase in their life cycle while picornaviruses like poliovirus do not (11). In prokaryotes, cloned DNA copies of the genome from the single-stranded RNA bacteriophage Q $\beta$  generate infectious phage when transfected into bacteria (6).

The availability of infectious, cloned poliovirus DNA will permit the study of this virus in many new ways. It will now be possible to specifically mutagenize the cloned DNA and generate defined poliovirus mutants with defects in any part of the genome. Such a mutant collection will enhance the study of poliovirus genetics. In addition, it may now be possible to construct effective, attenuated vaccine strains that are incapable of reverting to virulence. It is uncertain whether the application of recombinant DNA techniques to negative-strand RNA viruses (5) or viruses with multiple genome RNA's will also yield infectious cDNA. However, our results indicate that recombinant DNA methodology should markedly alter the study of positive-strand viruses with a genome of one RNA molecule.

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## Tetrahydrobiopterin in Striatum: Localization in Dopamine Nerve Terminals and Role in Catecholamine Synthesis

**Abstract.** *The hydroxylase cofactor, tetrahydrobiopterin, and its biosynthetic system are localized in dopaminergic nerve terminals in the striatum. This conclusion is based on the nearly equivalent loss of tyrosine hydroxylase and tetrahydrobiopterin and its initial biosynthetic enzyme, guanosine triphosphate cyclohydrolase, after injection of 6-hydroxydopamine into the substantia nigra. The role of the hydroxylase cofactor in the regulation of dopamine synthesis is reassessed.*

The initial and rate-limiting reaction in the biosynthesis of catecholamine neurotransmitters (dopamine, norepinephrine, and epinephrine) is catalyzed by tyrosine hydroxylase. This enzyme requires molecular oxygen and the reduced form of the hydroxylase cofactor, tetrahydrobiopterin (BH<sub>4</sub>), for the conversion of tyrosine to L-dopa, which is then decarboxylated to form dopamine in dopaminergic neurons of the central nervous system (CNS). Tetrahydrobiopterin is also required for tryptophan hydroxylase activity in the neurons that produce the other major biogenic amine neurotransmitter, serotonin. We previously reported that the distribution of hydroxylase cofactor across areas of the rat brain was positively correlated with the total (tyrosine plus tryptophan) hydroxylase enzyme activity (1). Since these enzymes are markers for aminergic neurons, our results suggested that BH<sub>4</sub> was at least highly concentrated if not exclusively localized in aminergic neurons in certain areas of the brain. We also reported that the hydroxylase cofactor content in cerebrospinal fluid (CSF) from patients with Parkinson's disease was reduced to 50 percent compared to control (2). Since Parkinson's disease is characterized by a degeneration of nigrostriatal dopaminergic neurons, a considerable portion of CSF cofactor appears to be derived from these cells.

In the brain BH<sub>4</sub> is synthesized de novo from guanosine triphosphate (GTP). Although the enzymatic steps in BH<sub>4</sub> biosynthesis have not been completely elucidated, it is accepted that the initial step is the conversion of GTP to

dihydroneopterin triphosphate. This reaction is catalyzed by the enzyme GTP cyclohydrolase (3). Just as tyrosine hydroxylase serves as a marker enzyme for catecholaminergic neurons, GTP cyclohydrolase should serve as a marker for cells with BH<sub>4</sub> synthesizing capability.

Tyrosine hydroxylase can be activated by such processes as direct protein phosphorylation (4) and limited tryptic digestion (5) as well as by anions (6) and phospholipids (7). Activation by protein phosphorylation is expressed in kinetic terms by a lower Michaelis constant (*K<sub>m</sub>*) of tyrosine hydroxylase for its cofactor BH<sub>4</sub> (4), and consequently the enzyme is more active in the presence of suboptimal BH<sub>4</sub> concentrations. The decreased *K<sub>m</sub>* for BH<sub>4</sub> is important because of evidence suggesting that intraneuronal concentrations of BH<sub>4</sub> are subsaturating and thus limit the activity of tyrosine hydroxylase in the dopaminergic neurons of the nigrostriatal system (8, 9). To develop greater understanding of the cellular localization and site of biosynthesis of BH<sub>4</sub> in the CNS, we studied the

nigrostriatal system of the rat as a model aminergic area because of the high concentration of dopaminergic neurons and the functional importance of this area in certain neurological disorders, notably Parkinson's disease.

The specific neurotoxin 6-hydroxydopamine (6-OHDA) was injected (10) into the left substantia nigra of male Sprague-Dawley rats to selectively destroy the dopamine neurons whose cell bodies, which are located in the nigra, project to and terminate in the corpus striatum. The contralateral striatum serves as a control since neurotoxic damage is limited to the nigra and striatum ipsilateral to the lesion. The success of the 6-OHDA lesion was tested by monitoring apomorphine-induced rotational behavior (11); only animals making three or more clockwise turns per minute were used in biochemical analyses. Striatal tyrosine hydroxylase activity was measured as described in (12). Reduced and oxidized forms of biopterin in the striatum were measured by native fluorescence after high-pressure liquid chromatography (13). Striatal GTP cyclohydrolase activity was measured by monitoring the conversion of GTP to dihydroneopterin triphosphate as described by Nixon *et al.* (14).

The results of the experiments (Table 1) show that tyrosine hydroxylase was depleted in the striatum on the lesioned side by 93 percent in comparison with the control activity in the contralateral striatum. This decrease in striatal tyrosine hydroxylase activity together with the clockwise turning exhibited by the same animals in response to apomorphine indicated almost complete destruction of nigrostriatal dopaminergic neurons. The 6-OHDA lesion also caused a 73 percent loss of total biopterin in the ipsilateral striatum as well as a 68 percent loss of striatal GTP cyclohydrolase activity (Table 1). Bullard *et al.* (15), who administered 6-OHDA intraventricularly, suggested that there was some association of reduced pterins with dopaminergic neurons, although the intraven-

Table 1. Effects of unilateral 6-hydroxydopamine injection in the substantia nigra on biopterin and related enzymes in rat striatum. Values are mean  $\pm$  the standard error of the mean.

Treatment	GTP-Cyclohydrolase (pmole/hour per milligram of protein)	Biopterin ( $\mu$ g/g, wet weight)	Tyrosine hydroxylase (nmole/min per milligram of protein)
Control ( <i>N</i> = 6)	3.85 $\pm$ 0.92	0.33 $\pm$ 0.01	2.50 $\pm$ 0.04
6-Hydroxydopamine ( <i>N</i> = 6)	1.22 $\pm$ 0.43*	0.09 $\pm$ 0.01†	0.17 $\pm$ 0.09†
Percent decrease	68	73	93

\**P* < .01 and †*P* < .001; significantly different from control values as determined by Student's paired *t*-test.