ed. Virus extraction procedures (*9*) yielded 50-nm spherical particles identical in morphology to standard CaMV (Fig. 2).

To determine whether the virus particles contain DNA characteristic of the original virus, we extracted the nucleic acid and subjected it to electrophoretic analysis on agarose gels. DNA from the particles comigrated with DNA from the standard virus, both in native and denatured form (Fig. 3). The native DNA's migrated as two bands, a faster sharp band composed of linear molecules and a slower diffuse band composed of a collection of circular forms (*7*). The denatured DNA from the standard CM4-184 virus contains two single-strand breaks (Fig. 1), and when it is denatured it falls apart into two linear single strands that comigrate as a single band on gels (*5*). The gel migration properties of the native and denatured DNA indicate that viral DNA molecules derived from the infection by cloned CaMV DNA have reacquired their secondary structure and their single-strand breaks. The Sal I digestion of the DNA obtained from virus particles isolated from plants infected with cloned CaMV DNA shows that the plant rejoined the Sal I cohesive ends of pLW408/Sal I DNA, thereby reconstituting the Sal I site.

It should be pointed out that the cloned CaMV DNA suffered no major insertions or deletions during reintroduction into the plant. The DNA retained all characteristics of the CM4-184 CaMV DNA from which it was derived, including the DNA fragment pattern resulting from the digestion with Sal I (Fig. 3) and several other restriction enzymes (data not shown). This is important because it means that the virus particles that arose from infection by cloned CaMV DNA did not, in fact, result from the accidental introduction of another strain of CaMV. Because CM4-184 DNA carries a large deletion, amounting to 5 percent of the total genome (*5*), it is clearly distinguishable from other CaMV types. The CM4-184 strain is unlikely to be accidentally introduced into our plants because the virus by itself cannot be transmitted by aphids, the natural vector for the virus (*8*). We routinely maintain another viral isolate, CaMV Cabb B-J.I. (an aphid-transmitted strain), in the same greenhouse with the test plants. The DNA from this strain can be clearly distinguished from CM4-184 DNA by gel electrophoretic analysis.

These experiments demonstrate that DNA obtained from plasmids constructed in vitro can infect plants and thereby open new opportunities for exploring the use of CaMV as a molecular vehicle in plants.

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8. The isolate was described by M. C. Y. Lung and T. P. Pirone [*Phytopathology* **63**, 910 (1973)]. DNA from CM4-184 has been used in cloning experiments because the virus itself is a deletion mutant that cannot be transmitted by its natural vector, aphids. Recombinant plasmids were propagated in *Escherichia coli* χ1776 under P2 containment conditions before 15 March 1979 and in *E. coli* SF8 under P1 conditions after 15 March 1979 following the issuance of the revised NIH guidelines (December 1978) and approval by the Institutional Biosafety Committee (IBSC). Plants were infected with recombinant plasmids containing CaMV DNA following IBSC approval (15 March 1979). Experiments were performed in greenhouses under P1 containment conditions as specified in the revised NIH guidelines (December 1978).
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Mitochondrial Water in Myocardial Ischemia: Investigation with Nuclear Magnetic Resonance

Abstract. *Nuclear magnetic resonance studies of mitochondria isolated from ischemic hearts after coronary vessel occlusion indicated a decrease in water proton relaxation times. This change coincided with a decrease in the hydration of the samples. It is suggested that in ischemia, changes in macromolecular hydration may be one of the first mechanisms to alter function in the mitochondria, which are vital to the energy-transducing process in heart muscle.*

Myocardial ischemia leads to rapid deterioration of mitochondrial function (*1-3*). This occurs even during the first 20 minutes of coronary vessel occlusion (the "reversible" stage). Irreversible cellular damage after 20 minutes of occlusion has been attributed to several factors (*4-8*). However, no conclusive evidence has been presented to account for the very early and progressive deterioration in organelle function that occurs with ischemia.

Nuclear magnetic resonance (NMR) studies have indicated the importance of cellular water and its exchange for several biological systems in normal and certain pathological states (*9-11*). In light of morphological evidence that mitochondria become swollen and ultimately disrupted during ischemia (*12-14*), we chose to ascertain whether changes in macromolecular hydration are associated with the altered function. Mitochondrial water content was investigated by measuring the NMR relaxation times of water protons. We report that the hydration of mitochondrial pellets and the spin-lattice (T_1) and spin-spin (T_2) relaxation times of water protons decrease as the period of ischemia increases. In fact, these changes begin to appear after just 3 minutes of coronary occlusion.

A loose ligature was placed on the circumflex coronary artery of surgically prepared and monitored dogs (15 to 25 kg) (*13*). The ligature was tightened until flow ceased entirely, and the heart was removed after a set period and immediately chilled to 4°C. Control animals received sham surgery; no occlusion was performed and the heart was removed after periods as long as 60 minutes. Mitochondria were isolated from 10- to 15-g samples of both the anterior and posterior papillary muscle regions of the left ventricle by using the Polytron procedure with either KCl-EDTA (*15*) or mannitol; additionally, two populations of mitochondria were isolated by the Polytron and Nagarse protocol of Palmer *et al.* (*16*). Protein was measured by the biuret method (*17*).

The mitochondrial suspension was diluted to 20 mg/ml with isolation medium, and conical plastic NMR tubes were filled with 0.45 ml of the suspension and centrifuged at 10,000*g* for 10 minutes. The supernatant fraction was aspirated, and any liquid adhering to the inside of the tube was absorbed with a cotton

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swab. Each tube was kept at 4°C until analyzed by NMR spectroscopy within 4 hours after the final centrifugation. As an indication of the stability of the preparation, NMR spectra from several pellets analyzed during the first hour did not vary significantly when reanalyzed 4 hours later. Water content of each sample was determined gravimetrically after the NMR measurements; samples were weighed before and after 24-hour incubation at 104° ± 1.0°C. All NMR measurements were made at 30.3 MHz with a Bruker SXP pulse spectrometer equipped with a 12-inch Varian electromagnet, and the spin-echo procedure was utilized (*18*). The sample temperature was 27° ± 0.2°C, and all data were computer-analyzed with a curve-fitting program (*19*).

After 5 minutes of occlusion the T_1 and T_2 water proton relaxation times decreased 15 and 30 percent, respectively, and then remained relatively constant for 25 minutes (Fig. 1). Then the relaxation times decreased again, and after 60 minutes of occlusion the T_1 and T_2 times were 30 and 50 percent of the control times, respectively. The hydration of the mitochondria (grams of H_2O per gram of dry tissue) also decreased with a similar time course (Fig 1).

To explain these decreases, we had to consider the possibility that mitochondrial fragility increased as the ischemic interval lengthened (*1*). For example, the final centrifugation at 10,000*g* in small NMR tubes may have destroyed greater numbers of mitochondria that were prepared from hearts that had endured longer ischemic periods. Numerous studies have indicated that the relaxation times are often dependent on the volume of water in the preparation. We therefore designed a series of experiments to detect any changes in extrasubcellular fluid volume in the mitochondrial pellet that might be indicative of damage during the last centrifugation. We found that the distribution of [^{14}C]inulin was essentially the same in mitochondrial pellets prepared from normal and ischemic myocardium (Table 1). The average volume occupied by [^{14}C]inulin was 88.5 μl in mitochondrial pellets from control hearts and 93 μl in mitochondrial pellets from ischemic hearts. Therefore, the extramitochondrial volume in the two preparations was very similar, and the magnitude of the difference between the hydration of the control and ischemic pellets cannot be explained in terms of extramitochondrial H_2O.

Another possible explanation of the decrease in mitochondrial pellet T_1 and T_2 relaxation times as ischemia progresses is that the isolation protocol selectively extracts certain types of mitochondria that contain different amounts of H_2O. We investigated this possibility by deliberately extracting two populations of mitochondria, using a combination of the Polytron procedure and treatment with the proteinase Nagarse (*16, 20, 21*). This enzyme causes the release of an intermyofibrillar pool of mitochondria that is not extracted by the Polytron method, which instead causes the release of predominately subsarcolemmal mitochondria (*16*). As is shown in Table 2, the T_1 and T_2 values differ depending on which mitochondrial fraction is isolated. The T_1 and T_2 values of non-Nagarse preparations (primarily subsarcolemmal mitochondria) were substantially higher than the corresponding values for the Nagarse preparation containing myofibrillar mitochondria; this was seen in both the control and ischemic preparations. However, in the ischemic preparation, all relaxation times were lower than those of the control preparations. Although it is likely that the subsarcolemmal mitochondria are more hydrated than the myofibrillar mitochondria, both types, when isolated from ischemic myocardium, show decreased water proton relaxation times. Thus it appears that the ischemic changes are independent of the population of mitochondria. The data suggest that the early changes in ischemic mitochondria are accompanied by dramatic changes in water distribution.

During brief period of ischemia (under 20 minutes), mitochondrial swelling has not been consistently observed. Recent evidence suggests that there are profiles of fusing mitochondria, condensation of matrix material, and increase in amount of dense material and granules (*22*). If swelling does occur, there may be a structural rearrangement of the mitochondrial matrix, since the mitochondrial pellets contain less water as ischemia progresses. The loss of protein and free H_2O and the gain of lipid molecules or granular material should result in lower hydration values and lower T_1 and T_2 values, and this may be one pos-

Table 1. Mitochondrial content of [^{14}C]inulin and estimate of inulin volume. A determination of extramitochondrial volume changes in the pellets was made by adding [^{14}C]inulin carboxyl to mitochondrial preparations just before final centrifugation in the NMR tubes. After centrifugation, 200 μl of the supernatant and of the resuspended pellet were counted for radioactivity. The pellets contained 29 and 31 percent of the total counts in control mitochondria for the Polytron and Nagarse preparations, respectively, and 31 and 30 percent of the total counts in ischemic mitochondria.

Mitochondrial fraction	Polytron		Nagarse	
	Volume (μl)	[^{14}C]-inulin (count/min)	Volume (μl)	[^{14}C]-inulin (count/min)
Control				
Supernatant		6391		5698
Pellet	90	2672	87	2592
Total		9063		8290
Ischemic				
Supernatant		6144		6406
Pellet	94	2792	92	2733
Total		8936		9139

Table 2. Water proton T_1 and T_2 relaxation times for two populations of mitochondria prepared with the Nagarse or Polytron procedure. Each value is the mean ± standard error for three to six pellets from a nonischemic (control) dog or from a dog made ischemic for 15 minutes.

Group	T_1 (msec)	T_2 (msec)
Polytron (mannitol)		
Control	1135 ± 20	110 ± 4
Ischemic	915 ± 11	88 ± 3
Nagarse		
Control	930 ± 6	71 ± 5
Ischemic	684 ± 8	48 ± 2

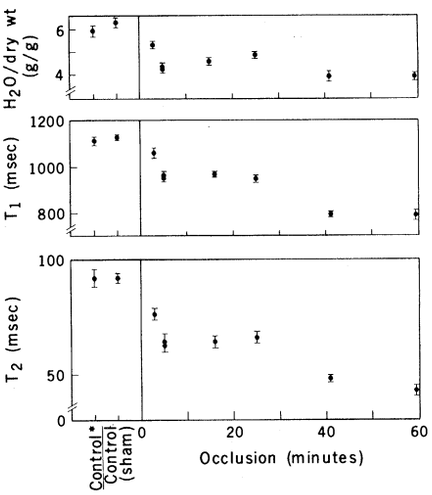

Fig. 1. Water proton relaxation times and hydration values for mitochondria isolated from control and acutely ischemic dog hearts. All data points are means ± standard errors. Control* data are for 11 mitochondrial pellets from five nonischemic dogs whose hearts were excised immediately under sodium pentobarbital anesthesia (30 mg/kg). Control (sham) data are for seven mitochondrial pellets prepared from two dog hearts, the coronary arteries of which were ligated but not occluded. Occlusion (3 to 59 minutes) data are for three to six mitochondrial pellets analyzed from a single dog; double points representing two dogs are shown at 5 minutes of occlusion.

sible explanation. It has been suggested that myocardial ischemia induces alterations in membrane lipids, lipid fluidity, and altered distribution pattern of intramembranous particles in the mitochondrial membranes (*23*, *24*). Certainly, changes in the hydration and physical properties of water (as measured by proton relaxation times) in mitochondria occur early in the response to ischemic insult. These observations are well correlated with those of some previous studies in which we demonstrated impairment of fatty acid oxidation and accumulation of long-chain acyl ester intermediates early in ischemia.

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Electrical Activity in an Exocrine Gland: Optical Recording with a Potentiometric Dye

Abstract. *Propagated action potentials in the salivary gland of a freshwater snail were detected by optical means. Voltage-dependent absorption changes from acinar cells stained with a merocyanine-oxazolone dye faithfully reproduced the time course of electrical activity in this tissue. Such signals may provide a useful tool for the study of endocrine tissue as well.*

A number of exocrine and endocrine gland cells display regenerative voltage changes in response to neural or hormonal stimulation or both (*1*). It is believed that these regenerative electrical events are causally related to the coupling of excitation to secretion (*2*), although the underlying mechanisms remain to be elucidated. Attempts to understand the excitation-secretion process have been impeded in part by the relatively small size of gland cells, which makes intracellular microelectrode recording very difficult (*3*). Electrophysiological study of glands might therefore be facilitated if an alternative technique for monitoring membrane potential could be developed that did not require cell impalement.

Vital dyes that respond optically to rapid changes in membrane potential have been used to monitor electrical activity in a variety of systems including squid giant axons (*4*), invertebrate central neurons (*5*, *6*), and vertebrate cardiac (*7*) and striate (*8*) muscle. To our knowledge, no attempt has been made to utilize these potentiometric probes in any electrophysiological studies of glandular tissue (*9*–*11*). We now report that propagated action potentials in a stained invertebrate salivary gland generate optical "spikes" that are clearly visible in a single oscilloscope sweep (*12*).

To establish the feasibility of an approach to gland electrophysiology based on optical measurement of membrane potential, we focused our efforts on the bilaterally paired salivary glands from the freshwater snail *Helisoma trivolvis*. This exocrine gland was selected since it was recently shown that individual acinar cells generate large overshooting action potentials when electrically or neurally stimulated and that these regenerative responses are propagated throughout the gland because of extensive electrotonic coupling between acinar cells (*13*). Another important technical advantage of this preparation is the relative ease with which intracellular microelectrode recordings can be made from individual acinar cells. This allows changes in the optical signal to be compared directly with membrane potential changes recorded intracellularly.

Surgically isolated snail salivary glands were stained for 20 minutes with a merocyanine-oxazolone dye (NK 2367, Nipon Kankoh-Shikiso Kenkyusho Co., Okayama, Japan) (*6*). To measure extrinsic voltage-dependent changes in absorption, an extended region of the gland was viewed under a water-immersion objective (×20; 0.33 numerical aperture) on a Reichert Zetopan binocular microscope. An adjustable slit in the objective image plane was positioned so that only light that had passed through a selected region (0.05 to 0.1 mm^2) of the gland reached a silicon diode photodetector (PV 444, EG & G, Inc.) (*6*).

Relatively large optical signals could be obtained from glands that had been superfused or luminally perfused with Ringer solution (*14*) containing the dye (Fig. 1). In Fig. 1A, an optical signal from a gland that had been luminally perfused with dye at 200 μg/ml is compared with the signal from another gland that had been superfused with dye at 25 μg/

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