third largest is centered on an obvious geothermal feature and volcanic center. An Rn peak and $Hg^0$ flush zone lies over the area of the greatest rate of deformation, the epicentral region of the January 1983 seismic swarm, and a postulated dike. This suggests that ground gas anomalies are reflecting deep-seated perturbations and not near-surface hydrology (*20*). The magma bodies are postulated to reach to 4.5 km (average depth, approximately 8 km) below the caldera floor (*17*) and the dike to 3 km (*19*). The near-surface hydrologic system extends to depths of 0.5 to 1.5 km and is dominated by flow from northwest to southeast (*20*).

Geophysical, geodetical, and geochemical data indicate that magmatic resurgence has taken place in Long Valley. Various gas signatures indicate an addition of magmatic gas to the geothermal system (*21*). The data are consistent with a model of dike emplacement triggering an increase in the flux of rising gases. Some gases may be directly magmatic but most are probably swept from pore spaces by the convection-induced "plume." The relatively mobile and/or inert gases H, He, and Rn create the surface anomalies observed, but $Hg^0$ is apparently fixed at depth. Mixing with cold, oxidized, or strongly acidic surface waters would promote HgS precipitation (*13*), or relatively sulfur-rich plume gases could cause formation of $Hg^0$ complexes. At Kilauea caldera (*3*), strongly acidic caldera soils were high in Rn and low in $Hg^0$, but peripheral areas had less acidic soil, lower Rn, and high $Hg^0$. Casa Diablo, the principal caldera fumarole, has both $Hg^0$ and Rn peak values. Apparently $Hg^0$ does not have a sufficient rise time to be fixed. The broad-scale Rn and $Hg^0$ anomalies reflect a steady-state convective geothermal pattern over older degassed magma. The $Hg^0$ anomaly of the Inyo craters (*2, 7*) is associated with background Rn or negative anomalies, indicating that no active plume exists there.

One may conclude that combined $Hg^0$ and Rn analysis offers an additional tool for understanding the process of magmatic resurgence taking place at depth. The large-scale geothermal signature of $Hg^0$ and Rn has been significantly perturbed. The model proposed can explain these perturbations in terms of a plume of gas over the newly intruded dike postulated on the basis of geophysical and geodetical evidence. A relatively small area of the caldera has been confirmed as the site of rising, possibly gas-rich, new magma.


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22. I thank the Terradex Corporation, the National Geographic Society (grant 2685-83), C. Williams, and the Department of Earth Sciences at Dartmouth College for financial support; K. Hudnut, E. Lawrence, and J. Lytle for their work; the Sierra Nevada Aquatic Research Laboratory for use of facilities; and M. Clark and the U.S. Forest Service for access to the study area.

5 February 1985; accepted 30 May 1985


# Cytosolic-Free Calcium Transients in Cultured Vascular Smooth Muscle Cells: Microfluorometric Measurements


Abstract. *Microfluorometric recordings were made of changes in the concentration of cytosolic-free calcium in cultured rat vascular smooth muscle cells treated with quin 2, an intracellularly trapped dye, under several conditions. Nitroglycerin decreased calcium in both the presence and absence of extracellular calcium and strongly and progressively decreased the extent of transient increases in calcium induced by repeated applications of caffeine in the absence of extracellular calcium. Therefore nitroglycerin probably decreases cytosolic-free calcium by accelerating the extrusion of calcium through the sarcolemmal membrane.*



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Contraction of vascular smooth muscle seems to be regulated by changes in the concentration of cytosolic $Ca^{2+}$. However, changes in $Ca^{2+}$ during contractile activity of vascular smooth muscle cells (VSMC's) have not been measured, except in a few studies in which the $Ca^{2+}$ indicator aequorin was administered intracellularly by microinjection or by making cells hyperpermeable (*1, 2*). We report here the successful recording of $Ca^{2+}$ transients for a given small area (<1 $\mu m^2$) in the cytosol of VSMC's by using the fluorescent, $Ca^{2+}$-sensitive dye quin 2, applied physiologically as the acetoxymethyl ester (quin 2/AM) (*3*). Nitroglycerin induces vasodilation, but the effect of nitroglycerin on the calcium homeostasis of VSMC's is debatable (*4–9*). Using the microfluorometry of quin 2, we investigated the effect of nitroglycerin on the concentration of cytosolic $Ca^{2+}$ in VSMC's.

Primary cell cultures of rat aortic medial VSMC's were established (*10*). On days 5 to 6, just before reaching confluence, the cultured cells on Lux chamber slides were incubated with growth medium containing 50 $\mu M$ quin 2/AM (DOTITE) for 60 minutes at 37°C and then washed three times with physiological saline solution (PSS) at 25°C to remove dye in the extracellular space. The "normal" PSS (*p*H 7.4 at 25°C) consisted of 135 m*M* NaCl, 5 m*M* KCl, 1m*M* $CaCl_2$, 1m*M* $MgCl_2$, 5.5 m*M* glucose, and 10 m*M* Hepes. A high-potassium version of this solution was prepared by replacing NaCl with KCl isosmotically. Unless otherwise indicated, the experiments were carried out in normal PSS at 25°C, and high cellular viability (>95 percent)

was maintained during each procedure, as assessed by the trypan blue exclusion test (*10*).

Quin 2 stained the cytosol; fluorescence was never observed in the extracellular space (Fig. 1A). In addition, myofilaments were not stained with quin 2 (Fig. 1, A and B). These observations are consistent with the earlier finding that quin 2 remains free in the cytoplasm and is not bound to macromolecules (*3*). Given the open communication of the nuclear matrix with the cytosol through nuclear pores, it is plausible that the concentration of $Ca^{2+}$ in the nucleus is about equal to that in the cytosol (*11*). Since the nuclei did not fluoresce, quin 2 was probably not sequestered in them. Changes in the concentration of extracellular $K^+$-induced concentration-dependent increases in fluorescence intensity in the cytosol, but this was not the case in either the nuclei of quin 2–treated cells or in the cytosol of cells without quin 2. The fluorescence of these spots remained unchanged (Fig. 2). Under the resting condition (5 m*M* extracellular $K^+$), there was no difference in fluorescence between cells in which dye was incorporated 5 minutes before the measurements and cells in which dye was incorporated 60 minutes before the measurements, indicating that the concentration of quin 2 was stable in the cytosol.

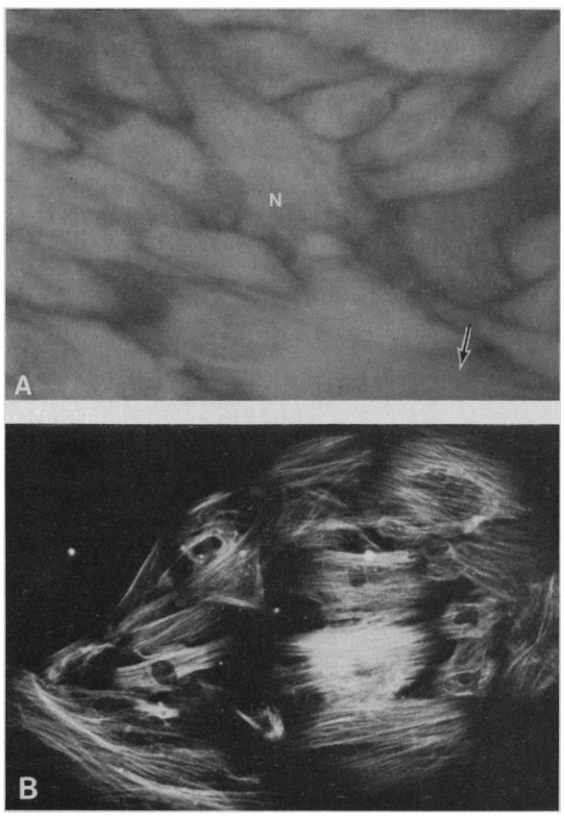


Fig. 1. (A) Fluorescence photomicrograph of a primary culture of VSMC's treated with quin 2, as obtained with a fluorescence microscope (model standard 18, Zeiss) equipped with a water-immersion objective system without a cover glass (Plan-Neofluor 63, Zeiss), an exciter filter (UV-D35, Toshiba), a chromatic beam splitter (FT395, Zeiss), and barrier filters (LP470 and KP560, Zeiss). The cells were excited at 350 nm and analyzed at wavelengths between 470 and 560 nm. Marked fluorescence was observed in the cytosol, but nuclei (N) and myofilaments [arrow; see also (B)] stained negatively. When cells had not been treated with quin 2, cellular components were not visible in the microscopic field. The microfluorometry was carried out within 2 seconds after exposure of the cells to the excitation light, since the fluorescence intensity of the cytosol of all the cells in the field was the same during this period (standard deviation, <5 percent). The rate of reduction in cytosolic fluorescence by photobleaching varied from cell to cell. Since it was necessary to expose the cells to the excitation light for about 30 seconds to obtain a photograph with a sharp contrast, the cytosolic fluorescence intensity in some cells is partially reduced. (B) Myofilaments of rat aortic smooth muscle cells in primary culture and stained with fluorescein isothiocyanate–labeled antibodies to smooth muscle myosin. The nucleus (negative staining) and myofilaments (positive staining) correspond to those negatively stained in (A). Direct immunofluorescence staining was carried out as described by Yamamoto *et al.* (*10*).

Nitroglycerin ($10^{-5}$*M*) rapidly and markedly reduced the increased cytosolic $Ca^{2+}$ induced by 55 m*M* extracellular $K^+$ (Fig. 3A). When nitroglycerin was washed out in the presence of 55 m*M* extracellular $K^+$, fluorescence rapidly returned to a high level, although it was significantly lower than the value obtained when 55 m*M* $K^+$ was first added. During the experiment, neither contraction nor swelling of cells with or without quin 2 was noted, as determined by phase-contrast microscopy at ×400. As shown in Fig. 3B, the inhibition by nitroglycerin was dose-dependent, and the minimum concentration of nitroglycerin required to reduce cytosolic $Ca^{2+}$ was $10^{-7}$*M*. Furthermore, nitroglycerin decreased cytosolic $Ca^{2+}$ in cells in the resting state in a dose-dependent manner [$F(4, 208) = 78.2$, $P < 0.05$]. These results indicate that nitroglycerin reduces cytosolic $Ca^{2+}$ not only in stimulated (high extracellular $K^+$) VSMC's, but in nonstimulated (5 m*M* extracellular $K^+$) VSMC's as well.

When VSMC's were incubated in $Ca^{2+}$-free PSS with 2 m*M* EGTA and 5 m*M* $K^+$, cytosolic $Ca^{2+}$ decreased gradually, reaching a steady state in 5 minutes (Fig. 4A). It took 1.73 ± 0.31 minutes (mean ± standard error, $n = 6$ experiments) for the quin 2 signal to fall to $1/e$ (time constant), and the steady-state level (asymptote value) was 17.7 ± 0.8 fluorescence units ($n = 6$), as determined by the nonlinear least-squares approximation with a correlation coefficient of at least 0.95. When $10^{-5}$*M* nitroglycerin was added to the incubation medium, the decline in cytosolic $Ca^{2+}$ was more rapid and reached a steady state in 2 minutes: the time constant and the steady-state level were 0.78 ± 0.02 minutes ($n = 6$) and 13.7 ± 0.6 fluorescence units ($n = 6$), respectively, or significantly lower than the values obtained in the absence of nitroglycerin (*P*

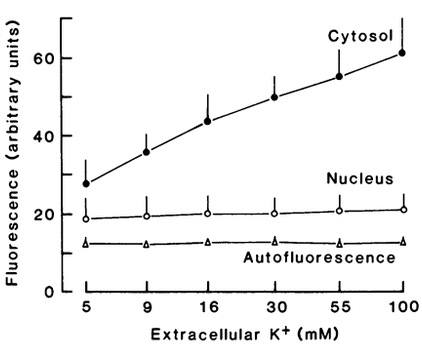


Fig. 2. Effect of extracellular $K^+$ concentration on the fluorescence signal from the cytosol (●) and nucleus (○) in quin 2–treated VSMC's and from cytosol in untreated cells (△). The VSMC's appeared as hills and valleys in the culture dish. Cells in the valleys were selected for the optical measurement to avoid possible fluctuations in the light signals produced by a pileup of the cells. Each cell was exposed to the excitation light only once for not longer than 2 seconds to avoid photobleaching. By using a 50-μm pinhole diaphragm (Zeiss) in the light axis and a photon-counting system with a photomultiplier (HTV R928, Zeiss) and a digital voltmeter (Zeiss), the fluorescence in a small circular area (<1 $\mu m^2$) could be measured. To read the fluorescence intensity, an input-output calculator (model 97S, Hewlett-Packard) was used. In each cell, a spot (<1 $\mu m^2$) 3 μm from the nucleus was chosen for the measurement of the cytosolic fluorescence to avoid possible fluctuation caused by the uneven thickness of the cell. Data are means ± standard deviations; at least 27 cells were used for each determination. Relative fluorescence was determined by assuming the fluorescence intensity in the cytosol of cells exposed to 135 m*M* extracellular $K^+$ to be 100 and that of the unexposed cells (background autofluorescence) to be 0.

$< 0.05$, paired Student's $t$-test). These results indicate that nitroglycerin actively decreases cytosolic $Ca^{2+}$ in VSMC's, even in the absence of extracellular $Ca^{2+}$.

Caffeine increases cytosolic $Ca^{2+}$ by releasing it from intracellular storage sites (*4*). To investigate the effect of nitroglycerin on $Ca^{2+}$ release from or uptake into the intracellular storage sites, we repeatedly applied 10 m*M* caffeine to VSMC's in $Ca^{2+}$-free PSS containing 2 m*M* EGTA. The level of cytosolic $Ca^{2+}$ was reduced progressively by each application of caffeine, and the fifth application produced only a minute cellular response in the absence of nitroglycerin (Fig. 4, A and B). The degree of fluorescence in the nucleus remained unchanged in the presence of caffeine.

The first application of caffeine in the presence of $10^{-5}M$ nitroglycerin with $Ca^{2+}$-free PSS containing 2 m*M* EGTA also induced a transient increase in cytosolic $Ca^{2+}$ to the level attained without nitroglycerin, suggesting that nitroglycerin has no effect on the caffeine-sensitive release of $Ca^{2+}$ from the intracellular storage sites. However, in the presence of nitroglycerin, the transient increase in cytosolic $Ca^{2+}$ caused by the second application of caffeine was reduced, and there was little or no increase in cytosolic $Ca^{2+}$ after the third and subsequent applications. The finding that nitroglycerin markedly decreases the transient increase in cytosolic-free $Ca^{2+}$ induced by the second and the subsequent applications of caffeine in the absence of extracellular $Ca^{2+}$ indicates that cytosolic $Ca^{2+}$ may be actively extruded from the cell as a result of nitroglycerin treatment; the amount of $Ca^{2+}$ restored to the intracellular storage sites for the subsequent release by caffeine is markedly and progressively reduced. Thus, nitroglycerin may decrease cytosolic $Ca^{2+}$ in VSMC's by accelerating extrusion of $Ca^{2+}$ through the sarcolemmal membrane.

The vasodilation induced by nitroglycerin has been attributed to acceleration of $Ca^{2+}$ extrusion (*4*), suppression of $Ca^{2+}$ release from the intracellular storage sites, stimulation of $Ca^{2+}$ uptake into the sites (*5, 6*), or a combination (*7*). Harder *et al.* (*8*) suggested that nitroglycerin blocks the $Ca^{2+}$ influx, and Kreye and Schlicker (*9*) indicated that nitroglycerin has no effect on the $Ca^{2+}$ pump in vascular microsomal fractions. Using quin 2–microfluorometry, we recorded changes in cytosolic $Ca^{2+}$ in VSMC's and found that nitroglycerin actively decreases $Ca^{2+}$ both in the presence and in the absence of extracellular $Ca^{2+}$. We

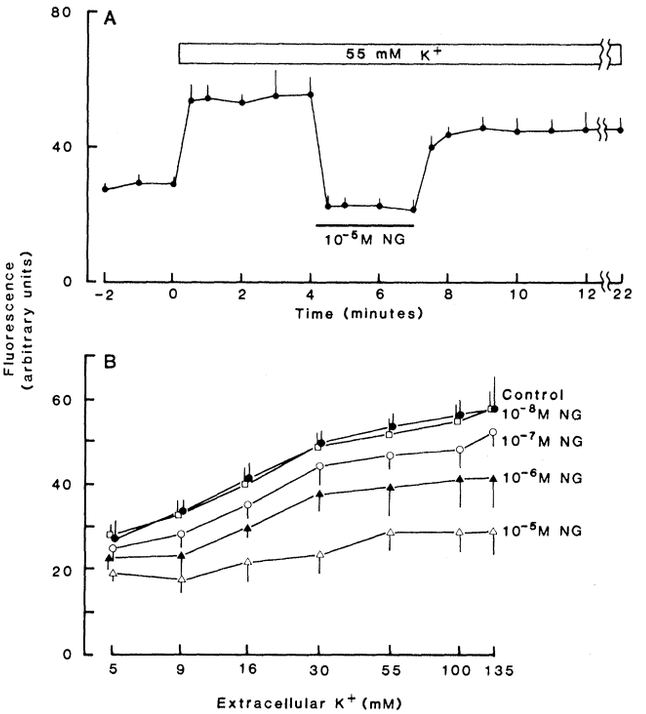


Fig. 3. Effects of nitroglycerin (NG) and extracellular $K^+$ on cytosolic $Ca^{2+}$. (A) Effect of $10^{-5}M$ nitroglycerin (indicated by bar) on the increase in cytosolic $Ca^{2+}$ induced by 55 m*M* $K^+$. Data are means ± standard deviations; at least eight cells were used for each determination. After addition of 55 m*M* $K^+$ and $10^{-5}M$ nitroglycerin, fluorescence intensities reached steady-state values within 30 seconds. (B) Dose-dependent effect of nitroglycerin on the increased cytosolic $Ca^{2+}$ induced by various concentrations of $K^+$. Microfluorometry was carried out 5 minutes after incubation medium containing various concentrations of $K^+$ and nitroglycerin was added to the cells. Nitroglycerin was not added to control cells (●). Data are means ± standard deviations; at least 40 cells were used for each determination.

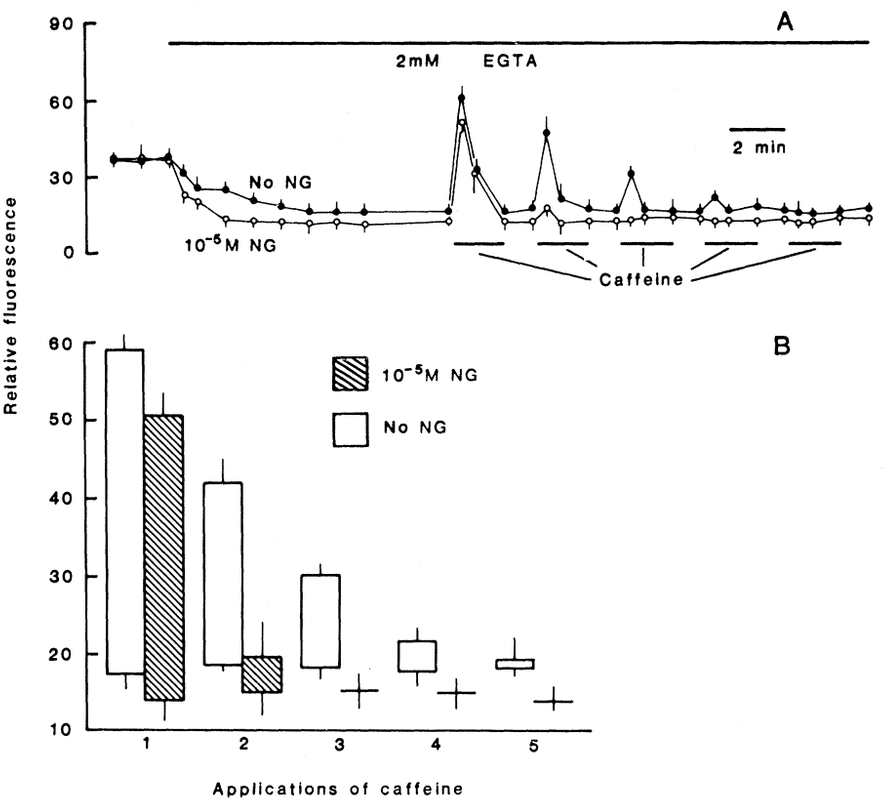


Fig. 4. Effect of repeated applications of 10 m*M* caffeine on cytosolic $Ca^{2+}$ in VSMC's in $Ca^{2+}$-free PSS containing 2 m*M* EGTA in the absence (●) and presence (○) of $10^{-5}M$ nitroglycerin. (A) Representative results, expressed as means ± standard deviations; at least eight cells were used for each determination. A gradual decrease in cytosolic $Ca^{2+}$ in the absence of extracellular $Ca^{2+}$ was enhanced by nitroglycerin. (B) Mean values for six experiments carried out as described for (A). The bottom and top of each column indicate the fluorescence intensities just before and 30 seconds after each application of caffeine, respectively. In the presence of $10^{-5}M$ nitroglycerin the caffeine-induced transient increase in fluorescence (equivalent to the increase in cytosolic $Ca^{2+}$) was markedly reduced by the second and subsequent applications of caffeine. Vertical lines indicate standard deviations.

believe that these decreases may be due to the acceleration in the extrusion of $Ca^{2+}$ through the sarcolemmal membrane.


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12. We thank H. Yamamoto and M. Hasegawa for help with the experiments, K. Sunagawa and Y. Harasawa for advice on the statistical analysis, A. Nishi and N. Hayashi for secretarial services, and M. Ohara for comments on the manuscript. We are also grateful to Nippon Kayaku Company for supplying nitroglycerin. Supported in part by grants-in-aid for scientific research 58570395 and 59440044 from the Ministry of Education, Science, and Culture, Japan.

30 January 1985; accepted 17 May 1985


## Seal Lungs Collapse During Free Diving: Evidence from Arterial Nitrogen Tensions


Abstract. *Arterial blood nitrogen tensions of free-diving Weddell seals (*Leptonychotes weddelli*) were measured by attaching a microprocessor-controlled blood pump and drawing samples at depth to determine how these marine mammals dive to great depths and ascend rapidly without developing decompression sickness. Forty-seven samples of arterial blood were obtained from four Weddell seals during free dives lasting up to 23 minutes to depths of 230 meters beneath the sea ice of McMurdo Sound, Antarctica. Peak arterial blood nitrogen tensions of between 2000 and 2500 millimeters of mercury were recorded at depths of 40 to 80 meters during descent, indicating that the seal's lung collapses by 25 to 50 meters. Then arterial blood nitrogen tensions slowly decreased to about 1500 millimeters of mercury at the surface. In a single dive, alveolar collapse and redistribution of blood nitrogen allow the seal to avoid nitrogen narcosis and decompression sickness.*



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To learn how marine mammals tolerate deep diving and rapid decompression, we studied the Weddell seal, which can dive to great depths (500 m) and ascend rapidly without encountering either nitrogen narcosis or decompression sickness (the bends) (*1*). Others have described common morphological adaptations of deep diving mammals (*2*) and obtained radiographic studies of restrained Weddell seals in hyperbaric chambers (*3*) and photographs of dolphins taken at depth with analysis of exhaled air after dives (*4*). These studies provided indirect evidence to suggest a mechanism of alveolar collapse at depth to protect these animals from the high arterial blood nitrogen tensions ($P_aN_2$) which would have otherwise occurred with compression. Measurements of muscle $PN_2$ in dolphins at the surface after repeated dives (*5*) and of $P_aN_2$ during forced chamber diving of seals (*6*), suggested that the rise in $P_aN_2$ is limited to 2000 to 3000 mmHg. However, no direct measurements of $P_aN_2$ have been made on freely diving animals to verify these estimates. Since both circulatory reflexes and respiratory responses are markedly different between seals that are free swimming and those in forced diving (*7*), we measured blood $P_aN_2$ during voluntary diving to learn how Weddell seals safely cope with rapid compression to and decompression from high pressures.

Four male Weddell seals (350 to 420 kg) were studied near McMurdo Station, Antarctica (77°S, 166°E). Seals were captured at nearby colonies and sledged to a field research site at which two holes, 1 m in diameter, had been drilled through the 3-m thick ice of McMurdo Sound. The field site was chosen to be at least 6 km from the nearest natural crack in the ice sheet so that when a seal was released into the water, it was obliged to return to the field site to breathe. An observation hut with a hole in its floor was placed over one of these holes; the other allowed the seal to enter and exit from the water. Instruments were placed on anesthetized seals at the field site, a catheter was introduced into a foreflipper artery and advanced to the aorta for blood sampling, and electrocardiogram (ECG) leads were placed for heart-rate monitoring. The ECG leads were connected to an 8-bit microcomputer backpack system (*8*) which also controlled a pressure-resistant submersible peristaltic pump that was used to withdraw blood from the arterial catheter. The microcomputer and blood sampling equipment were glued to the seal's dorsal fur. After 3 to 5 days of monitoring, the arterial catheter and ECG were removed, and the animals were released and were returned to their native colonies.

During the experiment, the instrumented seal was released and surfaced to breathe at the hole in the floor of the observation hut. This hut contained a Zenith Z-90 computer to which data on heart rate, diving depth, swimming velocity, and aortic blood temperature were transmitted while the seal rested at the surface (*8*). The backpack computer was programmed to pump blood into vinyl bags for 90 seconds at indicated depths during descent or ascent, or after a specified diving time. An alternative technique consisted of sequentially filling a sampling device containing nine plastic syringes. Each syringe was filled for 30 seconds; 5 ml of each blood sample was transferred to a glass syringe and, within 2 hours of sampling, injected into a Van Slyke apparatus for the determination of inert gas content (*9*). The few blood samples that could not be analyzed immediately were stored at 0°C. Serial determinations of inert gases from single samples revealed a maximum loss of inert gas content of 8 to 10 percent after 2 hours, presumably due to diffusion of nitrogen through the 1.0-mm thick sampling bag wall.