(Academic Press, New York, 1968), vol. 3, p. 137.
17. R. Pearcy, A. Harrison, H. Mooney, O. Björkman, *Oecologia* **17**, 111 (1974).
18. O. Björkman, R. Pearcy, A. T. Harrison, H. A. Mooney, *Science* **172**, 786 (1971).
19. C. C. Black, *Adv. Ecol. Res.* **7**, 87 (1971).
20. The leaf area index is the surface area of leaves in a canopy per unit surface area of ground.
21. D. N. Baker and R. Musgrave [*Crop Sci.* **4**, 127 (1964)], as cited in Zelitch (*8*, p. 279).
22. D. N. Baker, *ibid.* **5**, 53 (1965).
23. H. A. Mooney and E. L. Dunn, *Am. Nat.* **104**, 447 (1970).
24. R. S. Loomis and W. A. Williams, *Crop Sci.* **3**, 67 (1963).
25. Supported by NSF grants BMS 75-19510 and BMS 75-20640. We thank the National Park Service personnel at the Death Valley National Monument for their considerable assistance. This is Carnegie Institution of Washington Department of Plant Biology Publication No. 576.

27 May 1976; revised 23 July 1976

Bounds on "Bound Water": Transverse Nuclear Magnetic Resonance Relaxation in Barnacle Muscle

Abstract. *Relatively mobile protons that do not exchange with D_2O exist in barnacle muscle cells. These are not part of the nonfreezing "bound water" that does exchange. Ninety-seven percent of the muscle water exhibits a single transverse relaxation time of 35 milliseconds: one water molecule per thousand, which is briefly and irrotationally bound, will produce the observed relaxation properties.*

Most of the water in muscle tissue has a nuclear magnetic resonance (NMR) transverse relaxation time (T_2) of about 1/50 that of the pure liquid, an effect known for 20 years (*1*) without an interpretation of consensus (*2*, *3*), despite the proposed use of NMR relaxation measurements for cancer diagnosis (*4*, *5*). Nuclear magnetic resonance data in rat (*3*) and frog (*6*) skeletal muscle suggest that a small observed proton fraction with a T_2 of about 1 msec exchanges at an intermediate rate (*7*, *8*) with the major portion of cell water, and thus lowers the relaxation time of this major fraction below that of pure water. We report here that single muscle cells from the giant barnacle, *Balanus nubilus*, also exhibit a small proton fraction with a millisecond T_2. However, within experimental limits, these protons do not exchange with D_2O in times as long as 24 hours, and cannot be the cause of the proton relaxation in the major portion of tissue water. On the other hand, our proton and deuteron T_2 values for the major portion of the muscle water do fit an intermediate exchange rate model that requires only 0.1 percent of the water molecules to be in an "irrotationally bound" state at room temperature. The nonfreezing water protons that we observe at −34°C [attributed to "bound water" in studies of protein solutions and tissues (*9*, *10*)], do exchange with D_2O and are clearly different from the nonexchanging protons that we observe in these cells.

In this study, single muscle cells were dissected from the depressor muscles of the barnacle, blotted on filter paper, and gently placed into an NMR sample tube. Some specimens were partially deuterated by immersion for 2 to 3 minutes in artificial seawater (Instant Ocean) made from pure D_2O (*11*), or by placing an ani-

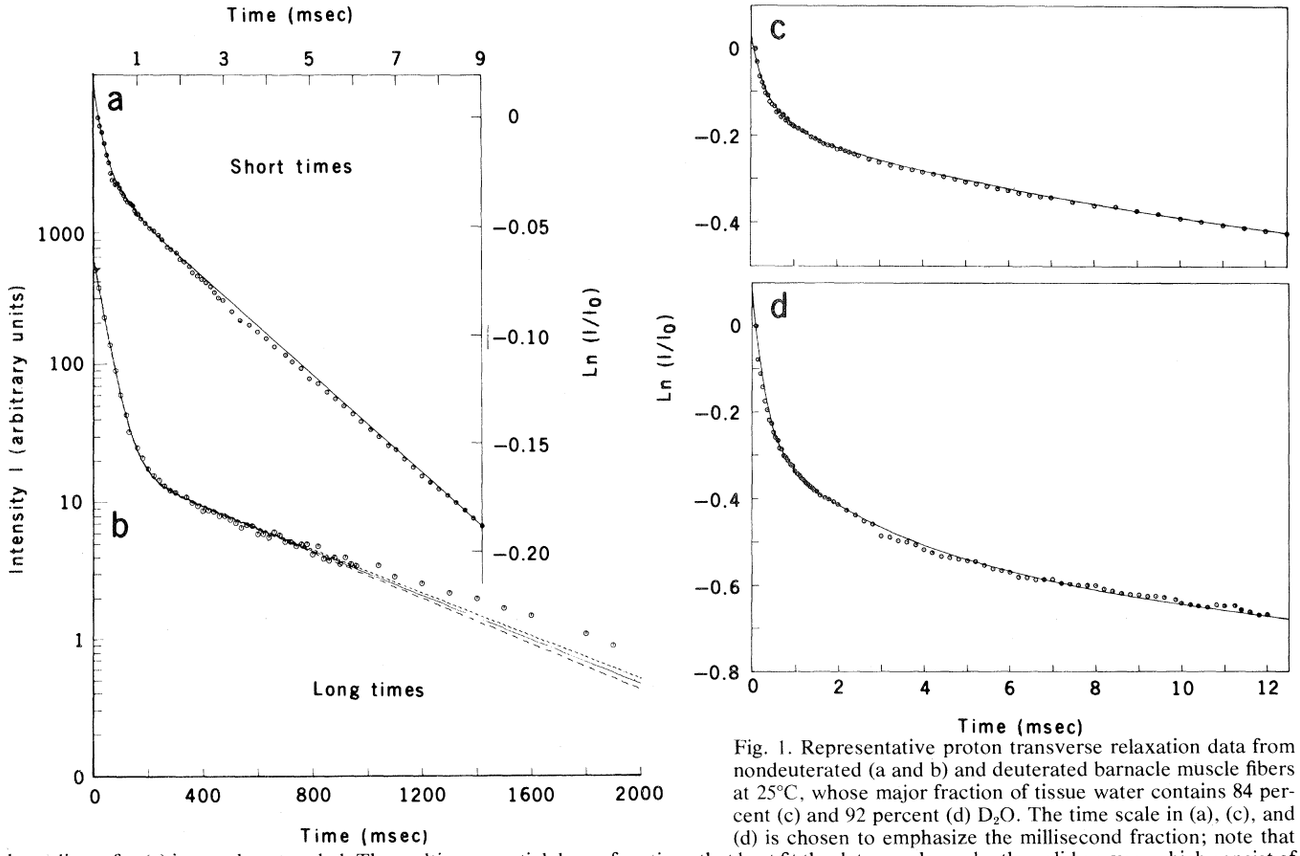

Fig. 1. Representative proton transverse relaxation data from nondeuterated (a and b) and deuterated barnacle muscle fibers at 25°C, whose major fraction of tissue water contains 84 percent (c) and 92 percent (d) D_2O. The time scale in (a), (c), and (d) is chosen to emphasize the millisecond fraction; note that the ordinate for (a) is greatly expanded. The multiexponential decay functions that best fit the data are shown by the solid curves, which consist of the sum of two (a and b) or three (c and d) exponential functions. For clarity, many of the data points are omitted. In these experiments, the CPMG pulse sequence was employed, with the pulse spacing 2τ equal to 50 μsec (a, c, and d) or 500 μsec. The fitted equations are: (a) $I/I_0 = 0.051 \exp(-t/0.80) + 0.949 \exp(-t/56.4)$; (c) $I/I_0 = 0.179 \exp(-t/0.39) + 0.099 \exp(-t/4.3) + 0.722 \exp(-t/84.5)$; and (d) $I/I_0 = 0.320 \exp(-t/0.29) + 0.184 \exp(-t/3.28) + 0.496 \exp(-t/102)$ where t is the time in milliseconds.

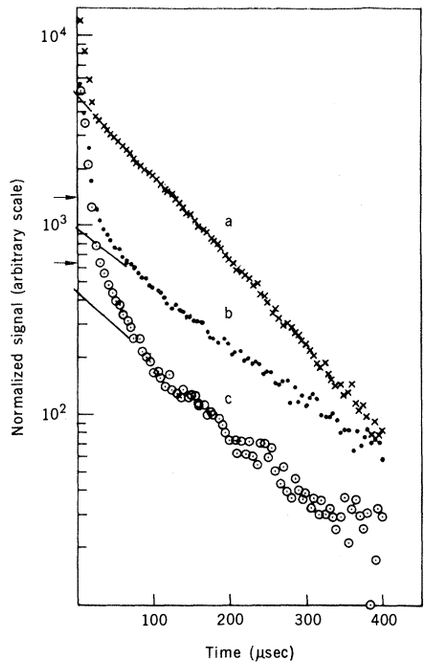

Fig. 2. Proton transverse relaxation data in nondeuterated (a) and deuterated barnacle muscle fibers whose major fraction of tissue water contains 71 percent D_2O (b) and 87 percent D_2O (c), at −34°C. The data had been normalized by the weight of the tissue. The arrows indicate the expected amplitude of the nonfreezing water signal at time $t = 0$, assuming that all of this water exchanges with the deuterated seawater.

mal overnight in seawater containing about 20 percent D_2O (*12*). Since each muscle cell weighs about 30 mg, one nondeuterated fiber (or four or five deuterated fibers) produces an adequate NMR signal, with less of the signal arising from extracellular fluid than is the case with vertebrate muscle tissue. The proton transverse relaxation was measured at 60 Mhz with a Bruker model SXP pulsed NMR spectrometer, and the signal was recorded by either a Nicolet 1074 or a Biomation 610 signal averager. Depending on the required time resolution, either the free induction decay was recorded, or the Carr-Purcell-Meiboom-Gill (CPMG) experiment was performed (*13*). Deuteron transverse relaxation was similarly recorded at 9.2 Mhz. Selected transverse magnetization data were fitted to a sum of exponential time decay functions by using a computer least squares routine. The variability in the calculated parameters from experiment to experiment greatly exceeded the calculated standard errors for each fit, even though the cells had been treated uniformly and the nonfrozen cells had remained contractile upon completion of the experiments. The deuterium fraction in each fiber was calculated from the relative amplitudes of the proton and deuteron signals corresponding to the major fraction of tissue water at 9.2 Mhz.

Transverse proton relaxation from nondeuterated muscle fibers is shown over two different time scales in Fig. 1, a and b. As in rat skeletal muscle (*3*), at least three proton fractions with greatly different T_2's appear in the time scale of Fig. 1, which we designate [following (*3*)] as millisecond, major, and extracellular (*14*), in order of increasing T_2. A fourth proton fraction (attributed to tissue protons) exhibits a T_2 of ~ 20 μsec and is not evident in Fig. 1. The apparent amplitudes and T_2's of these four proton fractions are summarized in Table 1; only the two intermediate fractions (accounting for 97 percent of the nonprotein NMR signal) are considered in detail below.

Proton relaxation data from heavily deuterated fibers are shown in Fig. 1, c and d, on a time scale chosen to emphasize the millisecond fraction, which, in the cell with the highest deuterium content (Fig. 1d), accounts for nearly half of the observed protons. Clearly, many of these protons are not removed when the cell is deuterated. Since a minimum of three exponential relaxation functions is needed to fit the data of Fig. 1, c and d, and their respective extensions to 50 msec, the millisecond fraction probably includes several proton populations with T_2's ranging from 0.3 to 4 msec. If we assume that none of these millisecond protons exchange, our data from the deuterated fibers predict a millisecond fraction of 6.2 percent in the nondeuterated fibers, compared to the estimates of 3.3 to 5.6 percent that we derive from the relaxation data from the nondeuterated fibers (Table 1). Neither standing overnight at room temperature, nor being subjected to several freeze-thaw cycles, affects the amplitude of the millisecond proton signal in deuterated fibers. These protons are probably not part of the cell water, and certainly do not determine the relaxation properties of the major fraction of tissue water by any exchange mechanism (*15*).

The relatively mobile protons observed by NMR in frozen protein solutions and tissues are often ascribed to bound water (*9, 10*). Figure 2 shows proton relaxation data for a constant weight of nondeuterated and deuterated fibers at −34°C. Part of the signal lasts for several hundred microseconds, much longer than the 6-μsec T_2 of ice at that temperature. In the nondeuterated fibers, the relative signal amplitude of this nonfreezing water is equivalent to about 0.07 g of water per gram of wet tissue, somewhat less than estimates of the bound water content of muscle tissue, but consistent with estimates of water bound to fibrous proteins (*2, 9, 10*). Since the signal from these nonfreezing water protons is inversely proportional to the tissue deuterium concentration, they readily exchange with D_2O (Fig. 2) and are clearly not to be identified with the millisecond

Table 1. Summary of transverse relaxation data for single barnacle muscle cells and rat gastrocnemius muscle tissue at room temperature. The relative amplitudes are normalized so that they sum to unity for the three fractions with slowest relaxations. Data for rat muscle are from (*3*).

Proton fraction designation	T_2 (msec)	Relative amplitude	Tentative proton source
		Rat	
Protein	~ 0.02	~ 0.2	Tissue protein
Millisecond	0.4	0.08	Bound water
Major	45	0.82	Intracellular water
Extracellular	200	0.10	Extracellular water
		Barnacle	
Protein	~ 0.02	~ 0.2	Tissue protein
Millisecond	0.75 ± 0.25	0.033 ± 0.006*	Relatively mobile protons in tissue protein and lipid
	0.3 to 4	0.056 ± 0.014†	
		0.062 ± 0.003‡	
Major	35	0.91 − 0.94	Intracellular water
	45§		
	55‖		
	20¶		
Extracellular	400	0.03	Extracellular water

*The relaxation data from nondeuterated tissue between 100 μsec and 50 msec could be adequately fitted by a sum of two exponential decay functions, corresponding to the millisecond and major proton fractions. The values given here are the averaged results from several fibers, assuming a two-exponential decay function. †The relaxation data from nondeuterated fibers between 100 μsec and 50 msec were also fitted to a sum of three decay functions, where the two shortest decay times (corresponding to the millisecond proton fraction) were arbitrarily set equal to those calculated for the heavily deuterated tissue. The averaged results are given here. ‡Extrapolated from the amplitude of the millisecond fraction in deuterated tissue samples, assuming that none of the millisecond protons exchange. §Rotating frame relaxation time $T_{1\rho}$ in an effective field $H_1 = 2.4$ gauss, measured using the CPMG pulse sequence with pulse spacing $2\tau = 50$ μsec. ‖Same as in § but at 5°C. ¶Deuteron T_2 at 25°C; the barnacle was deuterated in vivo. All other data in Table 1 are from nondeuterated tissues.

proton fraction observed at room temperature in these cells.

Our goal of understanding the relaxation properties of intracellular water in relation to those of pure water thus is reduced to that of understanding relaxation in the major fraction without using the millisecond proton fraction as a relaxation source, as was done previously (*3, 6*). We continue to assume that relaxation is caused by an exchange process. To include deuteron relaxation as well as the effects of variable isotopic composition on proton relaxation, we introduce a variant of the well-understood expression (*3, 7, 8*) that holds for a system in which exchange occurs between two states differing in relaxation time but having the same chemical shift

$$T_{2obs} = (x\ T_{2b}/P_b) + \tau_a \qquad (1)$$

where T_{2obs} is the transverse relaxation time observed for the major proton (or deuteron) fraction, T_{2b} is the intrinsic proton T_2 in the bound state for an undeuterated specimen, $P_b << 1$ is the fraction of protons plus deuterons in the bound state, $\tau_a(\tau_b)$ is the mean lifetime of a particle in the free (bound) state, and x is defined below. Detailed balance requires (*7*) that $\tau_a/P_a = \tau_b/P_b$, where $P_a + P_b = 1$. Other assumptions are: (i) the intrinsic T_2 of the free water is too long to contribute to the observed relaxation; (ii) the bound particle is an irrotationally bound (*16*) water molecule (so there is no dipolar or quadrupolar motional averaging in the bound state and the correlation time for the bound molecule is its lifetime in the bound state, τ_b); (iii) therefore $T_{2b}^{-1} = \sigma_H^2\tau_b$, where $\sigma_H^2 = 1.6 \times 10^{10}$ sec^{-2} is the intramolecular second moment (*8, 17*); and (iv) the lifetimes τ_a and τ_b are independent of isotopic composition and isotopic species (*8*). Under these assumptions the parameter x equals $\sigma_H^2/\sigma_D^2 \sim 0.1$ for deuteron relaxation (*18*) and f_H^{-1} for proton relaxation in partially deuterated tissue. Here the ratio σ_H^2/σ_D^2 gives the relative strengths of the proton and deuteron coupling interactions in the water molecule (*8, 18*) and f_H is the isotopic fraction of protons in the major fraction of tissue water (*19*).

In the fast exchange regime the first term of Eq. 1 is larger than the second, and T_{2obs} is proportional to x; in the intermediate exchange regime the lifetime term (τ_a) dominates and relaxation is independent of isotopic species and composition. From Fig. 3, the variation of T_{2obs} with x is somewhere between these two extremes; evidently the muscle water is, at room temperature, in the borderline region between fast and intermediate exchange (*20*). A linear regression of T_{2obs} against x yields $P_b = 0.08 \pm 0.02$ percent and $T_{2b} = 3.5 \pm 1.5$ μsec (or equivalently $\tau_b = 20 \pm 10$ μsec). Thus, about one water molecule per thousand, irrotationally bound for only tens of microseconds, can account for the transverse relaxation times of at least 97 percent of the water in barnacle muscle cells. This tiny water fraction is too small to be evident in a transverse relaxation plot such as Fig. 1. Other interpretations of the available NMR data perhaps exist, and this model must be tested by further NMR studies in tissue; it does, however, predict the dispersion in rotating frame relaxation observed in vertebrate muscle tissue (*21*).

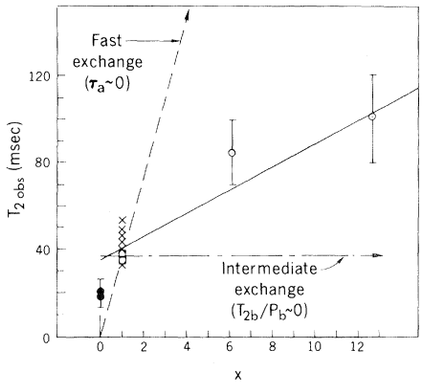

Fig. 3. Proton and deuteron transverse relaxation times for the major fraction of water in barnacle muscle fibers, plotted against the parameter x (see text). A small fraction of irrotationally bound water molecules is assumed to exchange with the remaining tissue water, causing relaxation in the major phase. The variation of T_{2obs} with x in the fast and intermediate exchange limits is shown; evidently the water in this tissue is in a borderline region between these two limiting exchange conditions. (●) Deuteron T_2 ($x = 0.1$). (□) Proton T_2 ($x = 1$). (×) Proton $T_{1\rho}$ ($H_1 = 2.4$ gauss; $x = 1$). (⊙) Proton $T_{1\rho}$ in partially deuterated fibers ($x = f_H^{-1}$, where f_H is the proton isotope fraction in the major fraction of tissue water).

KENNETH R. FOSTER*
Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20014

HENRY A. RESING
ALLEN N. GARROWAY
Naval Research Laboratory, Washington, D.C. 20375

References and Notes

1. E. Odeblad, B. Bhar, G. Lindstrom, *Arch. Biochem. Biophys.* **63**, 221 (1956).
2. R. Cooke and I. D. Kuntz, *Annu. Rev. Biophys. Bioeng.* **3**, 95 (1974).
3. C. F. Hazlewood, D. C. Chang, B. L. Nichols, D. E. Woessner, *Biophys. J.* **14**, 583 (1974).
4. R. Damadian, *Science* **171**, 1151 (1971).
5. R. R. Knispel, R. T. Thompson, M. M. Pintar, *J. Magn. Reson.* **14**, 44 (1974).
6. E. D. Finch and L. D. Homer, *Biophys. J.* **14**, 907 (1974).
7. In the intermediate exchange rate regime the observable relaxation rate for the major fraction is determined by its lifetime against exchange into the bound state rather than by the relaxation time of the bound state. For fundamental details see J. R. Zimmerman and W. E. Brittin [*J. Phys. Chem.* **61**, 1328 (1957)] and H. A. Resing [*ibid.* **78**, 1279 (1974)]; for a particularly complete example see (*8*).
8. J. S. Murday, R. L. Patterson, H. A. Resing, J. K. Thompson, N. H. Turner, *ibid.* **79**, 2674 (1975); H. A. Resing, *ibid.* **80**, 186 (1976).
9. I. D. Kuntz, Jr., T. S. Brassfield, G. D. Law, G. V. Purcell, *Science* **163**, 1329 (1969); P. S. Belton, K. J. Packer, T. C. Sellwood, *Biochim. Biophys. Acta* **304**, 56 (1973).
10. D. R. Woodhouse, D. W. Derbyshire, P. Lillford, *J. Magn. Reson.* **19**, 267 (1975).
11. This suggests a simple NMR method for studying diffusion of protons out of an individual muscle fiber. The proton T_2 of the intracellular water is 35 msec, and that of the extracellular water is at least tenfold greater. On immersion of a fiber into seawater made from 100 percent D_2O there are initially no protons with a long T_2; as H_2O diffuses from the cell, a slowly relaxing proton signal appears and grows. For a barnacle muscle fiber mounted axially in a cylindrical tube, this signal increased with a half-time of 3.5 seconds, as expected from a previous study of water diffusion from barnacle muscle fibers [I. L. Reisin and G. N. Ling, *Physiol. Chem. Phys.* **5**, 183 (1973)].
12. These two methods of deuteration resulted in slightly different NMR data from tissue water. Barnacle fibers that had been deuterated by incubating the intact animal in deuterated seawater showed the same relative proton fractions as the nondeuterated tissue. Single fibers that had been immersed in deuterated seawater exhibited a relatively larger proton signal with a long T_2 than did untreated fibers, presumably because more water remained on the fiber surface.
13. S. Meiboom and D. Gill, *Rev. Sci. Instrum.* **29**, 688 (1958). Because of the short pulse spacings required to observe the millisecond fraction, the CPMG pulse sequence actually measures the rotating frame relaxation time $T_{1\rho}$ rather than T_2 (that is, $2\tau = 50$ μsec gives an effective H_1 of 2.4 gauss). See E. D. Ostroff and J. S. Waugh [*Phys. Rev. Lett.* **16**, 1097 (1966)]. An example is given in (*5*). We did not correct our T_2 data for this effect. The spectrometer linearity had been verified for signal strengths varying by more than three orders of magnitude by using a doped water sample with a T_2 of 40 msec to provide a signal with known exponential decay.
14. The identification of the protons with the fastest and slowest relaxations with proteins and extracellular water, respectively, follows (*3*).
15. For fibers deuterated in vivo the deuteron transverse relaxation appeared from 50 μsec to several hundred milliseconds as a single exponential decay with a T_2 of about 20 msec, with no counterpart to the millisecond proton fraction (within an experimental uncertainty equivalent to 0.5 percent of the total tissue water). This is additional evidence that the millisecond proton signal arises from nonexchanging protons.
16. The concept of irrotationally bound water was introduced to explain NMR relaxation in protein solutions [see H. A. Resing and J. J. Krebs, *Abstr. 140th Meet. Am. Chem. Soc.* (1961), p. 13C] but disagrees with a recent interpretation of NMR data in protein solutions [see S. H. Koenig, K. Hallenga, M. Shporer, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2667 (1975)]. See also (*2*). In contrast to the work in protein solutions, we assume that the substrate in tissue that binds the water molecule is stationary.
17. N. Bloembergen, E. M. Purcell, R. V. Pound, *Phys. Rev.* **73**, 679 (1948).
18. D. E. Woessner, *J. Chem. Phys.* **40**, 2341 (1964); J. G. Powles and M. Rhodes, *Proc. Colloq. AMPERE* **14**, 757 (1967).
19. W. A. Anderson and J. T. Arnold, *Phys. Rev.* **101**, 511 (1956).
20. In this borderline region, T_2 undergoes a minimum as a function of temperature (*7, 8*). Such minima occur in agarose gels [W. Derbyshire and I. D. Duff; *Discuss. Faraday Soc.* **57**, 243 (1974)] and polylysine solutions (*10*). Our own data show that $T_{1\rho}$ rises as the temperature decreases (Table 1), which is consistent with the presence of this minimum in the barnacle muscle tissue as well.
21. H. A. Resing, A. N. Garroway, K. R. Foster, in *Proceedings of the Symposium on Magnetic Resonance in Colloid and Interface Science*, H. A. Resing and C. G. Wade, Eds. (American Chemical Society, Washington, D.C., in press).

* Present address: Department of Bioengineering, University of Pennsylvania, 200 South 33 Street, Philadelphia 19174.

12 May 1976; revised 23 July 1976