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During the past period new strategies have been brought to bear on the study of NMR relaxation of water protons in living muscle and in related biological systems, and new information as well as more precise measurements have been obtained. The simple model originally proposed by our research group, ⁽¹⁾ of two rapidly exchanging fractions of water, one essentially free, and the other essentially bound has been confirmed in several new ways. New data we have obtained suggest a more elaborate mechanism, which is currently undergoing definition in experiment.

Early C.W. work of Berendsen ⁽²⁾ at 60 Mc on collagen, had showed a splitting of the NMR proton line at moderate dehydration; and unpublished work by Pollak ⁽³⁾ on composite tissues at kilocycle frequencies had also showed two distinct relaxation times, indicative of incompletely equilibrated fractions of water. As a check, studies of T_1 and T_2 of frog gastrocnemius were therefore made at 60 Mc, under conditions of moderate to severe dehydration. The relaxation curves obtained by the Carr-Purcell, and Hahn spin-echo techniques confirmed the conclusions of our earlier measurements at 4 Mc and 27 Mc: the bound and free fractions are equilibrated in muscle, and give rise to a simple exponential trace, the characteristic time of which varies linearly with fractional dehydration over the entire range. The conclusion we draw from the proportionality of T_1 and T_2 to relative loss of weight is that dehydration reduces the proportion of free water without seriously affecting the amount of bound water, thus altering the relative weights with which the characteristic rates of bound and free fractions are combined in the observed rate. Qualitatively as well as quantitatively, these results confirm the original two-phase model.

A second new departure was an extensive study of the behavior of T_1 and T_2 as a function of temperature over a range from about -7.5°C to $+43.5^{\circ}\text{C}$. The values

$\ln T_1$ increase linearly with temperature T as $.016T$ in this range, which can be compared with the measurements of Simpson ⁽⁴⁾ for pure water. The values $\ln T_2$ do not, however, vary appreciably with temperature. We explain this difference of behavior by pointing to the fact that T_1 is influenced to a great extent, according to our model, by the free fraction, which has a behavior similar to that of pure water, the viscosity of which diminishes with increasing temperature in the manner described. The bound fraction, on the other hand, which dominates the value of T_2 , behaves like a solid, and is effectively unexcited at these temperatures.

To clarify the nature of the binding sites of water in muscle, an intensive search was made for an effect of orientation of muscle fibers relative to the main magnetic field, on the observed values of T_1 and T_2 ^{(1), (5), (6)}. A special probe was built (by Dr. J. Hofland) which enabled the orientation of a sample to be changed without altering its coupling to the radio frequency field. The detection efficiency of the system was checked by repeating on canine tendon the experiments of Berendsen on bovine achilles tendon, where it was found that when the fibers were at an angle of 55° ; i.e., $\cos^{-1} (1/3)$, to the main field, the C.W. line width was single and relatively narrow. Since two parallel dipoles oriented at this angle relative to their line of centers have zero interaction, Berendsen concluded that water was bound in such a way that the line joining adjacent hydrogen atoms was principally parallel to the fibers. Using Dr. Hofland's apparatus, and the method of spin-echoes, changes in T_2 were observed as a function of orientation that quantitatively confirmed Berendsen's observations on the second moment of his resonance line. Under moderate dehydration over P_2O_5 , it was further found that the curve of relaxation of spin-echoes was best fitted by a sum of exponentials, the characteristic times of which, were in good agreement with the triplet Berendsen

observed, under these conditions.

A study of frog sartorius muscle, chosen for this purpose because its fibers are fairly closely parallel, and because it is small enough to fit into the orientable probe, did not show any orientation effect outside experimental error. The curve of relaxation of spin-echoes did not depart appreciable from the form of a single exponential. This result emerged from a series of measurements at various degrees of dehydration, in which the total muscle weight was eventually reduced to 30% of its initial value.

We conclude that the bound fraction of water in muscle fibers is not aligned to any significant degree with the main fiber axis. Our finding is compatible with the suggestion that the cross-links between actin and myosin fibers observed by Huxley (7) are the sites of binding of water, since those links are distributed, more or less uniformly in azimuth in planes perpendicular to the fiber axis. Our result is thus in accord with the models of Davies (8) which associate muscle contraction with repeated helix-coil transitions of the cross-links. The reversible liberation of water is the expulsion of bound water sheathing an extended link when it contracts. We expected a negative orientation effect; and we regard it as additional evidence for the relevance of the liberation of water to the contraction process.

The relation between the initial static tension of a muscle, the rate at which work is done, and the additional tension developed during stimulation, is well known from the classic studies of A.V. Hill and his associates. We expect, from those results, that the application and development of tension will be significantly related to the reversible liberation of water on contraction which is the object of our study. To clarify these, Sr. Charles Zinn a graduate student with us, has built a device to apply various controlled initial tensions to the muscle samples in our probes, and

to measure more precisely the tension developed during stimulation at the same time as the NMR relaxation is being determined. Preliminary measurements in frog gastrocnemius muscle at rather low tensions and stimulation rates show a pattern different from that of our earlier measurements, which were characterized by much greater static tensions, rigorously isometric conditions, and more complete tetanus than has been the case in the new series, up to this moment. Though it is too early to give definite description or interpretation to the dependence of NMR relaxation on the initial and developed tension, we expect the experiments now in progress to make a contribution of the greatest importance to our picture of the contraction process.

Throughout this project we have always regarded work on model systems as an essential phase of our research effort.

The well known fact that acto-myosin preparations in vitro can be made to contract by the addition of ATP and calcium ions suggests, that a mechanism essentially similar to that of contraction of living muscle, may be involved, and that the reversible liberation of bound water may prove to be involved to that process as it is in living muscle.

With the valuable assistance of Professor Love of Biochemistry, extracted myosin of considerable purity has been prepared for study in our apparatus, on which has been performed an extensive series of measurements of T_1 and T_2 similar to those we have reported in living muscle and on the colloidal silica model.

Muscle transforms chemical energy into work and heat by splitting ATP with an enzyme (ATP-ase) located on myosin rods. In our model experiments, ATP energy is converted into heat by a process parallel to muscle contraction. A significant change in T_2 was found, the magnitude of which was essentially proportional to the

ATP-ase activity of the model. This illustrates the relevance of T_2 changes in the process by which muscles work.

ATP-ase was activated by the introduction of calcium ions into the myosin preparations at a pH 9.5, chosen so as to avoid significant changes of viscosity with activation. The use of calcium for this purpose is probably parallel to the situation in muscle. The pH, however, was unphysiological, in order to minimize the effects of viscosity changes on nuclear magnetic relaxation times.

The studies on the muscle protein myosin are an attempt to correlate the effect of the calcium-activated ATP-ase activity on the nuclear magnetic relaxation times of water, with those effects found for living muscle. We have studied a number of myosin preparations of varying ionic strength, pH, concentration and purity with the following general results.

The transverse relaxation time T_2 for the water protons is shorter than the longitudinal relaxation time T_1 . Also, T_2 depends on protein concentration, but T_1 shows little dependence. We assume these facts to be partial evidence for "binding" of water molecules by the protein.

In high purity solutions at pH 9.5, where light scattering indicates that the protein monomers do not aggregate, T_1 does not change with protein concentration or with the presence of calcium ions. It is, however, sensitive to temperature. These results show that there is little change in viscosity of the solutions as a result of chemical maneuvers.

Those solutions containing ATP to which calcium ions are added, show a reduction of T_2 which depends on ionic strength and pH. High ionic strength solutions were abandoned in favor of low ionic strength, high purity solutions.

The highest purity myosin solutions are those which have been chromatographed on a G-200 Sephadex column, treated with RNase and/or EDTA, then chromatographed on a DEAE-cellulose column. Of the five fractions obtained by this procedure, fractions II, III, IV are myosin. The ATP-ase activity, active affinity and the amount of bound nucleic acid decreases with increasing fraction number. Fraction IV has no detectable nucleic acid bound to the protein or in solution. (9)

Since the protein molecules in fraction II have a tendency to aggregate in direct competition with their ATP-ase activity, relaxation time measurements, so far, have been made mostly on fractions III and IV. The most interesting result is that the effect on T_2 is correlated with ATP-ase activity, i.e., fraction III shows a greater reduction of T_2 than fraction IV upon the addition of calcium ions to solutions of essentially equal concentrations of protein. This result suggests that bound nucleic acid may be a cofactor for the enzymatic activity of myosin, and that the change in T_2 is correlated with the ATP-ase activity of the myosin.

Finally, our work on diffusivity measured by NMR which culminated in the determination of the apparent diffusivity of water in colloidal silica suspensions as a function of concentration, temperature, NMR field gradient, and pulse repetition rate. To interpret these results required a theory of diffusion among obstacles, densely spaced at random. Initial theoretical work, was based on the properties of an inhomogeneous Boltzmann equation, describing the asymptotic relaxation towards equilibrium of a tagged material injected at a given time and place which led to a simple integral equation for the diffusivity of a dilute gas, studied as a test case. This was eventually recognized (10) as quite equivalent, but much simpler in form and derivation, to the familiar Chapman-Enskog theory. Our new methods have now been extended to dense systems in which the molecules are described as rigid spheres of finite radius, -- a model much closer in behavior to a liquid. As a test, a

classic unresolved problem, namely the molecular correction to the viscous drag on a sphere moving through the dense gas, is under study. This problem is actually closely related to the obstacle effect on diffusivity. As soon as it accounts successfully for the known, but as yet unexplained experimental behavior of the viscous drag, it will be applied to the interpretation of the NMR measurements on obstacle-limited diffusion in liquids. We expect, when this is done, to be able to use the methods we developed for NMR diffusivity measurement to help define the nature of the binding of water in living muscle. In particular, we expect it to discriminate between rotational and translational restriction of motion.

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