PROTON MAGNETIC RESONANCE STUDIES OF WATER IN SLIME MOULD PLASMODIA

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Abstract

Steady-state and pulse n.m.r. techniques have been applied in a study of water in the cytoplasm of slime mould plasmodia (*Physarum polycephalum*). The former method has been used to confirm that the signal detected was from protons in water molecules, and to estimate the fraction of the total water in the sample that was contributing to the observed signal. Pulse techniques have enabled direct measurement of the self-diffusion coefficient D of the bulk water in the cytoplasm, and the proton spin–lattice and spin–spin relaxation times T_1 and T_2 respectively. The measurements of D can be accounted for if most of the water is in a "free" state, similar to water in a dilute ionic solution, but with a lower value of D due to the obstruction effect of macromolecules and cytoplasmic structures. The values of T_1 and T_2 indicate that a small "bound" fraction of the water molecules has more restricted motion. The assumption of a two-state model, with exchange of water molecules between "free" and "bound" phases in a time $\ll 10^{-3}$ sec, yields a representative correlation time $\tau \sim 10^{-8}$ sec for the bound fraction. This model is the simplest compatible with all the above results. The underlying assumptions, the extent to which it is likely to be an approximation, and the implications regarding some theories of cellular functions are discussed. Similar results have also been obtained from samples of toad leg muscle, cells from the meristematic region of pea roots, and from agar and gelatin gels.

I. INTRODUCTION

The aim of these studies has been to use nuclear magnetic resonance (n.m.r.) techniques to determine some of the characteristics of water in cytoplasm. Plasmodia of the slime mould *Physarum polycephalum* are convenient for these experiments as they provide large quantities of living, exposed cytoplasm, without rigid cell walls.

The state of water in cytoplasm is relevant to many biophysical theories of cellular functions, and is a subject of controversy. Previous n.m.r. evidence relating to this topic has recently been reviewed (Walter and Hope 1971).

II. MATERIALS AND METHODS

(a) *Slime Mould*

A plasmodium of *P. polycephalum* is easily cultivated on oatmeal and may be harvested free of external water and exuded slime. Under normal conditions, in air, rhythmic streaming of fluid cytoplasm within a gelled layer is observable throughout the plasmodium. Upon exposure to an atmosphere containing an appreciable concentration of CO_2, streaming is found to stop within a few seconds due to the apparent gelation of the fluid cytoplasm, and to resume some time after the CO_2 is removed. External pressure applied to the plasmodium also causes gelation. The streaming properties have been reviewed by Kamiya (1959), and the internal structure of *P. polycephalum* has been studied by Rhea (1966).

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(*b*) *High-resolution N.M.R. Measurements*

Proton resonances from slime mould samples were recorded using a Varian A60 spectrometer at a frequency of 60 MHz, the temperature being controlled at 20°C. In order to avoid spurious line broadening due to magnetic field inhomogeneities arising from the diamagnetism of the sample, it is necessary that the samples be spherical, or long and cylindrical, of uniform composition, and free from bubbles. These requirements hinder the application of high-resolution techniques to many biological specimens. Samples of slime mould were either taken from the front of a plasmodium advancing over oatmeal and crammed down standard 5 mm O.D. glass n.m.r. tubes, or long strands of reticulate plasmodium growing over water were lifted gently into a hollow teflon cylinder, split longitudinally to allow the specimen to be inserted. External water was removed by blotting and the cylinder pushed into an n.m.r. tube which it fitted closely. Both of these methods caused some injury to the sample, so recordings were taken as soon as possible (within 10 min). Samples which had been through the latter treatment for a few minutes only, and did not darken or exude water, showed signs of migration if left overnight on agar, indicating that at least part of the plasmodium was alive during the measurements. The sample tubes were spun, and reproduceable Lorentzian lines were obtained. Alternatively, if samples were allowed to retain their original clumped shapes, the lines obtained were distorted and meaningless due to field inhomogeneities.

The integrated areas of the n.m.r. spectra were measured by a planimeter and compared with those obtained from pure water samples of the same shape, volume, and temperature. The radiofrequency field strength was kept below the level required for saturation of either resonance. Under these conditions, the relative areas of the lines should equal the relative number of water molecules, per unit volume, contributing to the *observed* signal. Chemical shifts of resonances from slime mould were compared with those from a distilled water sample in an identical container.

(*c*) *Pulse N.M.R. Experiments*

Studies of slime mould, toad skeletal muscle, meristematic cells from pea roots, and gels have been undertaken with pulse n.m.r. techniques. The procedures originated by Carr and Purcell (1954) have been applied in the following work, and the terms used below are defined in their paper. The spin–lattice relaxation time T_1 for the water resonance has been measured by the "null-method" in which a "180° pulse" of radiofrequency power is followed at a time t_1 later by a "90° pulse". The decay following the 90° pulse disappears when $T_1 = t_1/\ln 2$.

The rate of decay of "spin echos" in a sequence of one 90° pulse at time $t = 0$ and a train of 180° pulses at times t_1, $3t_1$, $5t_1$, ... has been used to determine the spin–spin relaxation time T_2 for the water resonance and the self-diffusion coefficient D of the water molecules. Carr and Purcell (1954) showed that

$$A_t = A_0 \exp\left[-(t/T_2) - (\gamma^2 G^2 D t^3/12n^2)\right], \quad (1)$$

where A_t is the spin–echo amplitude at time t, G is the magnetic field gradient across the sample in the direction of the external magnetic field, and n is the number of 180° pulses in the time t. If n is large and G is small, the second term in the exponent is negligible, and the decay is exponential with a characteristic time T_2. Thus the presence of small residual gradients in the external magnetic field does not affect the determination of T_2. These techniques do not put stringent restrictions on sample shape and uniformity, a great advantage with biological specimens. Alternatively, if n is small and a measured value of G is applied by passing a current through opposed Helmholtz coils on the magnet polefaces, then D may be determined from (1) if T_2 is known.

The experiments were undertaken with the pulse n.m.r. apparatus at the Division of Tribophysics, CSIRO, University of Melbourne. The external magnetic field was supplied by a permanent magnet, the proton resonant frequency being 20·77 MHz. The residual field gradient over the specimen volume was negligible for the purpose of these experiments. Known field gradients were applied by passing a measured direct current through a pair of opposed coils whose axis was along the direction of the external field. The coils were made to the specifications of Tanner (1965). A Bell incremental gaussmeter probe, clamped to the micrometer-driven support of a measuring microscope, was used to calibrate the field gradient over the sample volume. Field gradients used in experiments varied between 4·6 and 14·6 G cm^{-1}.

Sample temperatures were varied by passing a steady stream of heated or cooled dry air over the outside of a 5-mm O.D. thin-walled sample tube. Specimens were not directly exposed to the air stream to avoid drying. Temperatures were continuously monitored with a thermistor. Gas-flow rates were measured and held constant, and gas mixtures were proportioned, with previously calibrated flow-meters. Low rates of flow (50 ml gas/min) were used whenever gas mixtures were blown directly over specimens. At other times the samples were sealed in a mixed-gas atmosphere and a flow of the gas mixture was kept up outside the sealed tube.

Traces of spin echos were displayed on a Tektronix storage oscilloscope and photographed for later analysis. Values of T_2 were obtained from plots of $\log_{10} A_t$ versus t, where the spin-echo amplitude A_t was measured after each 180° pulse in a train of 6–25 pulses, in the absence of an applied magnetic field gradient. In all cases these spin-echo decays were exponential within error, indicating a single value of T_2. Knowing T_2 for a given sample, a measured field gradient was applied and a new trace was recorded by varying the time between a 90° pulse and a single ($n = 1$) 180° pulse, and superimposing the spin echos on the storage oscilloscope. D was then obtained from a plot of $\ln[(A_t/A_0)]+(t/T_2)$ versus t^3, utilizing the relation

$$(\mathrm{d}/\mathrm{d}t^3)\{[\ln(A_t/A_0)]+(t/T_2)\} = -(\gamma^2 G^2 D/12n^2) \qquad (2)$$

which follows directly from (1).

The samples were prepared as follows:

(1) *Slime Mould.* Clumps from the advancing front of a slime mould plasmodium growing on oatmeal were harvested free from external water, with minimum injury, and placed against the inside wall of a sample tube. After about 12 hr, reticulate branching of the plasmodium was apparent, indicating that the specimen was alive during experiments. Samples which were injured or deprived of air for long periods would soon darken, lose their clumped shape, and exude water. Results from such specimens were rejected. Measurements were taken at times up to 11 hr after the samples were placed in the tubes.

(2) *Toad Muscle.* Sartorius and adductor longus muscles were removed from the legs of a decapitated and pithed toad (*Bufo marinus*), and kept in Ringer's solution until just before the experiment. Individual muscles were then blotted and suspended in the apparatus. The muscle was in a relaxed state and was not stretched or injured. Experiments were continued up to 5 hr after decapitation without any significant change in the results. The muscles were returned to the Ringer's solution between experiments.

(3) *Meristematic Cells.* The meristematic region (1–1·5 mm of the root tip) was excised from approximately 100 pea roots (1–3 cm long), and immediately placed in water. The excised tips were blotted, placed in a tube in the apparatus, and kept under humid conditions by means of a water-soaked wick at the other end of the tube. The experiments were performed within 2·5 hr of excision of the tips. Mature root tissue was also examined.

(4) *Agar and Gelatin Gels.* Gels of various concentrations were made up with distilled water and placed in sample tubes. These gels do not appear to have been previously examined with pulse n.m.r. techniques.

III. Results

(a) *High-resolution N.M.R.*

Samples of slime mould always yielded a single, symmetrical, Lorentzian resonance at a frequency very close to the signal from pure water in the same magnetic field. From comparisons of signal areas it was concluded that a given volume of slime mould contained $82\pm5\%$ as much water, contributing to the observed signal, as the same volume of pure water. Within error the same result was obtained regardless of the way in which the sample was placed in the tube, provided accurately cylindrical shapes were obtained. It is highly probable that all the slime mould samples were gelated during these measurements, and injury was unavoidable.

TABLE 1

VALUES OF VARIOUS PARAMETERS OBTAINED BY PULSE N.M.R. TECHNIQUES

Number of experimental determinations of each parameter given in parenthesis. Errors marked with an asterisk indicate that these are standard deviations. Otherwise errors have been estimated from the scatter of points on graph plots and the signal: noise ratio of original oscilloscope traces. The quantities τ_b and p_b were calculated as described in Section IV

Sample and Conditions	Temp. (°C)	T_1 (msec)	T_2 (msec)	$10^5 D$ ($cm^2 sec^{-1}$)	$10^8 \tau_b$† (sec)	p_b† (%)
Slime mould						
In air	27–28·5	131±13* (8)	30·7±2·2* (13)	1·26±0·7* (3)	1·0±0·2	7·4±0·1
	23–23·8	156±50* (4)	24±6 (2)	1·20±0·2 (2)	1·9±0·9	8·7±0·6
	10	203±30 (1)	30·4±2 (1)	1·03±0·2 (1)	2·0±0·4	7·4±0·2
	6·2	203±30 (1)	33·5±4 (2)	1·35±0·3 (1)	1·9±0·5	7·0±0·2
	2·5–3·0	226±33* (4)	33·5±7 (2)	0·51±0·15 (1)	2·04±0·5	7·0±0·2
	1·2–1·5	208±55* (3)	32·1±4* (6)	0·58±0·1 (3)	2·0±0·6	7·0±0·3
Results obtained 5 months earlier	20	90±23* (7)	34±9* (12)	0·84±0·10* (6)	0·92±0·5	9·7±0·5
In CO_2–air (1 : 1 or 4 : 1)	23–23·8	96·5±36* (6)	36·4±11* (7)	1·12±0·20* (5)	0·92 (0·30–1·35)‡	9·8±0·5
Pea roots						
Meristematic region	20	180±10 (3)	47±4 (1)	0·59±0·15 (1)	1·35±0·2	5·5±0·1
Mature cells	20	300±50 (3)	56±4 (2)	0·68±0·17 (1)	1·78±0·3	4·0±0·2
Toad muscle						
Sartorius	21	230±20 (3)	37±3 (2)	1·20±0·3 (2)	1·9±0·3	5·7±0·1
Adductor	21	125±5 (2)	29·7±2 (1)	1·50±0·25 (2)	1·44±0·2	8·5±0·1
Gelatin gel						
2·0%	24	1160±40 (1)	295±20 (1)	2·2±0·1 (1)	1·6±0·2	0·76±0·05
5·0%		1160±40 (1)	274±20 (1)	2·4±0·3 (1)	1·72±0·2	0·81±0·05
12·0%		725±30 (1)	226±15 (1)	2·0±0·2 (1)	1·25±0·15	1·10±0·1
25·0%		580±25 (1)	204±15 (1)	1·44±0·06 (1)	1·10±0·15	1·40±0·1
Agar gel						
0·7%	24	1160±40 (1)	117±8 (1)	2·41±0·15 (1)	3·05±0·3	1·2±0·1
1·4%		1160±40 (1)	72±5 (1)	2·1±0·3 (1)	4·10±0·5	1·6±0·1
6·0%		986±35 (1)	21·3±2 (1)	2·26±0·3 (1)	6·9±0·9	3·4±0·2

† The errors shown for τ_b and p_b result from the experimental errors in T_1 and T_2 only. Allowance for additional intermolecular interactions would increase p_b by a factor $\simeq 1 \cdot 35$. The range of values of r which could be assumed ($1 \cdot 50$ Å $< r < 1 \cdot 60$ Å) would cause a corresponding variation in p_b by a factor $\simeq 0 \cdot 75$ to $1 \cdot 25$.

‡ Extremes of range of τ_b corresponding to error range in T_1 and T_2.

The fractional weight of water in several samples of the plasmodium was found by weighing before and after oven-drying at 100°C. Assuming that water was the only volatile substance lost, the fractional weight of water lay in the range 80–86%. If the average density of the remaining constituents is close to that of water then it can be concluded that at most *c.* 10% of the water in the plasmodium does not contribute to the observed high resolution n.m.r. signal.

The resonances from slime mould plasmodia were considerably broader than those from pure water and corresponded to values of T_2 in the region 3×10^{-3} to 10^{-2} sec. Little weight can be attached to these results because of the probable occurrence of spurious line broadening, as mentioned earlier. Similarly, values of T_1 calculated from the saturation behaviour of these lines are unreliable, as prior knowledge of T_2 is required.

(*b*) *Pulse N.M.R.*

The measurements of T_1, T_2, and D obtained by pulse n.m.r. techniques are shown in Table 1. The quantities τ_b and p_b (respectively, the rotational correlation time of bound water molecules and the fraction of bound water molecules) were calculated from the results in the manner described in the next section.

IV. Interpretation

(*a*) *Slime Mould Plasmodia*

The high-resolution n.m.r. results have shown a signal which represents at least 90% of the water in the plasmodium. There is no reason to doubt that the signal detected by pulse n.m.r. has the same origins. The fact that values of T_2 measured in the pulse experiments are longer than those obtained from steady-state linewidth measurements can be attributed to the effect of residual magnetic field inhomogeneities in the latter.

The self-diffusion coefficients for water in the slime mould plasmodium (see Table 1) are reduced by an approximate factor of 2 from the value for pure water, which is $2\cdot57\times10^{-5}$ cm^2 sec^{-1} at 25°C (see Eisenberg and Kauzmann 1969). A similar result has been found in n.m.r. experiments by Abetsedarskaya, Miftakhutdinova, and Fedotov (1968) for water in frog muscle and liver, in maize and bean leaves, and in maize roots and by Douglas, Frisch, and Anderson (1960), Abetsedarskaya *et al.* (1967), and Bezrukov (1968) for water in macromolecular solutions. Abetsedarskaya, Miftakhutdinova, and Fedotov (1968) have pointed out that such a decrease in D in biological material can be accounted for by the theory of Wang (1954). In this case the main effect is an increase in the average diffusion path length of water molecules due to the presence of hydrated macromolecules. Wang (1954) has shown that, for a macromolecular solution,

$$D = D_o(1-\bar{\alpha}\phi)(1-C_h/C_o), \tag{3}$$

where D_o is the self-diffusion coefficient of pure water at the same temperature, ϕ is the total volume fraction occupied by hydrated macromolecules, C_h is the concentration of bound water of hydration, and C_o is the total water concentration. The theory assumes that the macromolecules are ellipsoids of revolution, $\bar{\alpha}$ being a function of the axial ratio. As the axial ratio varies from 1 to ∞, $\bar{\alpha}$ varies from 1·500

to 1·667 for prolate ellipsoids, and from 1·500 to ∞ for oblate ellipsoids. An oblate ellipsoid of 10 : 1 axial ratio has $\bar{\alpha} = 3 \cdot 107$. The term $(1-C_h/C_o)$ accounts for bound water of hydration which is exchanging rapidly with the free water. For n.m.r. measurements of D, "rapid" exchange would mean an exchange time $\ll T_2$.

In view of the variety of cytoplasmic structures known to exist down to a macromolecular scale, and the macromolecular concentrations in cytoplasm, a reduction of D by a factor of order 2 is easily accounted for. It is unnecessary to postulate the formation of any long-range quasi-crystalline "structure" or any increase in the microviscosity of the bulk water.

The values measured for T_1 and T_2 in slime mould are such that $T_1 > T_2$. Both are small compared to the values for pure water, for which $T_2 \sim T_1 = 3 \cdot 57$ sec at 25°C (Krynicki 1966). Relaxation in pure water is well described by the following equations, derived by Bloembergen, Purcell, and Pound (1948) and modified by Kubo and Tomita (1954):

$$1/T_1 = \tfrac{3}{10}(\gamma^4\hbar^2/r^6)\{[\tau/(1+\omega_0^2\tau^2)]+[4\tau/(1+4\omega_0^2\tau^2)]\}, \tag{4}$$

$$1/T_2 = (3/20)(\gamma^4\hbar^2/r^6)\{3\tau+[5\tau/(1+\omega_0^2\tau^2)]+[2\tau/(1+4\omega_0^2\tau^2)]\}, \tag{5}$$

where γ is the nuclear gyromagnetic ratio, $\hbar = h/2\pi$ where h is Planck's constant, r is the distance between protons in a water molecule. τ, the rotational correlation time of a water molecule, is approximately the average time taken to rotate through 1 rad and is equal to $2 \cdot 6 \times 10^{-12}$ sec at 25°C (see e.g. Eisenberg and Kauzmann 1969, Table 4.6).

These equations attribute relaxation solely to intramolecular interactions among nuclear dipoles. Further improvement has been obtained by incorporating intermolecular interaction terms. The resulting equations have a similar functional form to (4) and (5), with a translational correlation time replacing the rotational τ above. The intermolecular contribution to relaxation is found to be approximately half the intramolecular contribution (see e.g. Carrington and McLachlan 1967, p. 193). It is not possible to fit the experimental values of T_1 and T_2 for water in slime mould to equations (4) and (5), assuming a value of r in the range 1·50–1·60 Å, and allowing for intermolecular contributions in the same proportion as for pure water. Thus an overall reduction in the degree of motional freedom of water molecules (i.e. an increase in "structure") corresponding to a uniformly reduced τ is not compatible with the results. If extraneous sources of relaxation such as paramagnetic impurities can be discounted, which requires paramagnetic ion concentrations $\ll 10^{-3}$M (see Carrington and McLachlan 1967, p. 196), the results are most simply explained by proposing exchange of water molecules between a large "free" phase and a small "bound" phase, probably representing water of hydration of macromolecules. Each phase is described by a rotational correlation time for the water molecules. This model has been used previously to explain similar results by Bratton, Hopkins, and Weinberg (1965), Abetsedarskaya, Miftakhutdinova, and Fedotov (1968), Clifford, Pethica, and Smith (1968), and others. In view of the measurements of D, the free phase has been assumed to have the same τ, and hence T_1 and T_2, as pure water at the same

temperature. If the average residence time of a molecule in either phase is small compared to T_1 and T_2 for either phase (i.e. if "rapid" exchange occurs) then:

$$(1/T_1) = (p_a/T_{1a})+[(1-p_a)/T_{1b}], \quad (6)$$

$$(1/T_2) = (p_a/T_{2a})+[(1-p_a)/T_{2b}], \quad (7)$$

where the subscripts a and b refer respectively to the free and bound phases, and $p_{a,b}$ to the fraction of water molecules in phase a or b ($p_b = 1-p_a$). These equations are a particular case of the general relations for rapid exchange among many phases (Zimmerman and Brittin 1957). It is assumed that the act of exchange among phases is not in itself an important relaxation mechanism. Equations (6) and (7) in conjunction with (4) and (5), when applied to the experimental results for T_1 and T_2, yield the values for τ_b and p_b shown in Table 1. There is an absolute error of about $\pm 2\%$ in p_b for slime mould due to the range of possible values of r. When calculated from bond lengths and angles measured for water vapour (Eisenberg and Kauzmann 1969), $r = 1{\cdot}514$ Å, whereas $r = 1{\cdot}58$ Å has been obtained from the n.m.r. dipolar splitting for water of hydration of $CaSO_4.2H_2O$ (see e.g. Carrington and McLachlan 1967, p. 32). The values in Table 1 assume $r = 1{\cdot}539$ Å, the value used by Clifford and Sheard (1966). Allowance for intermolecular interactions would increase p_b by a factor of about 1·35 if the intermolecular nuclear dipolar interaction in the bound phase is similar to that in the free phase.

It is possible to explain the values of T_1 and T_2 in terms of other rapid-exchange models, e.g. exchange among more than two states, or a continuous distribution of states (cf. Clifford and Sheard 1966; Lynch and Marsden 1966, 1969; Lynch, Marsden, and George 1969). There does not appear to be sufficient data to obtain information about extra states without further unsupported postulates. Because of the observed values of D, distributions of τ would need to be skewed towards values in the range 10^{-11}–10^{-12} sec, thus log-normal distributions as used by the above authors would not be appropriate. In addition these models were applied to hydrated keratin, where the water content is very low compared to that of cytoplasm. For these reasons the simple "bound–free" exchange model is regarded as most appropriate for our results. It is possible that τ_b represents a mean of a distribution of correlation times, within the bound phase. On the other hand a relatively narrow distribution of τ for the free phase is consistent with our observed values of D.

(*b*) *Toad Leg Muscle and Meristematic Cells from Pea Roots*

The results shown in Table 1 are similar to those obtained from slime mould and have been interpreted in the same manner.

(*c*) *Gelatin and Agar Gels*

The bound–free exchange model is again applicable. The value of D for the most dilute gels is the same within error as for a sample of distilled water at 24°C. The calculated trends to larger bound fractions with increasing gel concentration favour the model. Gelatin showed a decrease in D with increasing gel concentration,

which is expected in view of Wang's theory of diffusion discussed earlier. This behaviour was not evident in agar, where the gels were more dilute.

V. Discussion

Various models of cellular functions have explicit or implicit requirements regarding the state of water, ranging from a semi-solid structure with long-range molecular ordering ("association" theories, see e.g. Ling 1962; Fenichel and Horowitz 1963; Horowitz and Fenichel 1965; Troshin 1966; Cope 1969; Ling and Bohr 1970) to a "free" liquid state as in an ionic solution ("membrane" theories, see e.g. Katz 1966). Much of the experimental data that has been used as a basis for discussion of the water structure is indirect or ambiguous.

The finding for water in slime mould plasmodia, that self-diffusion coefficients for the detected water molecules are reduced by a factor compatible with increased diffusion path length around macromolecules (equation 3), is generally favourable to membrane rather than association theories. At most, 10% of the water is in a state where T_2 is too short, and the water structure is too rigid, for the resonance to be detected by high-resolution or pulse techniques. Diffusion coefficients for intracellular ions (Hodgkin and Keynes 1953; Kushmerick and Podolsky 1969) are consistent with the presence of a substantial free water phase. Values of D calculated for water diffusing into frog eggs by Ling, Ochsenfeld, and Karreman (1967) were taken to indicate long-range ordering of the water structure in the eggs, apparently without consideration of an alternative explanation as above, and after rejecting membrane limitation to diffusion.

Ultrastructural studies of *P. polycephalum* (Rhea 1966) have shown the presence of irregularly shaped vacuoles with longest dimensions up to *c.* 20 μm, although most are in the region 3–10 μm. These vacuoles are evenly distributed throughout the cytoplasm and occupy approximately 20–30% of the total volume. The n.m.r. results show no sign of a separate, slowly exchanging phase; thus, rapid exchange of water molecules across vacuolar membranes is required. A minimum exchange time is set by diffusion in the absence of membrane limitation. For a sphere of radius r the exchange half-time $t_{0.5} = r^2/32D$ (see e.g. Briggs, Hope, and Robertson 1961), where D is the self-diffusion coefficient inside the sphere. The vacuoles found by Rhea (1966) in *P. polycephalum* have many invaginations and the average width is around 3 μm. Assuming $D \sim 2\times10^{-5}$ cm^2 sec^{-1}, $t_{0.5} \sim 3\times10^{-5}$ sec for a sphere of radius 1·5 μm. Thus rapid exchange should occur if membrane limitation is small, but it is not possible to estimate this quantity. The relative volume of cytoplasm occupied by nuclei and other organelles is comparatively small (*c.* 5%). The relative volume of vacuoles increases towards the posterior end of a plasmodium, and is apparently related to the cytoplasmic streaming cycle (Park and Robinson 1967). All the samples used in n.m.r. experiments were taken from the anterior end.

The bound phase in our interpretation is most reasonably identified with water in the vicinity of macromolecules and cellular structures, e.g. membranes. More detailed inferences about its nature must be made with caution because it is probable that coupling between nuclear dipoles within water molecules is not the only source of relaxation in the bound phase. Other contributing factors may include interactions

between protons in water and adjacent macromolecules, and exchange of these protons. The calculation of τ_b does not require assumptions about the interactions, and the values of τ_b could reflect self-diffusion of water within the bound phase, proton exchange, macromolecular motion (both rotational and segmental), and relative motion of water molecules and macromolecules. Thus it is likely that τ_b for cytoplasm represents the average of a distribution rather than a single well-defined correlation time.

Calculations of p_b require a knowledge of the relaxation mechanism within the bound phase. The values in Table 1, which result from the assumption of an intramolecular dipolar interaction only, should therefore be regarded as approximate rather than exact. The assumption of an additional intermolecular (water–water) contribution has been shown earlier to increase p_b by a factor of about 1·35.

Relaxation in the bound phase of protein solutions has been studied and discussed by Abetsedarskaya *et al.* (1967). In view of their conclusion that τ_b was determined by internal motion in the system of protein and bound water, rather than by rotation of the complete system, it is very unlikely that the bound phase in our experiments, where $\tau_b \sim 10^{-8}$ sec, is a truly "ice-like" state.

The cessation of streaming in the plasmodium in an atmosphere of CO_2 and air has been found to correspond to no measurable change in D. This is to be expected if a gel network is formed as is shown by comparison with the results from agar and gelatin gels. The most pronounced effect was a decrease of T_1 with time after administration of the CO_2. T_1 decreased from 145 msec to 58 msec in 35 min, which indicates a slight increase in p_b and a small decrease in τ_b. This may be a result of injury to the plasmodium which was apparent after prolonged exposure to high CO_2 concentrations, as a similar effect was found in samples injured by other means. The value of D was found to be independent of the time of exposure. After a few minutes exposure to CO_2 the plasmodium, although gelated, recovered in air without injury.

The reversible cessation of streaming upon exposure to CO_2 has been regarded as an "anaesthetic" action (Seifriz 1941) and it might appear to have some relevance to the theories of Pauling (1961) and Miller (1961) that anaesthesia is due to the stabilization by proteins of clathrate-type "cages" of water molecules around non-polar anaesthetic molecules. The n.m.r. results provide no evidence for this effect in the bulk cytoplasm of slime mould, but it must be pointed out that Pauling and Miller respectively envisaged the formation of such structures at nerve synapses and cell membranes, in which case the proportion of water in these structures would probably be too small for detection by n.m.r. Nevertheless, from chemical considerations (Catchpool 1966) there is no obvious reason why such structures should not be expected to form around proteins and membranes in bulk cytoplasm.

It should also be noted that the results with gelated plasmodia eliminate the possibility of a spurious contribution to D due to cytoplasmic streaming in ungelated samples.

The results for slime mould in Table 1 show a general decrease in D with decreasing temperature, apart from two inconsistent results at 10 and 6·2°C. The scatter of the results is too large to obtain an accurate value for the activation energy for self-diffusion of water. Possible values lie in the range 1·4–7·6 cal mole^{-1}, the

most likely value being *c.* 5·0 cal mole^{-1}, which agrees reasonably well with the value for pure water (see e.g. Eisenberg and Kauzmann 1969).

The results for D in toad leg muscle agree well with those obtained for frog muscle by Abetsedarskaya, Miftakhutdinova, and Fedotov (1968). By contrast, the values of D obtained from both meristematic and mature pea root tissue are approximately equal, and are a factor of 2–3 lower than Abetsedarskaya's results for bean leaves and maize leaves and roots. The estimates of relative vacuolar volume in mature plant cells used by Abetsedarskaya, Miftakhutdinova, and Fedotov (1968) are probably too low. Cells from meristematic regions have undeveloped or small vacuoles. The similarity of D from both meristematic and mature pea root tissue indicates that the vacuolar water also has a reduced value of D, an unexpected result.

The measurements of D for water in agar and gelatin gels, which again show that most of the water is free, appear to contradict the conclusion of Hechter *et al.* (1960), based on steady-state n.m.r. linewidth measurements, that water in agar gels is in a state intermediate between ice and free water. This conclusion would result if it were assumed that all the water is in the same state described by a single correlation time. Rapid exchange between a bound and a free phase, as above, was not considered by Hechter *et al.* but will equally well account for their observations.

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