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DIFFUSION OF WATER IN BIOLOGICAL TISSUES

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Abstract

A method is presented for obtaining simple approximate solutions for the problem of self-diffusion in an ordered array of obstacles. Our results are compared with some previous exact and approximate solutions, and we find that our method agrees well with the exact results over a large range of the volume fraction of the obstructions. It is shown that there is an important distinction between measurements of the diffusion coefficient by the capillary flow method and the spin-echo method. The modifications for the spin-echo case are given and applied to recent measurements on the anisotropy of the self-diffusion of water in striated muscle and to measurements on cysts of the brine shrimp. The analysis shows that very large volume fractions of obstructive barriers are required in order to account for the reduction in the diffusion coefficient in biological systems. Thus this model analysis leads to the supposition that a substantial fraction (20-40%) of the cell water is hydration water, or that the diffusion coefficient of the cytoplasmic water is reduced substantially from the free water value. In either case, the conclusion that a substantial fraction of cell water has diffusive properties that are altered by the macromolecules of the cytoplasm seems inescapable. In the case of NMR methodology, the measuring times are such that the values for diffusion are often influenced by the presence of macromolecular structures (obstructions) within the cells. This suggests that obstructions make a significant contribution to the value of the NMR diffusion coefficient and that NMR may have practical value for the evaluation of obstruction effects.

Key Words: Water, diffusion, water dynamics, cytoplasmic water, Artemia.

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Introduction

The application of nuclear magnetic resonance (NMR) techniques to the study of biological systems has led to much new information about the properties of cellular constituents. It is generally agreed that changes in the NMR parameters of water protons (i.e. the chemical shifts, the longitudinal and transverse relaxation times, T_1 and T_2, and the self-diffusion coefficient, D) when compared with pure water are due to the presence of the cellular macromolecules. The nature of the water-macromolecular interaction and its influence on the structure and dynamics of cellular water are still unresolved. Studies of the NMR parameters of water have been particularly important. The relaxation times for water play a major role in NMR imaging considerations [24]. The relaxation times have also been important in the evaluation of competing theories of cellular water and its role in cellular phenomena. NMR measurements of the diffusion coefficient have several advantages over the classical (chemical, isotopic) methods of studying diffusion [9, 15]. The classical methods all involve measurements on a time scale from a few seconds to many hours. The diffusion coefficient measured by these methods is thus influenced by many rate-limiting cellular structures (e.g. macromolecular obstructions, permeable membranes, protein adsorption sites). The NMR measurements, however, can be carried out over a variable time scale down to a few milliseconds, thus allowing a study of the influence of some of these structures on the diffusion process [37]. The NMR parameters, when compared with the values for pure water, are affected in a similar fashion in a wide variety of biological systems: T_1 is smaller by a factor of 2-20, T_2 by a factor of 5-200 and D by a factor of approximately 2 (the factor can be larger in some systems--see below) [11].

A proper understanding of the diffusion mechanism in biological systems is also important for the application of NMR imaging techniques to the study of biological transport properties. Suggestions to form images based on diffusive properties [19] are now being realized with the application of imaging techniques to the study of flow, diffusion, and perfusion in physiological systems [21]. Studies of the influence of barriers and obstructions on diffusion are thus important if these images are to be related to cellular function.

In the conventional view, the cell is an aqueous gel of water, ions and macromolecules bounded by a physiological membrane in which the interaction between the water and the solutes and macromolecules is limited to the "hydration" layer surrounding them, which, due to the strength of the Van der Waals forces, must be regarded as only a few molecules in thickness. The fraction of the water which has properties substantially different from those of pure water would be small in this model--of the order of 10% or less. In this model, the reduction of the relaxation times is explained by a rapid

exchange between the molecules of "free" water and those in the hydration phase[43, 10, 12]. The strong relaxation in the hydration layer leads to a shortened relaxation time for the entire liquid, and the experimental results can be explained by this model if the relaxation times of the two phases and their exchange times are chosen appropriately. Measurements of the frequency-dependence of the T_1 relaxation time have shown that a spectrum of correlation times is required [2], and models have been proposed that ascribe this spectrum to the dynamics of the diffusive motion of the proteins [29], or to the dynamics of the surface diffusion of water over the irregular surface of the protein [31].

The reduction of the diffusion coefficient in the two-fraction model is ascribed to a hydrodynamic interaction of the "free" water with the macromolecules [41]. This interaction, referred to as the "obstruction effect," can be separated into two contributions: 1) the cross-section for diffusion is reduced due to the volume occupied by the macromolecules; and 2) the paths of the diffusion currents are increased (sometimes referred to as the "tortuosity factor"). It has been suggested that there is also a further interaction associated with an individual molecule diffusing according to its Brownian motion [8]. The normal hydrodynamic "backflow" that would be produced by the motion of the molecule diffusing in an infinite medium will be retarded by the viscous interaction with the stationary macromolecules. This resistance to the "backflow" will reduce the mobility of the diffusing molecule.

In recent years, alternate models of the cell have questioned the assumption that the region of correlation of the water with the solute ions and the macromolecules is confined to a small hydration layer. In these models, the cytoplasm of the cell has properties that differ appreciably from those of the watery solution assumed in the conventional view [17(Chapt. 9)]. The ionic selectivity of the cytoplasm is assumed to involve adsorption by mechanisms similar to those occurring in exchange resins [7, 17(Chapt. 8); 22;], solids [6], or hemoglobin [16]. An extensively tested hypothesis describing the adsorption of ions to fixed charges on macromolecules within living cells is the Association-Induction (AI) hypothesis of Ling [17(pp 145-225)]. A major feature of the AI hypothesis is that the cytoplasmic water of cells is strongly polarized by macromolecular fixed charges and that the water-macromolecular correlations extend far outside the normal hydration layer, thus involving a substantial fraction of the water in the cells--of the order of 80% or more.

The AI hypothesis does not, at present, provide a means for the quantitative calculation of the relaxation times or the diffusion coefficient for the cytoplasmic water. A fundamental expectation arising from the hypothesis, however, is that the correlation time associated with the "free" water phase would produce an increase in the longitudinal relaxation rate associated with that phase, and a decrease in the diffusion coefficient - particularly in the rotational motion of water molecules [17(pp 171-173)]. There is now evidence from neutron scattering and NMR relaxation measurements that there is an increase in the translational and rotational correlation times for the "free" water, although the relaxation appears to be primarily due to the water-macromolecular interaction [29, 30]. It was originally hoped in the case of NMR, that measurements of the diffusion coefficient would be free of the interpretive difficulties of the relaxation time measurements, since the diffusion is dominated by the "free" fraction. These measurements, however, present their own difficulties, since the interpretation depends on the evaluation of the obstruction effects. In this paper we present a survey of the calculations on which our evaluation of the results for biological systems is based, and we discuss the applications to NMR measurements on biological systems.

One of the principal uncertainties in the interpretation of the NMR measurements of the diffusion coefficient is in the estimation of the obstruction effects. NMR diffusion measurements require a few milliseconds, even with the most advanced available technology. During this time, the water molecule diffuses a distance $\sim \sqrt{2Dt}$, which is of the order of a micron or more. The measured diffusion coefficient is thus an average over this distance, and can include the influence of encounters with many macromolecules as well as with cell membrane structures. Thus it is of considerable importance to have models whereby the obstruction effect may be evaluated, and the primary thrust of this paper is to present a model for examining the obstructive effect in biological systems. On the other hand what is needed is a method to reduce this measuring distance so that a "local" value of the diffusion coefficient can be obtained for "free" water. The technique of quasi-elastic neutron scattering (QNS) offers such a possibility and only a brief summary of this technology will be presented.

Neutron spectroscopy is a relatively new technique for the study of biomolecular dynamics [20]. Inelastic scattering from proteins gives insight on the modes of motion of the entire molecule, while QNS has been used for the study of the diffusion motion of proteins and water.

The QNS method has great advantages for the study of the slow diffusive motion of protons. The large incoherent neutron cross-section of protons (~ 80 barns) means that the scattering from a macromolecular-water system will be almost entirely due to protons, since the incoherent cross-sections for carbon, oxygen and nitrogen are negligibly small. The macromolecular protons, which are part of the globular proteins or other polymer-like structures, will participate in the dynamical motion of these structures, which will broaden the elastic line that would be obtained if the scattering centers were stationary. The scattering from the water protons will also be affected by their molecular motion which will produce a characteristic broadening that can be related to the molecular properties through a theory due to Van Hove [40] which relates the incoherent scattering to the space-time Fourier transform of the self-correlation function $G_S(\mathbf{r},t)$ of the water protons. (The scattering from the water protons can be separated from that due to the protein protons by various methods, for example by selective deuteration.) This method is now coming into use for the characterization of the interaction of water with molecular surfaces, such as are found in clays, polymers and proteins [39]. The self-correlation function contains information about the diffusive properties. Different models for the diffusion process [34] (e.g. Brownian motion, jump diffusion, oscillatory diffusion) lead to different dependencies of the line width Γ of the quasi-elastic line on the momentum transfer $\mathbf{Q}$ of the scattered neutron. A fit of the line width $\Gamma(Q)$ with a given model then gives information on the parameters of the model (diffusion coefficient, jump residence time, oscillatory amplitudes).

The QNS method has a great advantage over the NMR measurements of the diffusion coefficient: the "measuring length" is of the order of the wavelength of the scattered neutrons. This is of the order of 1-10 A for a typical experiment, so that a measurement of the "local" diffusive properties of the water is possible, independent of the presence of barriers or obstructions. In addition, the small size of the "measuring length" permits a study of the dynamics of the diffusion process on a microscopic scale, which is not possible with NMR methods. A review of the use of the QNS technology in the study of the diffusive properties of water in cells and model systems is presented elsewhere [30]. In this reference, it is shown that the value of the diffusion coefficient obtained by QNS measurements in biological systems is reduced from the value for pure water, but the

reduction is not as great as that observed by NMR methods on the same systems. This suggests that obstructions make a significant contribution to the value of the NMR diffusion coefficient and that NMR may have practical value for evaluation of obstruction effects.

Diffusion in an Array of Obstacles

Basic equations

The analysis of the self-diffusion of water in a system with heterogeneous boundaries requires the solution of the diffusion equation for a two-component mixture. The modification of the hydrodynamic equations for this case has been discussed in detail by Landau and Lifshitz [13], and we summarize below the results needed in this paper.

We consider a fluid composed of two components whose mass fractions are c and 1-c. The density of each component is ρc and $\rho(1-c)$, where ρ is the total density of the fluid. (In the general case, ρ would be a function of c, but for the self-diffusion coefficient determined by the spin-echo method, where the "components" are distinguished by their spin orientation, ρ is independent of c.)

In reference [13], a rather general treatment is given that includes diffusion, baro-diffusion and heat conduction. We will consider a system at constant pressure and temperature so that only diffusion occurs. In that case, the equations that govern the motion of the fluid are:

$$\frac{\partial \rho}{\partial t} + \nabla \cdot \rho \mathbf{v} = 0 \qquad (1)$$

(Conservation of mass),
where $\mathbf{v}$ = momentum per unit mass of fluid,

$$\rho\left[\frac{\partial \mathbf{v}}{\partial t} + (\mathbf{v} \cdot \nabla)\,\mathbf{v}\right] = \eta\, \nabla^2 \mathbf{v} \qquad (2)$$

(Navier-Stokes equation),

$$\frac{\partial(\rho c)}{\partial t} + \nabla \cdot \rho c \mathbf{v} = \nabla \cdot (\rho D \nabla c) \qquad (3)$$

(Continuity equation for component of density ρc),

$$\rho = \rho(c) \qquad (4)$$

(Equation of state at constant temperature and pressure).

For small values of $\mathbf{v}$ and ∇c, these equations can be linearized. Equations (1) and (4) are unchanged, and Eq. (2) and (3) become

$$\rho \frac{\partial \mathbf{v}}{\partial t} = \eta\, \nabla^2 \mathbf{v} \qquad (2')$$

$$\frac{\partial(\rho c)}{\partial t} + c\nabla \cdot \rho \mathbf{v} = \rho D \nabla^2 c \qquad (3')$$

For steady state conditions, we obtain

$$\nabla^2 \mathbf{v} = 0 \qquad (2'')$$

$$\nabla^2 c = 0 \qquad (3'')$$

If the fluid is enclosed by boundaries on which $\mathbf{v} = 0$, then $\mathbf{v} = 0$ everywhere. Equation (3") governs the diffusive motion and has been used in the calculations summarized in part B. It should be noticed that in the linearized theory there is no backflow correction, as there would be for a colloidal particle in a fluid. In the continuum approximation, which is the basis of these calculations, the diffusing particles do not produce a first-order hydrodynamic flow.

Approximate Solutions for Steady-State Diffusion through an Array of Obstacles

The theory of the obstruction effect in the self-diffusion of water in protein solutions has been treated by Wang [41] for a random array of spherical and ellipsoidal molecules and by Rorschach et.al. [27] for a hexagonal array of cylinders. An exact solution to the diffusion problem for a cubic lattice of spheres has also been presented by Rayleigh [25]. Maxwell has given a solution for an analogous problem in electrical conduction: the flow of current in a medium containing a random array of spherical inclusions [18].

Wang's solution has been widely quoted in the biological literature. Results are usually given in terms of an "effective" diffusion coefficient D' defined by

$$\langle j \rangle = -D' \frac{\Delta C}{L} \qquad (5)$$

where

$\langle j \rangle$ = average diffusion current density through the array

$\frac{\Delta C}{L}$ = average concentration gradient in the array.

For spheres, Wang obtains the result

$$D' = D\left(1 - \frac{3}{2}\phi\right) \qquad (6)$$

where D is the unobstructed diffusion coefficient and ϕ = volume fraction occupied by the spheres. Errors in this result have been pointed out by Clarke et al. [4] and by Rorschach and Hazlewood [28].

Rorschach et al. [27] have employed a "cellular method" to evaluate the obstruction effect in a regular array of obstacles. (A complete account that corrects some errors in ref. [27] has been prepared for publication and is available from the authors.) For a hexagonal array of cylinders, the results for D' are:

1) Diffusion perpendicular to cylinder axes:

$$D' = D\,\frac{1 - \phi}{1 + 0.80\,\phi + O(\phi^4)} \qquad (7)$$

where $O(\phi^4)$ represents terms of order ϕ^4 and higher.

2) Diffusion parallel to cylinder axes:

$$D' = D(1 - \phi) \qquad (8)$$

For a cubic array of spheres, the "cellular method" gives

$$D' = D\,\frac{1 - \phi}{1 + .63\phi + O(\phi^{10/3})} \qquad (9)$$

which can be compared with the results obtained by Rayleigh [25] and Maxwell [18]:

$$D' = D\left(1 - \frac{3\phi}{2 + \phi - .392\,\phi^{10/3} + \ldots}\right) \quad \text{(Rayleigh)} \qquad (10)$$

$$D' = D\,\frac{1 - \phi}{1 + \phi/2} \quad \text{(Maxwell)} \qquad (11)$$

The cellular method agrees well with the exact solution of Rayleigh, deviating by a maximum of 3% for $\phi \le \frac{\pi}{6}$ (the maximum value of ϕ for close-packed spheres).

In the case of spin-echo measurements of the diffusion coefficient, some modifications of the above equations are necessary. This is most clearly seen for the cylindrical

obstacles for diffusion parallel to the cylinder axes. In this case, the obstruction effect is due entirely to a decrease in the effective cross-section for diffusion by a factor (1-ϕ). The measured spin-echo diffusion coefficient D_S, however, would not be changed, but would have the value $D_S = D$ that would be found in an infinite medium. A detailed analysis shows that the value of D_S is given by:

$$D_S = \frac{D'}{(1 - \phi)},$$

so that the result for a hexagonal array of cylinders in the "cellular method" is

$$D_S = \frac{D}{1 + .80\phi + \ldots} \qquad (12)$$

and for a cubic array of spheres,

$$D_S = \frac{D}{1 + .63\phi + \ldots} \qquad (13)$$

The Direct Hydration Effect

In the previous discussion, we have treated the obstructions as impenetrable obstacles. In a real biological system, these obstacles will be the proteins and other macromolecules and ions, but they will be surrounded by a layer of hydration water which will modify the equations given above. The direct hydration effect has been considered by Wang [41], and he gives the following expression for the total effective diffusion coefficient including hydration effects:

$$D_t = D'(1-f) \qquad (14)$$

where f is the fraction of water that is hydrated. There is some ambiguity in the application of this equation, since it is not clear whether or not the volume fraction f of the obstructions should include the hydration water. A treatment of this problem with the "cellular method" in which the cylindrical obstacle is surrounded by a sheath of non-diffusing hydration water shows that the influence of the direct hydration is to multiply Eqs. (7) and (8) by the factor (1-f), and that ϕ should include the volume of the hydration water. In this case, the right hand sides of Eq. (12) and (13) must also be multiplied by (1 - f):

$$D_S = \frac{D(1 - f)}{1 + .80\phi + \ldots} \quad \text{(cylinders)} \qquad (15)$$

$$D_S = \frac{D(1 - f)}{1 + .63\phi + \ldots} \quad \text{(spheres)} \qquad (16)$$

NMR Measurements of Water Diffusion

Basic Method

The measurement of diffusion coefficients with NMR is based on the diffusion of magnetization in an inhomogeneous magnetic field. Since the magnetization is carried by the nuclear moments, it is the self diffusion coefficient that is measured in a single-component system, designated above by D_S. Diffusion in a weak magnetic field gradient has two influences on the magnetization. It produces a small change in the longitudinal relaxation time [32], and it produces a decay in the transverse component of the magnetization that is easily observable in the spin-echo method [1]. The influence of the field gradient on the transverse decay cannot be described by a relaxation time. The form of the decay can be determined from

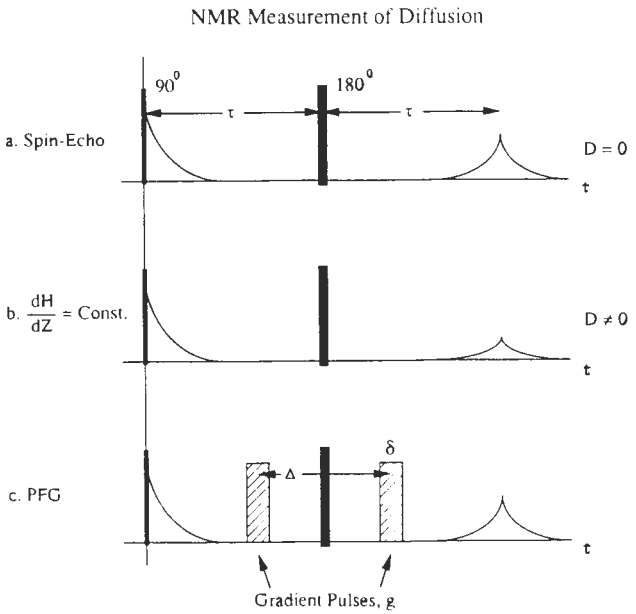

Figure 1. RF pulses (90° and 180°), gradient pulses δ and nuclear signals obtained in a spin-echo sequence. (a) Without field gradient and D = 0. (b) With applied steady field gradient and D ≠ 0. (c) Pulsed field-gradient sequence.

the Bloch equation to which a diffusive term has been added [38], and the solution shows that the amplitude of the echo at t = 2τ is governed by the equation

$$A(t) = A(0) \exp(-\gamma^2 G^2 D t^3/12 - t/T_2) \qquad (17)$$

where τ is the 90°-180° pulse separation, A(0) is the amplitude of the free-induction decay at t = 0, γ = gyromagnetic ratio, G the field gradient along the resonance field $\mathbf{H}_0$, D the (self) diffusion coefficient, and T_2 the transverse relaxation time. This is illustrated in Fig. 1, where the echo for a 90-180 pulse sequence is shown, without a field gradient in (a) and with a field gradient in (b). This method, referred to as "method A" by Abragam [1], is often used with a fixed time t = 2τ. The echo amplitude A(t) is measured as a function of the gradient G, from which D can be determined. A second method ("method B") makes use of a series of 180° pulses. Although the decay during the interval between pulses follows the t^3 relation, as in equation (17), the refocusing of each 180° pulse gives an overall decay for the n'th pulse given by

$$A(n) = A(0) \exp[-2\gamma^2 G^2 D n\tau^3 /3 - t/T_2] \qquad (18)$$

or, since t = 2nτ,

$$A(t) = A(0) \exp[-\gamma^2 G^2 D \tau^2 t/3 - t/T_2]. \qquad (19)$$

There are two disadvantages to the constant-gradient methods described above. Firstly, there is a limitation on the size of the gradient that can be imposed, since large gradients lead to a narrowing of the echo-width. Extremely narrow echoes are difficult to measure accurately, and so we are limited to relatively weak gradients, which means that D must not be too small. Secondly, the "measuring time" during

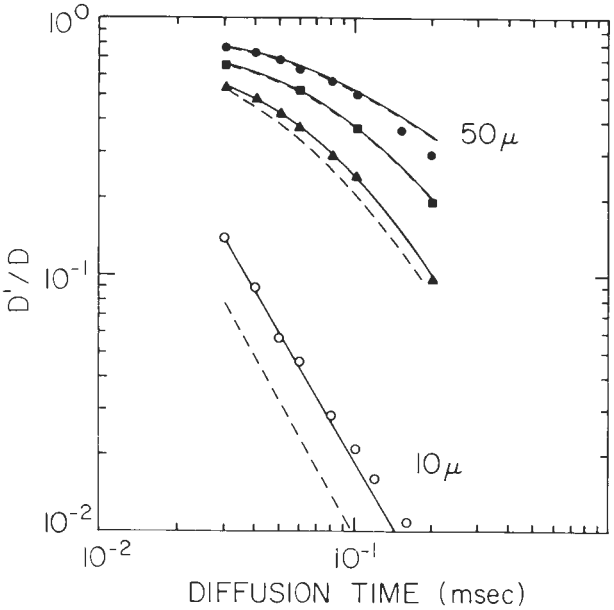

Figure 2. The experimental and calculated restricted diffusion coefficients displayed as a function of diffusion time. Experimental results: 10 μm sample, o; 27 μm sample, ▲; 37 μm sample, ▪; and 50 μm sample, ●. The solid curves are from the theory of Neuman [23] in which the capillary diameters are adjusted for the "best fit". The dashed curves are from Neuman's theory with the measured mean capillary diameters. (From Lauffer [14]. Reproduced from the Physical Review, 1974, v. 9A, pp 2792-2798, by copyright permission of the American Physical Society.)

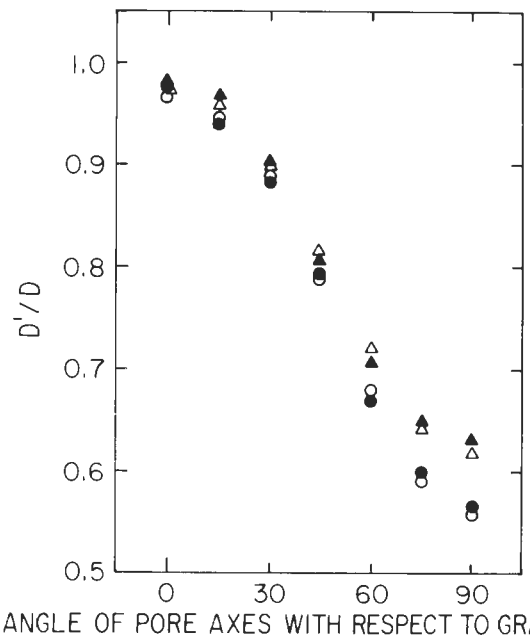

Figure 3. Restricted diffusion coefficients for the 27 μm sample at diffusion times of 30 ms (▲, exp.; Δ, calc.) and 40 ms (●, exp., o, calc.) displayed as a function of orientation. (From Lauffer [14]. Reproduced from the Physical Review, 1974, v. 9A, pp 2792-2798, by copyright permission of the American Physical Society.)

which diffusion occurs is approximately 2τ. (A detailed analysis [35] shows that the "measuring time" is actually $2\tau/3$, since the motion near the 180° pulse has more influence on the decay than that near the 90° pulse or the echo.) If we wish to study the dependence of D on diffusion time, we must vary τ. But τ is limited on the low side by the width of the free-induction decay and on the high side by T_2, so that there is not a wide latitude (in practice, $\tau \sim$ 10-100 msec).

In order to overcome the problems of the constant gradient methods, the pulsed-field-gradient (PFG) method [35] has come into use. In this method, the field gradient is applied during the intervals on each side of the 180° pulse, as shown in Fig. 1 (c). These field-gradient pulses have the same influence on the echo amplitude as a steady gradient, except that we now avoid the problems of the steady gradient method. The echo is now observed with no applied gradient so that it is not narrowed. The "measuring time," which is $\Delta - \delta/3$, is well-defined and can be varied over a wide range without changing the time τ. This method is very useful for the study of biological systems, since the measuring time can be reduced to approximately ~ 0.1 msec, which gives a "measuring distance" $d_m = \sqrt{2Dt} \sim 1/2$ μ. This permits a study of the influence of cell membranes as diffusion-limiting barriers.

Compartments and Obstructions

The interpretation of spin-echo measurements of the diffusion of water in cellular systems is complicated by the heterogeneous character of the water environment. The free diffusion of the water molecules is hindered by their interaction with the cellular macromolecules and membrane structures. The "effective" diffusion coefficient D' depends on the distance that the molecules diffuse during the measuring time 2τ, and this distance will be shortened if obstructions or barriers are present.

Barriers and Compartments. The influence of impermeable planar barriers and compartments with cylindrical and spherical symmetry has been investigated theoretically by Robertson [26] and by Neuman [23]. A calculation for permeable planar barriers has been presented by Tanner [36]. An experimental study of the impermeable planar-boundary case was made by Wayne and Cotts [42], and good agreement was found with Robertson's calculations. A careful study for cylindrical boundaries has been carried out by Lauffer [14]. These measurements were made by "method A" described above with a variable measuring time 2τ . Figure 2 shows some results of this work. The ratio D'/D of the "effective" diffusion coefficient to the "free" diffusion coefficient is plotted as a function of diffusion time 2τ for capillaries whose diameter is 10-50μ. Diffusion was measured perpendicular to the cylinder axes. The dotted lines are calculations from Neuman's expression for D'/D, and the agreement is good except for the smaller capillaries, for which there is some uncertainty in the size determination. The anisotropy of the diffusion in this restricted geometry is shown in Figure 3. The diffusion parallel to the cylinder axis is unaffected by the barriers.

Obstructions. The presence of small quasi-spherical obstructions within the cell (small organic molecules, globular proteins, etc.) will also influence the diffusion coefficient as

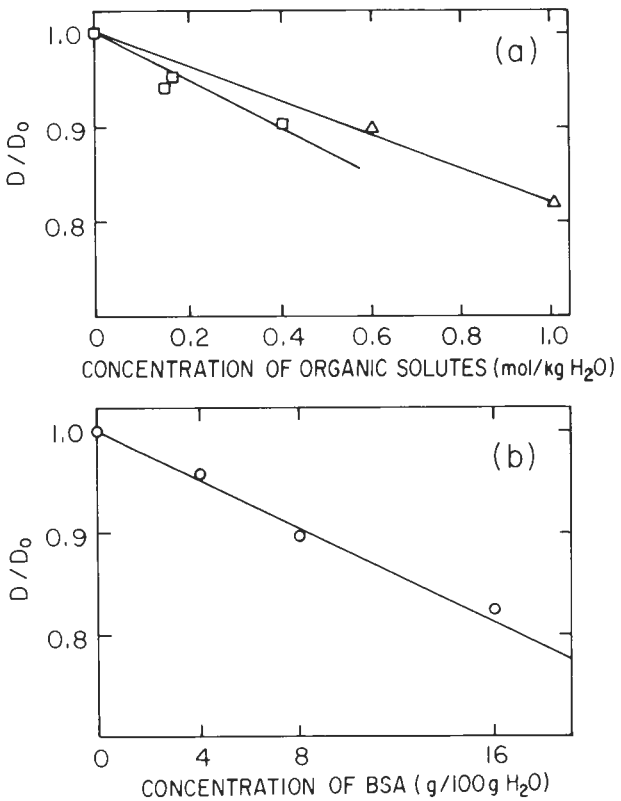

Figure 4. The relative self-diffusion coefficient of water, D/D_0, in artificial mixtures of solutes found in barnacle muscle as a function of concentration. (a) Salt-propionate solutions and small nitrogenous organic solutes. ▬, propionate-containing solutions. Δ, solutions containing small organic molecules. (b) bovine serum albumin. (From Clark, et. al. [4]. Reproduced from the Biophysical Journal, 1982, v. 39, pp 289-299, by copyright permission of the Biophysical Society.)

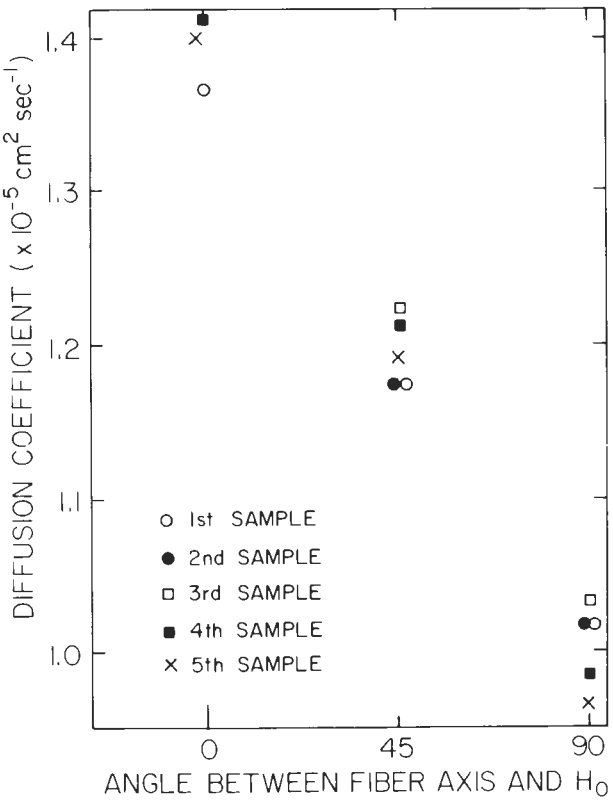

Figure 5. The spin-diffusion coefficient for oriented muscle fibers as a function of the angle in degrees between the fiber axis and the external magnetic field, H_0. The spin-echo measurements give the value of D_S along H_0. (From Cleveland, et. al. [5]. Reproduced from the Biophysical Journal, 1976, v. 16, pp 1043-1053, by copyright permission of the Biophysical Society.)

measured by the spin-echo method. The effective diffusion coefficient will be reduced by virtue of the obstruction of some of the possible diffusion paths, as described in detail earlier in this paper. These effects are not so dramatic as in the case of barriers, since the path length is increased only slightly. For an extreme case, we can consider closed-packed spherical obstructions. For diffusion through a diameter d, the path will be of length $\pi d/2$ along a circumference. The diffusion coefficient will be decreased by $\sim(2/\pi)^2 = 1/2.5$, in rough agreement with the value expected from Eq. (13) for $\phi = 1$.

A study of the influence of some small molecules and Bovine Serum Albumin (BSA) on D'/D has been carried out by Clark et al. [4] as part of a comprehensive study of water in barnacle muscle. Their results are shown in Figure 4. It is difficult to compare these results directly with the theory (Eq. (15) & (16)), since the (hydrated) volume fraction occupied by the solutes is not known. In fact, Clark et al. make use of the theory to estimate the hydration, on the assumption that the hydration water is non-diffusing and forms part of the obstruction. The effects are significant but not dramatic: a 20% reduction in D'/D is produced by 1 mole organic molecules per kg of H_20 or by 0.2 grams of BSA per gram of H_20.

Some Results on Biological Systems

A comprehensive survey of NMR measurements of the diffusion coefficient for water in biological systems has been given by Hazlewood [11]. The results given in Table IV show that $0.1 < D'/D < 0.8$, with the largest value for chicken egg white and the smallest for red blood cells [11]. Typical values for skeletal muscle are $D'/D \approx 0.5$. The main question concerning these measurements is the degree to which they can be explained with obstruction and compartment effects. We will discuss several recent measurements of diffusion which address this question.

Cleveland et al. [5] have studied the diffusion of water in skeletal muscle of the rat. The striated character of the muscle, due to the oriented action-myosin filament system, permitted a study of the anisotropy of the diffusion coefficient. The results are shown in Fig. 5. The diffusion coefficient for motion perpendicular to the fiber axes, $D(90^o)$, is less than the value for motion parallel to the axes, with the ratio $D(0^o)/D(90^o) = 1.4$. If this ratio is entirely due to the obstruction effect, then Eqs. (7) and (8) require that $D(0^o)/D(90^o) = 1+0.8\phi$, which requires that $\phi = 0.5$, where ϕ is the volume-fraction of the myosin-actin system. This is far larger than the value calculated from the actual dimensions of the filaments as determined e.g. by X-ray and electron-microscope studies, which give $\phi \approx 0.16$. So, there would have to be a

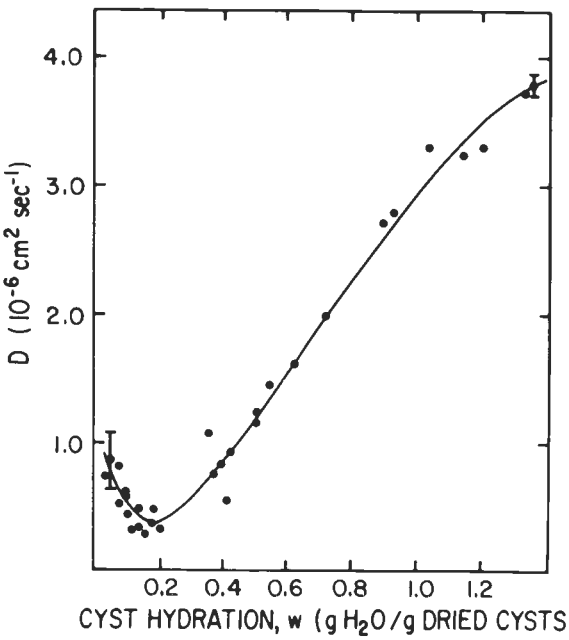

Figure 6. Values of the NMR-measured self-diffusion coefficient D of water as a function of hydration (g H_2O/g dry weight) for cysts of the brine shrimp Artemia. (From Seitz, et. al. [33]. Reproduced from the Archives of Biochemistry and Biophysics, 1981, v. 210, pp 517-524, by copyright permission of Academic Press.)

substantial fraction (~ 40%) of the cell water associated with the filament system if the obstruction model is to explain the anisotropy. For diffusion parallel to the axes, $D(0^o) = 1.4 \times 10^{-5}$ cm^2/s, which gives $D(0^o)/D(H_20) = 0.6$. If one attempts to ascribe this decrease to spherical non-filamentary obstructions, a value of $\phi \approx 1$ is required (see Eq. (13)). If we include the direct hydration effect and assume that there are no non-filamentary obstructions, then the volume fraction of hydration water associated with the filaments is $f \approx 0.40$. In either case, a large fraction (~40%) of the cytoplasmic water does not diffuse as "free" water.

A similar conclusion was reached by Clark et al. [4] in their study of water in barnacle muscle. The measurements were made with a modification of "method A" with $6 < 2\tau < 30$ msec. They attempted to isolate the influence of the filamentary structures by conducting a separate study of the reduction in diffusion by the other macromolecular obstacles in the cytoplasm. The obstruction effect of the filaments alone was estimated by use of an equation of the form

$$\frac{D'}{D} = \frac{(1 - f)}{1 + .67\phi + \ldots} \qquad (20)$$

correct to 1st order in ϕ, where D' is the measured diffusion coefficient in the muscle fiber-cytoplasm system and D is the diffusion coefficient in the cytoplasm without fibers. (Eq. (20) differs slightly from Eq. (15).) They obtain a filament hydration H in the range $0.65 < H < 1.46$ gm H_20/gm filaments. The smaller value is for fresh fibers, the larger for membrane damaged fibers swollen by saline. These values are about ten times larger than values previously estimated from NMR studies [3].

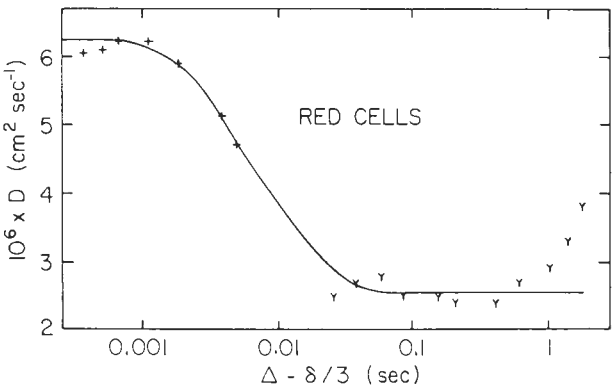

Figure 7. Self-diffusion coefficients of water in human red cells vs diffusion time at 27° C. Solid curve is drawn as a guide to the eye. +, alternating gradient sequence; Y, stimulated echo sequence. (From Tanner [37]. Reproduced from the Archives of Biochemistry and Biophysics, 1983, v. 224, pp 416-428, by copyright permission of Academic Press.)

Measurements of the diffusion coefficient over a wide range of hydration have been carried out by Seitz et al. [33] on cysts of the brine shrimp artemia. The cysts consist of about 4000 cells in a non-differentiated shell that is spherical when fully hydrated. The hydration can be varied over a wide range (0 - 1.3 gm H_20/gm dried cyst) while still retaining viability. The results of the measurement of the diffusion coefficient as a function of hydration are shown in Fig. 6. The measurements were made with "Method A" with $2\tau = 40$ ms. In this relatively "dry" biological system, there is a dramatic reduction in the diffusion coefficient. In the most highly hydrated samples (1.3 gm/gm), the value of D is reduced by a factor of 6 compared with the pure water value. For the lowest hydration for which reliable measurements could be made (~0.2 gm/gm), D is reduced by a factor of nearly 100. It is clear that these reductions cannot be explained by Eq. (16) for any reasonable choice of the parameters, if we assume that the only influence of the macromolecules is to provide obstructions. Even if $\phi = 1$, we require that $f \approx 0.99$, that is, all of the cytoplasm is hydration water. Yet normal metabolic activity occurs for hydration values greater than 0.6 gm/gm [33], so that enzymatic reactions must occur as in a solution. The diffusive properties of the entire cytoplasm appear to be strongly affected by the presence of the cellular macromolecules, and this influence increases as the hydration is reduced.

A study of water diffusion in human red cells, yeast and cysts of the brine shrimp (artemia) has been carried out by Tanner with the PFG method [37]. The measuring time

$$\Delta - \delta/3$$

was varied over the range 0.1 - 1000 msec, so that the influence of the cell membranes as permeable barriers could be studied. The diffusion coefficient for red cells as a function of measuring time is shown in Figure 7. As the measuring time is reduced below 1 second, there is a transition from $D = 2.5 \times 10^{-6}$ cm^2/sec to $D = 6.3 \times 10^{-6}$ cm^2/sec. This increase in D is ascribed to the influence of the permeable cell membranes, which are ineffective below $\Delta - \delta/3 \sim 1$ msec. From these results and his previous calculations on permeable planar barriers [36], Tanner obtains a permeability of P = 0.011 cm/s and an average barrier separation of 8 μm. At the shortest measuring time, the value of D is reduced by a factor of 4

compared with the "free" water value. This reduction cannot be explained with Eq. 13 as a simple obstruction effect with "free" cell water. If we include the direct hydration effect (Eq. (16)) and assume $\phi = 1$, we still require that $f \approx 0.6$, i.e. 60% of the cell water would have to be hydration water.

Conclusions

The NMR measurements of water diffusion have provided important information on the structure of water in biological systems. All of the measurements show that the diffusion coefficient is reduced, and in some cases the reduction is substantial. A part of this reduction can be explained by the presence of membrane barriers and macromolecular obstacles that hinder diffusion in the cytoplasm. It is not possible, however, to explain all of the reduction in diffusion by these mechanisms. It is also necessary to suppose either that a substantial fraction (20-40%) of the cell water is hydration water, or that the diffusion coefficient of the cytoplasmic water is reduced substantially from the free water value. In either case we are driven to the conclusion that a substantial fraction of the cell water has diffusive properties that are altered by the macromolecules of the cytoplasm.

There are two difficulties that lie in the way of a more detailed study of the diffusion process with NMR. Firstly, even the most modern PFG methods do not permit the reduction of the measuring time below about 0.1 m sec. This means that the influence of barriers and obstructions whose scale is below about 1 μm cannot be studied. The measured diffusion coefficient will be an average over the heterogeneous cellular structure at this scale. This leads to the second difficulty, which is the uncertainty in modeling this heterogeneous structure. Various models can be used, as described in this paper, but they are approximations whose uncertainties are hard to estimate. It would be most helpful if the measuring distance could be reduced to the scale of the macromolecular separation. It is possible to do this with the use of neutron scattering techniques.

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Discussion with Reviewers

L. Edelmann: It has been shown that certain protein solutions and polymer solutions have reduced solubility for sucrose, Na salts and other solutes; and it is assumed that most of the water in these solutions is affected by macromolecules in a similar way than the water in the cytoplasm of living cells. Since these solutions are stable systems without the heterogeneous character of the water environment as is usually the case in living cells, wouldn't it be worthwhile to investigate the diffusive properties of water in these solutions? Did you try or do you plan to model the structure of polymer solutions of different compositions and hence different solvency properties and compare the diffusion coefficients obtained with NMR and QNS measurements?

Authors: We agree that simultaneous NMR and QNS studies of polymer solutions would be useful, and some measurements have been made that bear on this comparison. We list below some neutron scattering and NMR results (some of them unpublished).

System	$\frac{D'}{D}$ (QNS)	$\frac{D'}{D}$ (NMR)
Pure water	1.0	1.0
Frog muscle	0.6	0.5
Artemia cysts(1.2g/g)	0.30	0.15
Agarose (20% sol.)	0.8	0.63
Polyox (38% sol.)	0.42	0.4-0.5

The agreement is good, with the NMR values being slightly lower, presumably because of the influence of barriers and obstructions. (It should be noted that both neutron and NMR values for D'/D will be influenced by water adsorption and desorption on the polymers or proteins.)

D. LeBihan: Preliminary studies of diffusion measurement in vivo have been published, including human studies, using NMR imaging (JMRI 1:7-28,1991). One important question is to find out whether water diffusion differs from that measured in vitro. A possible difference could reflect a particularity of the water molecular organization in living tissues, but also additional sources of water motion ("water streaming") associated with cell function, that could not be separated from actual diffusion in NMR experiments.

Another interesting issue regards the origin of the interaction between water and macromolecules. This question could be partially answered by measuring the activation energy associated with diffusion. The temperature-diffusion relationship for water diffusion gives an activation energy compatible with the energy required to break two hydrogen bonds. For water molecules interacting with macromolecules in the hydration layer, other types of interaction should be detected, such as Van der Waals interaction, for which another value of the activation energy should be found. Have the authors done some experiments in this direction?

Authors: We agree that the results from in vivo studies may differ from the in vitro results. In our own "in vitro" work on the diffusive motion of water in biological systems (both NMR and QNS), we have tried to insure that the living state was intact, in the sense that the condition of the cytoplasm, especially the cellular level of ATP, was the same as in the normal functioning cell. Any "water streaming" that is associated with intra-cellular function should be observable under these conditions. On the other hand, the perfusion essential for the maintenance of the living state that is always present in the "in vivo" measurements is absent in these experiments, and this inter-cellular environment undoubtedly makes an important contribution to the diffusive/perfusive transport that will affect MR images. We expect that eventually it will be possible to separate perfusion from diffusion, and we believe that the in vivo and in vitro measurements will provide complementary information that will help with the interpretation of MR images.

Additional work on the activation energies for diffusion as a function of hydration and of the non-frozen fractions of water would be most informative. We have not pursued this problem.

G. N. Ling: The reader may be puzzled by the statement of Drs. Rorschach and Hazlewood that "The nature of water-macromolecular interaction and its influence on the structure and dynamics of cellular water are still unresolved." I think the apparent conflict between my own view on the matter and that of the authors may lie in a different concept of what constitutes a "resolution". I am satisfied with a less exact but, on the whole, correct picture, believing that in biology another kind of "uncertainty principle" may operate. To wit, the precision and exactness that physicists have long become accustomed to from their highly successful study of the vastly simpler inanimate systems may not be achievable in the living systems, because they may not be there. The resolution which I believe that we have already achieved in regard to the water-macromolecular interaction is in choosing between (1) the conventional membrane-pump model in which the bulk of cell water is normal liquid water and there is little or no interaction between the bulk-phase water and cell macromolecules (2) the association-induction model in which the bulk-phase water in living cells exists as polarized multilayers, interacting strongly

and pervasively with certain intracellular macromolecules, i.e., fully-extended proteins. Having said this, I must add immediately that I do not mean to say that a more refined polarized-multilayer theory may not be achieved in the future. In fact, that is almost certain to happen. However, the resolution between that refined polarized-multilayer theory and its present cruder counterpart would be the resolution among different models of the same kind, and thus not to be compared in overall significance with the resolution having just been achieved: between the free-water theory and the polarized-water theory.

Authors: We agree with Dr. Ling that the conflict between us over the use of the world "unresolved" in referring to the nature of the water-macromolecular interaction is more apparent than real. From our point of view, in living cells we lack quantitative knowledge about the structural properties of the water molecules (e.g. the radial distribution function and the space-time correlation function.) Ling's AI hypothesis is not yet sufficiently detailed to permit a calculation of the NMR relaxation times nor the diffusive properties of cellular water. A more detailed microscopic picture will ultimately be required to resolve this problem. Nevertheless, there is sufficient data about the diffusive motion of water molecules in biological systems to make at least two qualitative statements: (1) within the cell, the amount of water experiencing reduced diffusive motion is substantial; and, (2) the rotational motion of the majority of the water molecules is reduced significantly from that of ordinary water. We feel it is important for any model of cellular function to account for these general observations about the diffusive motion of water molecules. Our findings support in a general way Dr. Ling's hypothesis that cellular water exists in polarized multilayers as over against the conventional view that cellular water properties are unaffected by the macromolecules, but they do not yet provide a basis for a definitive microscopic picture.