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THE STATE OF WATER IN LIVING TISSUES (RESULTS OF INVESTIGATIONS BY THE METHOD OF YaMR SPIN ECHO) *

[Following is the translation of an article by L. M. Abetsedarskaya, F. G. Miftakhutdinova, and V. D. Fedotov, Biological Institute of the Kazan State University imeni V. I. Ulyanova-Lenina, published in the Russian-language periodical Biofizika (Biophysics) Vol XIII, No 4, 1968, pages 630--636. It was submitted on 2 Feb 1967.]

The question of the state of water in living tissues is one of the fundamental problems of biology. Dependent on what state the water in tissues is found in are the approaches to the solution of many important problems of physiology, such as the transport of substances in the cell, transmission of energy, problems of permeability, etc.

There are two basic concepts about the state of water in tissues. According to the first concept the main part of water within the cell is assumed "free" and in essence in the same state within and outside of the cell, and only a small part of the intracellular water is "bound" due to hydration and differs in its properties from ordinary water. According to the second concept water, ions, and biopolymers within the cell form a highly regulated unique system. This system has the properties of a lattice in which the water component fundamentally differs in structure and properties from ordinary liquid water.

The present work is devoted to a direct experimental study of the state of water in living plant and animal tissues by the method of nuclear magnetic resonance (spin echo).

* YaMR - NMR, nuclear magnetic resonance.

Materials and Methods

A study was made of the time of spin-spin relaxation (T_2), spin-lattice relaxation (T_1), and coefficient of self-diffusion (D) of water in solutions and gels of proteins and in living plant and animal tissues. Investigations were made of bovine serum albumin (Lawson Firm, London), egg albumin (obtained by threefold reprecipitation of ammonium sulfate), the liver and gastrocnemius muscle

of a frog (Rana esculenta), leaves and roots of maize sprouts (Zea mays), and Russian beans (Vicia faba).

The spin echo method was used for studying the time of proton relaxation and coefficient of self-diffusion of water. The operational frequency of the unit was 20 MHz. The time of spin-spin relaxation was measured by the method of Carr-Purcell /1/. For plant tissues the influence of proton exchange on spin-spin relaxation was eliminated by the method of Allerhand and Gutovsky /2/. The time of spin-lattice relaxation was measured either by the 90—90° method of Hahn or with the help of the zero-method of Carr-Purcell /1/. Coefficient of self-diffusion was measured by the method of Hahn-Woessner /4/ and Ghosh and Sinha /5/. Accuracy of measurement was $T_1 \pm 7\%$, $T_2 \pm 10\%$; $D \pm 5\%$. The temperature of the specimens during measurement was maintained with the help of a liquid ultrathermostat U—8 with an accuracy of $\pm 1^{\circ}$. The time of heating at a given temperature together with the time spent for measurement equaled 40—50 minutes.

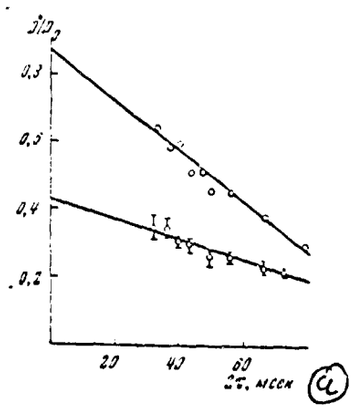
Results and Discussion

As our investigations showed, measurable coefficients of self-diffusion of water in various plant and animal tissues depend strongly on the time of observation (i.e., on the time 2τ between the first applied radio-frequency pulse and the echo), which in our experiments changed from 20 to 80 ms. According to Woessner /6/ such a dependence is observed in the presence of barriers which limit the diffusion of water, i.e., during diffusion which is not in an infinite reservoir. In this case the average displacement of molecules in time of observation 2τ will be less than in an infinite reservoir, and the measurable coefficient of self-diffusion D^* with a definite 2τ will be the function of displacement, traversed by a molecule of water in an infinite reservoir during time 2τ, and the function of average distance to the barriers. If one were to take the time of observation as lesser and lesser so that the average displacement which a molecule goes through during the time of observation becomes much less than the average distance to the barriers, i.e., so that fewer and fewer of the molecules experience the influence of the barriers, then at $2\tau \to 0$ the measurable coefficient of self-diffusion D^* will strive for the true value of the coefficient of self-diffusion in a medium which is inclosed between the barriers. The drawing shows the dependence of the measurable coefficient of self-diffusion D^* on the 2τ for roots of beans and the liver of a frog.

The distance between limiting barriers, evaluated from our experimental data for various tissues, turned out to be on an order of tens of microns. Such limiting barriers for animal tissues may be plasmalemma, and for plant tissues - plasmalemma and to some degree tonoplast. Consequently it can be assumed that the coefficients of self-diffusion of water cited in Table 1 for various

plant and animal tissues at $2\tau \to 0$ characterize the mobility of water directly in the cell. *

* The coefficients of self-diffusion D cited in works [7—10] for various plant tissues are not the coefficients of self-diffusion of intracellular water, since these were coefficients of self-diffusion which were measured at the same $2\tau = 80$ ms, and the influence of limiting barriers was not excluded. Besides this, for plant tissues they did not exclude the contribution of proton exchange in the coefficient of self-diffusion of water [11] which was measured by the method of Ghosh and Sinha [5].



Dependence of measurable coefficient of self-diffusion (D*) on the time of observation (2τ) for roots of beans (1) and for frog liver (2) at 35°C.
Key: (a) 2τ, ms.

As can be seen from Table 1, the coefficients of self-diffusion of water for plant cells is somewhat higher than for animal, which is apparently explained by the development of a vacuole in plant cells, the volume of which, according to the data from a number of authors [12—15], can reach 30—50% of the internal volume of the cells.

Since the coefficient of self-diffusion D in the protoplasm of animal cells is all told 1.3—1.8 times less than in plant cells, it is apparent that the coefficient of self-diffusion of water in the protoplasm of plant cells will be on a value of the same order, and this means that the coefficients of self-diffusion of water in protoplasm (D_1) and vacuoles (D_2) are sufficiently close. Therefore, even if there is no averaging of the coefficient of self-diffusion of water for the vacuole and protoplasm, then the amplitude of the echo is the sum of the two exponents

$$A(\tau, G) = A_1 e^{-\frac{1}{3}\gamma^2 G^2 D_1 \tau^3} + A_2 e^{-\frac{1}{3}\gamma^2 G^2 D_2 \tau^3},$$

where A - amplitude of echo; A_1 - amplitude of signal at $\tau = 0$ from protoplasm; A_2 - amplitude of signal at $\tau = 0$ from vacuole; γ - gyromagnetic ratio; G - gradient of magnetic field; 2τ - time of observation; D_1 - coefficient of self-diffusion of water in protoplasm; D_2 - coefficient of self-diffusion of water in a vacuole.

Table 1

Experimental and calculated values D, $T_{1,2}$, and ν for solutions of proteins and various living tissues

	C*	$D \cdot 10^5$ в клетке, см²/сек ±12% (a)	T_2, мсек ±10% (b)	T_1, мсек ±10% (c)	$D \cdot 10^5$ протоплазмы, см²/сек (d)	ν**
e Вода	—	2,4	2500	2500	—	—
f Яичный альбумин	9,1	2,06	600	1150	—	0,07
g Бычий сывороточный альбумин, раствор	20,0	1,93	150	340	—	0,065
h Бычий сывороточный альбумин, гель	20,0	1,90	40	440	—	0,24
i Листья кукурузы	10,5	2,0	96	250	1,82—1,60	0,32
j Цитоплазма + вакуолярный сок из листьев кукурузы	7,8	2,1	—	—	—	—
k Листья бобов	9,5	1,81	110	300	1,56—1,20	0,32
l Корни кукурузы	9,1	1,76	133	885	1,49—1,12	0,12
m Икроножная мышца лягушки	19,5	1,56	47	658	1,56	0,14
n Печень лягушки	22,1	1,02	46	270	1,02	0,20

* Concentration of dry substance, % by weight.
** Amount of "hydrate" water in 1 g of H_2O per 1 g of dry substance.
Key: (a) $D \cdot 10^5$ in a cell, $cm^2/s \pm 12\%$; (b) T_2, ms $\pm 10\%$; (c) T_1, ms $\pm 10\%$; (d) $D \cdot 10^5$ in protoplasm, cm^2/s; (e) water; (f) Egg albumin; (g) Bovine serum albumin, solution; (h) Bovine serum albumin, gel; (i) Maize leaves; (j) Cytoplasm and (+) vacular sap from maize leaves; (k) Leaves of beans; (l) Roots of maize; (m) Gastrocnemius muscle of frog; (n) Liver of frog

Thanks to the fact that values D_1 and D_2 are close, all the same the average coefficient of self-diffusion of water will be measured [15]. Since the value of coefficient of self-diffusion of water of the cytoplasm and vacular sap from the cells of maize leaves (where averaging of the coefficient of self-diffusion takes place deliberately) is very close based on the value of the coefficient of self-diffusion in intact cells of maize leaves (Table 1), one can consider

that the measurable coefficient of self-diffusion is the average-weight coefficient of self-diffusion of protoplasm and vacuole.

Stemming from what was said above and accepting that the coefficient of self-diffusion of water in vacuoles at 25° is equal to $2.4 \cdot 10^{-5}$ cm²/s (i.e., to the coefficient of self-diffusion of pure water) and the volume of a vacuole comprises 30—50% of the internal volume of cells, calculations were made of the coefficients of self-diffusion of water in the protoplasm of plant cells. These are presented in Table 1. It turned out that the coefficients of self-diffusion of water in the protoplasm of both animal and plant cells are only 1.3—2.4 times lower than in pure water and are comparable with the coefficients of self-diffusion of water in solutions and gels of proteins. Thus, for a 19% solution of egg albumin the coefficient of self-diffusion of water is 1.5 times lower than in pure water.

What are the possible causes of a lowering of the coefficient of self-diffusion of water in solutions and gels of proteins and in protoplasm?

According to Wang [16], in solutions of protein the self-diffusion of water is less than in pure water for two reasons.

1. Protein molecules have a by far greater volume and by far less self-diffusion than molecules of water. These large and almost immobile protein molecule-microbarriers prevent the movement of a molecule of water, i.e., molecules of water in the vicinity of protein molecules should diffuse along a longer path in order to appear on the other side of a molecule of protein. Therefore the coefficient of self-diffusion of water in solutions of protein will be less than in pure water.

2. The second cause, decreasing the coefficient of self-diffusion of water in protein solutions, is the hydration of proteins. But since hydration, according to Samoylov [17], can be both positive and negative, i.e., the structure of water in the immediate vicinity of protein molecules can be strengthened and broken down, then due to hydration there is also the possibility of a lessening of the coefficient of self-diffusion of water depending on which hydration predominates.

According to Wang [16], the coefficient of self-diffusion of water in solutions of macromolecules equals

$$D = D_0(1-\bar{a}\varphi)\left(1-\frac{c}{c_0}\right).$$

D_0 - coefficient of self-diffusion of pure water; $\bar{a}$ - dimensionless numerical coefficient, changing from 1.5 to 3 depending on the form

of the protein molecule; φ - total volumetric part, occupied by the hydrated protein molecule; C_h - concentration of hydrate water; C_0 - total concentration of water.

For a 10.6% solution of egg albumin (Table 2) the coefficient of self-diffusion of water D due to all causes is reduced from $2.4 \cdot 10^{-5}$ cm²/s for pure water to $1.97 \cdot 10^{-5}$ cm²/s. Due to only the effect of microbarriers the coefficient of self-diffusion would be reduced from $2.4 \cdot 10^{-5}$ to $2.02 \cdot 10^{-5}$ cm²/s, and due to the effects of hydration would be reduced from $2.4 \cdot 10^{-5}$ to $2.35 \cdot 10^{-5}$ cm²/s.

Table 2

Coefficients of self-diffusion of water in solutions of egg albumin (experimental values and values calculated with consideration of various effects)

	$D \cdot 10^5$, $D' \cdot 10^5$, $D'' \cdot 10^5$, cm²/s (a)		
(b) Вода	2,4	—	—
(c) Яичный альбумин, 10,6%-ный раствор	1,97*	2,02	2,35
(d) Яичный альбумин, 19%-ный раствор	1,62*	1,71	2,28
(e) Яичный альбумин, 9,1%-ный раствор	2,06	2,10	2,35

D' - value of coefficient of self-diffusion in a solution if the change in diffusion would be due only to the effect of microbarriers.

D'' - coefficient of self-diffusion of water in a solution if the change would be due only to the effect of hydration.

* Based on the data of Wang [16].

Legend: (a) cm²/s; (b) Water; (c) Egg albumin, 10.6% solution; (d) Egg albumin, 19% solution; (e) Egg albumin, 9.1% solution.

Thus a very significant part of the lowering of the coefficient of self-diffusion in protein solutions is conditioned by the effect of microbarriers, and only a small share - by the effect of hydration.

In exactly the same manner as in the case of solutions of protein, the effect of microbarriers for the protoplasm of plant and animal cells should be significant, since in the cell is found a multitude of microscopic and submicroscopic formations - chloroplasts, the nucleus, mitochondria, microsomes, endoplasmic reticulum, etc., and also dissolved macromolecules, which will exert an inhibiting action on the self-diffusion of molecules of water. Components of cytoplasm, based on approximate calculations stemming from the data of Vild'man and Kogen [17], for plant cells occupy 30% of the volume

of protoplasm, and for animal tissues, the liver in particular, - 50—60 [17]. If the Wang formula is used and a calculation is made of such a quantity of microbarriers, then only the effect of the microbarriers is more than sufficient to explain the resulting lowering of values for D for the protoplasm of cells of various tissues, since for the lowering of the value of D which was observed it is sufficient if the volume of microbarriers comprises 20—40% of the volume of the protoplasm. Besides this, a lowering of the coefficient of self-diffusion of water in the protoplasm can possibly be due to a lowering of the coefficient of self-diffusion of the fraction of water which is found in the organelles due to the action of the membranes of the organelles as limiting barriers, even with the complete preservation of the mobility of water within the organelles. Therefore, the lowering of the coefficient of self-diffusion of water in protoplasm due to hydration should be quite small, and the coefficients of self-diffusion of water in protoplasm reflect mainly the structural peculiarities in the protoplasm of cells of various tissues which are conditioned by the number and quality of microscopic and submicroscopic particles.

It follows from what has been said that the self-diffucion of water between microbarriers is very close to the value of self-diffusion of pure water.

As is known, conditions of translation movement in a pure liquid, mainly in solutions of low-molecular substances, are determined by the structure of the liquid [17, 20, 21]. Therefore the value of the coefficient of self-diffusion of water in the medium between microbarriers in a protein solution or in protoplasm is a characteristic of the structure of water of this medium. Consequently, on the basis of the conclusion made above that the coefficient of self-diffusion of water between microbarriers in protoplasm is close to the coefficient of self-diffusion of pure water at a given temperature, it is possible to assume that the structure of water of a hyaloplasm is very close to the structure of pure water.

The greater short-range orderliness which is inherent to water than to other liquids is conditioned by the ability of molecules of water to take part in four hydrogen bonds and by the geometry of these bonds. At temperatures which are not too high the structure of water can be characterized as a tetrahedral ice-like shell, the cavities of which are partially filled by molecules of water. In connection with the openness of the shell the translation movement of molecules of water takes place mainly through its cavities and leads to a less significant randomization than in liquids with a dense packing. It is necessary to stress that the term "structure" of water is used only in the sense of short-range orderliness, i.e., orderliness which is spread out for short distances from a certain selected molecule, since thermal movement constantly disrupts the order of mutual disposition of molecules which is formed due to hydrogen bonds.

Table 3

Energy of activation E_a of self-diffusion of water in solutions and gels of protein and in various live tissues at 20°

(a) Объект исследования	E, ккал/моль (b)
(c) Вода	4,5
(d) Яичный альбумин, 10%-ный раствор	4,5
(e) Яичный альбумин, 7%-ный гель	4,6
(f) Вирус табачной мозаики, 5%-ный раствор	4,5*
(g) Листья кукурузы	4,6
(h) Корни кукурузы	4,6
(i) Печень лягушки	3,7

* Based on the data of Douglas, Firsh, and Anderson [23].

Key: (a) Object of investigation; (b) E, Cal/mole; (c) Water; (d) Egg albumin, 10% solution; (e) Egg albumin, 7% solution; (f) Tobacco mosaic virus, 5% solution; (g) Leaves of maize; (h) Roots of maize; (i) Frog liver.

During diffusion a molecule of water is spasmodically shifted from one position of equilibrium to another. In order to shift to a different position of equilibrium it is necessary that a vacancy-vacuum be formed where a molecule could jump across, and it is necessary to break the bonds with its surrounding neighbors in the given position of equilibrium. Since due to the open structure in water diffusion is carried out mainly through voids in the structure, then the molecule should possess an energy of activation which is sufficient only for breaking the bonds with neighbors. Therefore the greater the number of bonds (hydrogen, dipolar, etc.) by which a molecule of water is joined, the greater should be the energy of activation of self-diffusion of water. The energy of activation of self-diffusion of water can serve as a measure for a comparison of the increase or decrease of bonds belonging to a molecule of water, i.e., it can serve as the characteristic of structure of water. At temperatures from 0 to 80° the energy of activation of self-diffusion in water changes from 5.0 to 4.2 Cal/mole [22].

Table 3 shows the values of energy of activation of self-diffusion of water in cells of various tissues and in solutions of protein. As can be seen, these values are sufficiently close to the energy of activation of pure water.

Thus, since the coefficient of self-diffusion of water between microbarriers is close to the coefficient of self-diffusion of pure

water and the energy of activation of self-diffusion of water in a cell is close to the energy of activation of self-diffusion in pure water, there are no bases to assume that in the cell there occurs a significant ordering of the structure of water, and the water of hyaloplasm essentially differs in its properties from pure water.

This conclusion is also confirmed by data relative to the times of proton relaxation T_1 and T_2 in live tissues.

During a study of relaxation in protein solutions it was shown [2] that in solutions of protein water which differs in its properties from ordinary water makes up a very small part of all the water of the solution and is water which is directly interacting with the protein. Behavior of relaxation time is conditioned by the existence of two fractions of water, differing in their mobility (fractions of water directly interacting with protein and the fractions of remaining water which do not differ in their properties from pure water), and by rapid exchange by molecules of water or protons (in a time of less than 10^{-4} s) between these fractions. The amount of water interacting directly with protein (hydrate) changed depending on the openness of the entire structure of protein and supermolecular formations, and the relaxation times of this water are connected directly with the movement of fractions of the protein molecule relative to each other.

During a study of times of proton relaxation in animal and plant tissues [7, 9] it was revealed that ratio $T_1/T_2 > 1$, and times of proton relaxation T_1 and T_2 are single-phase.

It is possible to point out, in like manner as is done for protein solutions in [2], that:

1) behavior of time of proton relaxation in tissues is conditioned by the existence of fractions of water with different properties and by exchange between them;

2) during slow exchange between fractions and considerable amounts of water in the fractions a two-phase nature should be observed in relaxation time;

3) at the observed values for the ratio of T_1/T_2 slow exchange is impossible with a small amount of water differing from the remaining water.

Thus, for tissue water there should exist a sufficiently rapid exchange by molecules of water or protons between the fractions of water which have a different mobility. And since, as was indicated above, the structure of water of a hyaloplasm is sufficiently close to the structure of pure water, it can be thought that the relaxation characteristics of hyaloplasm water differ little from those of pure water, therefore the time of relaxation can be presented in the form

$$\frac{1}{T_1} = \frac{P_a}{T_{1a}} + \frac{P_b}{T_{1b}}, \quad \frac{1}{T_2} = \frac{P_a}{T_{2a}} + \frac{P_b}{T_{2b}},$$

where T_{1a} and T_{2a} - times of proton relaxation of unchanged ordinary water; P_a - share of ordinary water (fraction a); P_b - share of water, differing strongly in its mobility and relaxation characteristics from ordinary water (fraction b); T_{1b} and T_{2b} - times of proton relaxation of water of fraction b. By using this concept a calculation was made, just as in work [24], of the amount of water which differed sharply from ordinary water in its mobility. It turned out that the amount of such water, relative to a gram of dry substance (resulting data are cited in Table 1), is not great and in all appearance this is water which is directly interacting with dissolved macromolecules and non-aqueous components of organelles. Apparently not only in hyaloplasm but also in the organelles there cannot be a considerable share of water which differs in its properties from ordinary water.

In this work main attention was given to the state of water in the hyaloplasm, and especially to the amount of water in the cell which differs strongly in its properties from ordinary water. We did not touch individually on the problem of the state of water in microscopic and submicroscopic structures of the cell and on the mobility of water in these structures, the volume of which, as was pointed out above, may make up a considerably share of the volume of protoplasm. These problems together with the concrete mechanisms, determining the role of hydrate water, the role of hydration level of the cell for maintaining the structure and functional condition of macromolecules and microscopic and submicroscopic structures of the cell and the cell as a whole, are the subject for future investigations.

Conclusions

1. Coefficients of self-diffusion have been obtained for water in cells of various live tissues.

2. The relatively low values for the coefficient of self-diffusion of water in the protoplasm of cells of various tissues in comparison with pure water are conditioned mainly by the effect of microbarriers and only to an insignificant degree by the effect of hydration.

3. The energy of activation of self-diffusion of water in a cell is close to the energy of activation of self-diffusion of pure water.

4. The structure of water in hyaloplasm is very close (or the same) to the structure of ordinary water in vitro.

5. An appraisal was made of the water in various tissues which differs strongly in its properties from ordinary water.

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