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T.V. Krupska [1, 2], Qiliang Wei [1], Jinju Zheng [1], L.P. Morozova [3], Weiyou Yang [1], V.V. Turov [1, 2]

INFLUENCE OF TEMPERATURE AND ADDITIVES OF ORGANIC SUBSTANCES ON PHASE TRANSITIONS IN PIG NERVOUS TISSUE

[1] Institute of Micro/Nano Materials and Devices, Ningbo University of Technology, Ningbo City, 315211, P.R. China
[2] Chuiko Institute of Surface Chemistry of National Academy of Sciences of Ukraine 17 General Naumov Str., Kyiv, 03164, Ukraine, E-mail: krupska@ukr.net
[3] Vinnytsia National Agrarian University 3 Sonyachna Str., Vinnytsia, 21008, Ukraine

Low-temperature ^{1}H NMR spectroscopy was used to study the pig spinal and brain tissues of the original samples at different temperatures, in chloroform, including that with the addition of hydrochloric acid, and in a mixture of chloroform and trifluoroacetic acid (TFAA) in a ratio of 6:1.

It has been found that water in the tissues of the pig's brain and spinal cord is bound and is part of polyassociates, the radius of which is in the range R = 1–100 nm. During the freezing-thawing process, only the signal of bound water is observed in the ^{1}H NMR spectra, while the protons of biopolymers and phospholipid membranes are not detected in the spectra, which indicates their low molecular mobility. Chloroform dissolves in the substance of cell membranes, increasing the mobility of aliphatic groups, and the membrane material passes from an ordered to a partially disordered state. This effect is much stronger for spinal cord tissue, which is due to the lower content of interstitial water.

The characteristics of interstitial water layers were calculated: the concentration of strongly and weakly bound water, the maximum decrease in the Gibbs free energy in the layer of strongly bound water and interfacial energies. It has been found that for spinal cord tissue, the introduction of chloroform into the system is accompanied by a decrease in the binding of water in the nervous tissue (the amount of highly bound water decreases from 180 to 250 mg/g), and the value of interfacial energy decreases from 24 to 19.6 J/g. However, these changes have little effect on the radius distributions of interstitial water clusters. The main maximum of the distribution does not change and is observed at R = 20 nm. In the presence of TFAA, the amount of strongly bound water increases to 1400 mg/g, and the value of interfacial energy increases to 77.6 J/g. For brain tissue, their value turned out to be significantly less. Thus, chloroform had virtually no effect on the binding energy of water in the tissue, and the effect of TFAA also turned out to be half that for spinal cord tissue.

Keywords: *^{1}H NMR spectroscopy, brain, spinal cord, phospholipid structures, strongly bound water, weakly bound water, association of water*

INTRODUCTION

Currently, the study of the phase state of nerve tissues is largely associated with solving the problems of their fusion, cryopreservation and transplantation [1–4]. Nervous tissue belongs to complex biological systems that ensure the interaction of the organism with the external environment and its response to changes in both the state of the environment and the organism. In addition to nerve cells (neurons and axons), the composition of nervous tissue includes neuroglia, which ensures the functioning and metabolic processes of nerve cells, as well as synaptic systems that allow the transmission of nerve excitations to other cellular structures [5, 6]. For experiments, one of the most accessible types of nerve tissue is pig tissue.

From a physicochemical point of view, nervous tissue, both the brain and the spinal cord, is a colloidal system consisting of nerve fibers immersed in a gel-like substance called neuroglia. Its basis is the aqueous system, consisting of sections of intracellular cytoplasm separated by cell membranes [7]. In this case, the number of neuroglial cells is approximately 10 times greater than the number of nerve cells, although their linear dimensions are many times smaller. Then nervous tissue can be represented as a system of compartments filled with an aqueous solution of low- and high-molecular substances formed by neuroglial cells, nerve cells and organelles of

different types of cells. Due to the small volume of these compartments, water is in a bound state due to interaction with cell walls, biopolymer molecules and mineral and organic substances dissolved in it. On the other hand, cell membranes are formed predominantly by phospholipid structures that are in a liquid crystalline state [8, 9].

The phase state of both water and phospholipid membranes is mainly influenced by temperature and the presence of substances that can penetrate the membranes or concentrate in them. A convenient experimental method that allows one to detect the transition of water from a liquid to a solid state is the method of low-temperature ^{1}H NMR spectroscopy [10–13]. In this case, the large difference in the transverse relaxation times of protons in solid and liquid water is used [14]. Freezing of part of the interstitial water leads to a decrease in the observed intensity of the water signal in liquid NMR spectra. Similar patterns apply when observing the spectra of phospholipid structures. The state of phospholipids with low molecular mobility is not recorded in high-resolution spectra. If organic substances that increase the mobility of aliphatic fragments are dissolved in the substance of the cell membrane, signals from individual phospholipid groups can be observed.

The purpose of this work was a comparative study of phase transitions in the nervous tissues of the spinal cord and brain of a pig during heating of samples cooled to 210 K, the influence of weakly polar chloroform and highly polar trifluoroacetic acid on this process. The choice of a non-polar medium and an acidic additive was determined by the desire to find out how they affect the structure of the network of hydrogen bonds of water in nervous tissues, its clustering and stability in a wide temperature range, during the process of thawing the samples. Deuterochloroform and deuterotrifluoroacetic acid were used as substances that did not produce extraneous signals in the ^{1}H NMR spectra. It was assumed that in real experiments with cryopreservation they could be replaced with non-polar hydrocarbons (fats) and organic acids.

MATERIALS AND METHODS

The measurements used post-mortem samples of pig brain tissue taken from the inside of the hemispheres at a depth of 30 mm. Spinal cord tissue was taken from the middle part of the spinal column. Before use for NMR studies, tissues were stored in a freezer at –18 °C 14 days. Measurements were carried out in 5 mm NMR ampoules. The samples were pieces of fabric with linear dimensions of about 3 mm^2. They were placed in a measuring ampoule in such a way that they filled approximately 60 % of the working volume of the ampoule, the rest of which could be filled with a liquid organic medium. The amount of tissue in the ampoules was 200–300 mg. Deuterated chloroform and deuterated trifluoroacetic acid (NMR spectroscopy grade) were used as organic media.

NMR spectra were recorded on a high-resolution NMR spectrometer (Varian "Mercury") with an operating frequency of 400 MHz. We used 90° probe pulses with a duration of 3 μs and a bandwidth of 20 kHz. The temperature in the sensor was regulated by a Bruker VT-1000 thermal attachment with an accuracy of ±1 degree. The signal intensities were determined by measuring the peak area using the procedure of decomposing the signal into its components, assuming a Gaussian waveform and optimizing the zero line and phase with an accuracy that was no less than ±5 % for well-resolved signals, and ±10 % for overlapping signals. To prevent overcooling of water in the studied objects, measurements of the concentration of non-freezing water were carried out by heating samples pre-cooled to the temperature of 210 K. The technique of NMR measurements and its use to determine the thermodynamic characteristics and radii of bound water clusters was described in detail earlier [10–13].

RESULTS AND DISCUSSION

In Fig. 1 *a*, *b* there are shown ^{1}H NMR spectra of a spinal cord sample taken at different temperatures in the case when the voids in the working gap of the measuring ampoule are filled with air. At temperatures $T \leq 270$ K, one broad signal is observed in the spectra, the chemical shift of which is $\delta_H = 5.8–6.5$ ppm. At higher temperatures it splits into two signals with different intensities. One (more intense) with a chemical shift $\delta_H = 5.5–5.8$ ppm, and the other with $\delta_H = 4.5–5.0$ ppm. Both of these signals can be attributed to interstitial water. As was shown in [10–13], the chemical shift of water is predominantly determined by the average number of hydrogen bonds in which each of its molecules

participates. For water that does not participate in the formation of hydrogen-bonded complexes, chemical shift values in the region δ_H = 1.0–1.5 ppm are characteristic. In the case of tetracoordinated water (hexagonal ice) – δ_H = 7.0 ppm. For liquid (bulk water), depending on temperature, the value of the chemical shift of protons is δ_H = 4.5–5.0 ppm. Then the signal in stronger magnetic fields (with a smaller chemical shift) should be attributed to that part of the interstitial water, the association of which is close to that of liquid water. That is, the properties of such water are close to its bulk properties. This is also evidenced by the fact that the signal in strong fields appears only at T > 270 K, which is typical for water experiencing weak disturbances from macromolecules or other cellular structures with which it can enter into adsorption interactions.

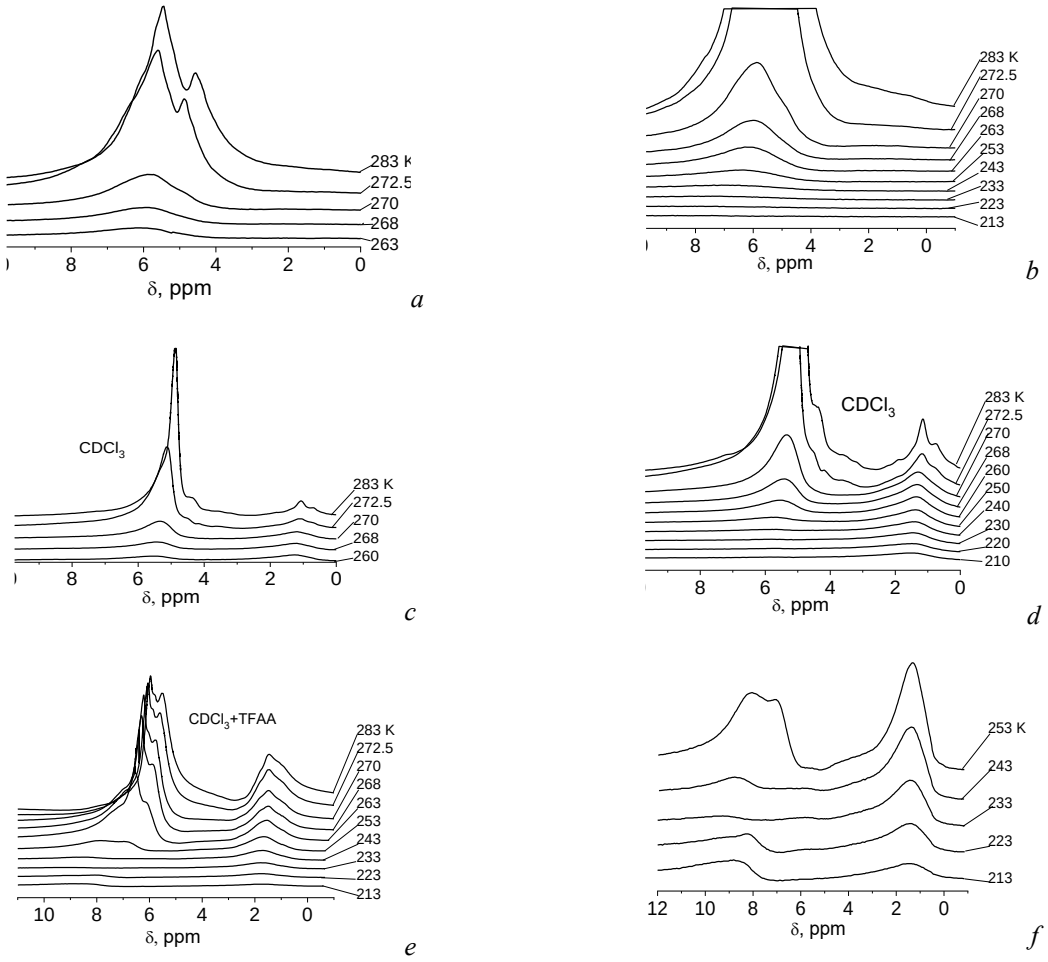

Fig. 1. [1] H NMR spectra of initial pig spinal cord tissue (*a*, *b*), in $CDCl_3$ medium (*c*, *d*) and in 6$CDCl_3$ + 1TFAA medium (*e*, *f*) taken at different temperatures

The large width of the water signal at temperatures T > 270 K can be due to two reasons: the low molecular mobility of water, which forms gel-like systems inside cells and in the intercellular space, as well as the existence of several states of water with different values of chemical shift, between which a slow (on a scale of NMR time) molecular exchange [14]. Signals of protein molecules, DNA, lipids and glycoproteins present in cellular tissues are not observed in the spectra due to the low mobility of their proton-containing groups. In a completely thawed sample (at T = 283 K, Fig. 1 *b*) at δ_H = 1.5–2.5 ppm. Low-intensity signals are observed, which may be due to unassociated forms of water or aliphatic groups, such as phospholipids [10].

Filling the space between pieces of the spinal cord with weakly polar $CDCl_3$ leads to a change in the appearance of the spectra (Fig. 1 *c*, *d*). The width of water signals decreases, and the signal intensity in strong magnetic fields (δ_H = 4–4.5 ppm) decreases. In addition, quite intense signals appear in the region δ_H = 1.5–2.5 ppm throughout the entire temperature range accessible to measurement. These can be signals from weakly ordered forms of water [10] or protons of aliphatic groups of lipid structures, concentrated predominantly in cell membranes [5]. To assign signals in this part of the spectrum, the sample was dried at the temperature of 376 K for 1 hour. The spectra of such a dehydrated sample are shown in Fig. 2.

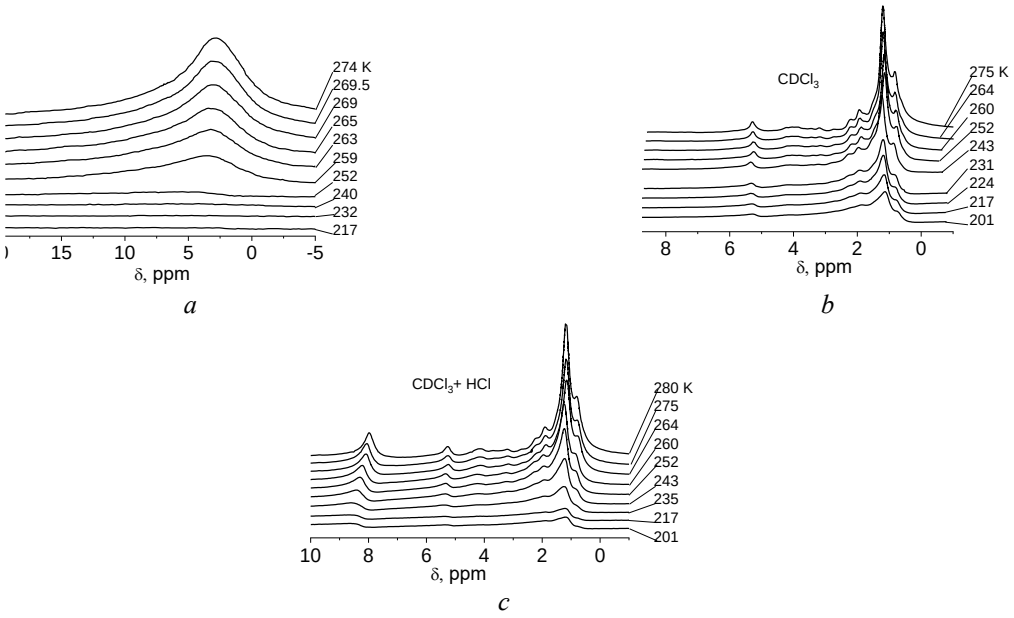

Fig. 2. ^{1}H NMR spectra taken at different temperatures of samples of dehydrated nervous tissue of the pig spinal cord in air (*a*), in $CDCl_3$ medium (*b*) and in $CDCl_3$ medium with the addition of HCl (*c*)

In air (Fig. 2 *a*), one broad signal is recorded in the spectra, the center of which has a chemical shift δ_H = 2.5 ppm. As the temperature decreases to T = 256 K, the signal intensity gradually decreases, and then in a small temperature range it abruptly decreases several times. This behavior of the NMR signal is typical for substances with a phase transition. Filling the gaps between particles of nervous tissue with chloroform leads to a sharp decrease in the width of the signals and the manifestation of its fine structure (Fig. 2 *b*). In this case, the shape of the spectrum is characteristic of the spectra of phospholipids [12]. If weakly associated forms of water are present in the system, they cannot be distinguished against the background of signals from aliphatic groups of phospholipids. The addition of 50 mg/g hydrochloric acid to the system (Fig. 2 *c*) is accompanied by the appearance in the spectra of a water-acid merged signal in the region δ_H = 8–8.5 ppm. The rest of the spectrum remains unchanged. It can be concluded that despite the high water content in the nervous tissue of the spinal cord, chloroform, which is poorly soluble in water, easily penetrates the tissue and is localized mainly in cell membranes, increasing the mobility of lipid structures in them. This entails a change in the parameters of phase transitions of lipids in the direction of reducing the probability of the formation of their sedentary, ordered forms.

In complex, hierarchically ordered heterogeneous systems, which include biological objects, water can be part of spatially separated structures formed by various types of water polyassociates (clusters or domains) [11]. The association of water in clusters of different sizes differs slightly, which causes similar values of the chemical shift of protons. However, as was shown in [15, 16], the solubility of strong acids in them can differ significantly. Since “acidic” protons in acids have chemical shifts in the range δ_H = 9–11 ppm, clusters with greater solubility with respect to acid molecules are observed in the

spectra in weaker magnetic fields (they have a larger chemical shift).

Fig. 1 *d*,*f* shows ^{1}H NMR spectra of a sample of spinal cord nervous tissue in a $6CDCl_3$ + 1TFAA medium. As can be seen from the figure, TFAA causes differentiation of water clusters located in the nervous tissue based on their capability to dissolve trifluoroacetic acid. Assuming that the chemical shift of water in clusters that do not dissolve acid is δ_H = 5 ppm, and for pure acid is δ_H = 10 ppm, we can estimate the acid concentration in different types of water clusters formed in the nervous tissue of the spinal cord, located in $CDCl_3$ medium (Fig. 1 *e*).

As the temperature decreases, the water present in the nervous tissue freezes and the acid concentration in the unfrozen part of the water system increases. In this case, a shift of water signals to the region of large chemical shift values is recorded. Between different types of water clusters containing dissolved acid, molecular or proton exchange reactions can occur. As can be seen from the data in Fig. 1 *e*, in the temperature range $253 < T < 213$ K with decreasing temperature, separation and then merging of signals is observed, which is typical for the case of acceleration of proton exchange [14] – a transition from the region of slow to the region of fast proton exchange. This probably occurs due to the preferential freezing of water in their clusters, which contain little acid. Then, with a decrease in temperature, exchange processes between clusters, which contain a lot of acid and differ less in the magnitude of the chemical shift, accelerate.

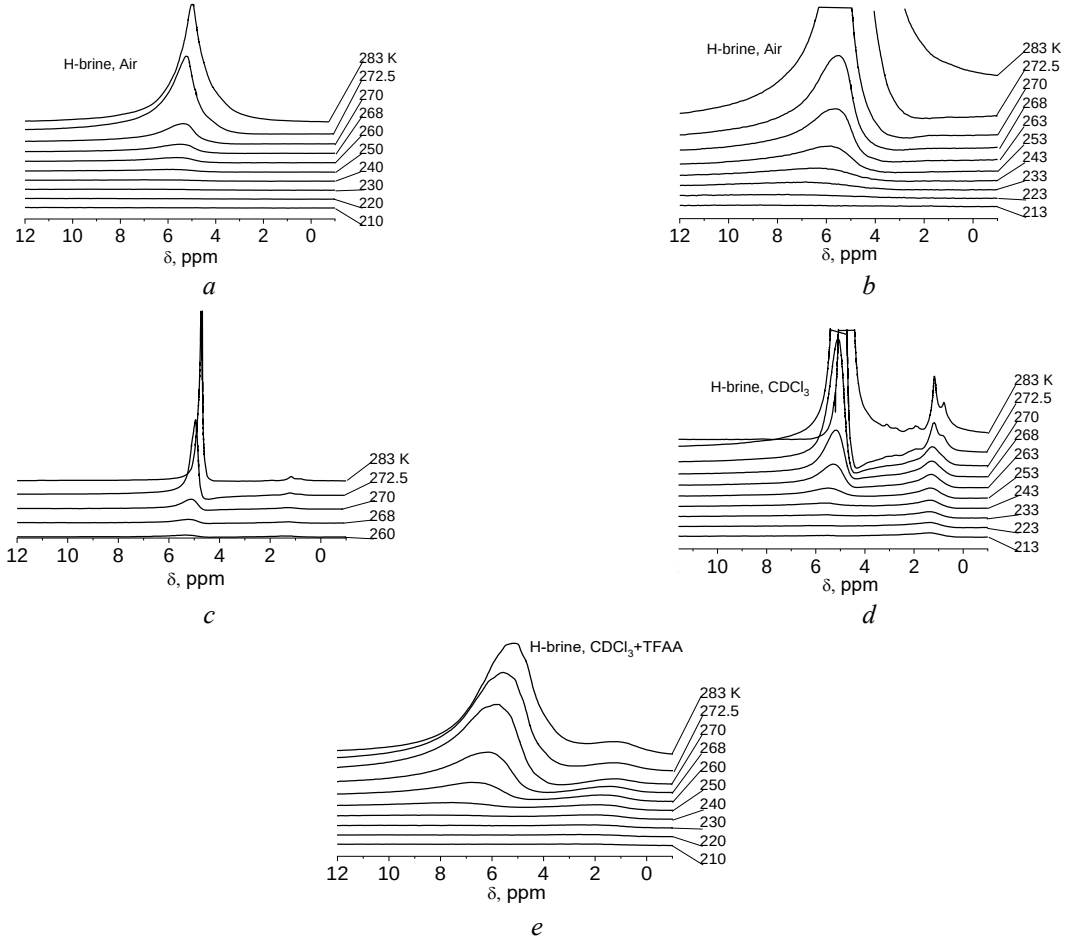

Fig. 3. ^{1}H NMR spectra of pig brain tissue taken at different temperatures in the original (*a*, *b*), in $CDCl_3$ medium (*c*, *d*) and in $6CDCl_3$ + 1TFAA medium (*e*)

Fig. 3 shows the results of a study of the nervous tissue of the pig brain. The shape of the spectra is similar to those shown in Fig. 1. The difference consists in a slightly larger signal width, which, in some cases, does not allow observing the fine structure of the spectra. In air,

even with high sensitivity of the spectrometer (Fig. 3 *b*), signals from lipid groups are not recorded. They appear in a chloroform environment (Fig. 3 *c*, *d*), but their intensity is significantly less than for the nervous tissue of the bone marrow. This is probably due to the high water content in the tissue. The addition of TFAA to the $CDCl_3$ medium does not lead to the receipt of resolved signals related to water clusters containing different amounts of dissolved acid, which may be due to the large widths of water signals related to different types of water clusters.

Based on temperature changes in the intensity of the water signal, its thermodynamic parameters and distributions along the radii of bound water clusters can be calculated [10–13]. To determine the geometric dimensions of clusters of adsorbed water, we used the Gibbs-Thomson equation, which relates the radius of the pore in which water is located with a decrease in the freezing temperature [17–18]:

$$\Delta T_m = T_{m,\infty} - T_m(R) = -\frac{2\sigma_{sl}T_{m,\infty}}{\Delta H_f \rho R} = \frac{k_{GT}}{R}\ , \qquad (1)$$

where $T_m(R)$ is the melting temperature of ice localized in pores of radius R, $T_{m,\infty}$ is the melting temperature of bulk ice, ρ is the density of the solid phase, σ_{sl} is the energy of interaction of a solid with a liquid, ΔH_f is the volume enthalpy of melting and k_{GT} – constant of the Gibbs-Thomson equation.

The influence of the solid surface extends to several molecular layers deep into the liquid phase [19]. The process of freezing (defreezing) of bound water corresponds to changes in the Gibbs free energy, caused by the effects of limited space and the nature of the phase interface. The differences from the process in volume are smaller, the further the water layer is from the surface. At $T = 273$ K, water freezes, the properties of which correspond to bulk water, and as the temperature decreases (without taking into account the effect of supercooling), layers of water closer to the surface freeze. To change the free energy of bound water (ice), the following relation is valid:

$$\Delta G_{\text{ice}} = -0.036(273.15 - T), \qquad (2)$$

where the numerical coefficient is a parameter associated with the temperature coefficient of change in the Gibbs free energy for ice [20]. By determining the temperature dependence of the concentration of non-freezing water $C_{uw}(T)$ from the signal intensity in accordance with the method described in detail in [6–9], the amount of strongly and weakly bound water and the thermodynamic characteristics of these layers can be calculated.

The interfacial energy of water at the interface with solid particles or in its aqueous solutions was determined as the modulus of the total decrease in the free energy of water due to the presence of a phase boundary [10–13] according to the formula:

$$\gamma_S = -K \int_0^{C_{\text{uw}}^{\max}} \Delta G(C_{\text{uw}})\, dC_{\text{uw}} \qquad (3)$$

where $C_{\text{uw}}^{\max}$ is the total amount of non-freezing water at $T = 273$ K.

The amount of water in tissues per unit mass of dry matter was determined by drying tissues at $T = 376$ K for 1 hour. Measurements showed that brain tissue contained 4000 and spinal cord tissue 3500 mg/g of water. Fig. 4 *a* shows the dependence of the concentration of non-freezing water on temperature for all studied systems. Based on these dependences, using formula (2), we calculated the dependences of the change in the Gibbs free energy of interfacial water on the concentration of non-freezing water (Fig. 4 *c*), and based on formula (1), the distribution along the radii of clusters of interstitial water (Fig. 4 *d*).

Table shows the characteristics of the layers of interstitial water: the concentration of strongly and weakly bound water (C_{uw}^{S} and C_{uw}^{W}, respectively), the maximum decrease in the Gibbs free energy in the layer of strongly bound water (ΔG^S) and interfacial energies calculated in accordance with formula (3). It was assumed that strongly bound water is that part of water for which $\Delta G < -0.5$ kJ/mol (temperature $T < 265$ K) [10–13].

For spinal cord tissue, the introduction of chloroform into the system is accompanied by a decrease in the binding of water in the nervous tissue. Thus, the amount of strongly bound water decreases from 180 to 250 mg/g, and the value of interfacial energy decreases from 24 to 19.6 J/g (Table). However, these changes have little effect on the radius distributions of interstitial water clusters (Fig. 4 *d*). The main maximum of the distribution does not change and is observed at $R = 20$ nm. The difference in distributions is observed in the relative increase in the amount of

water included in the R = 100 nm clusters. In the presence of TFAA, the amount of highly bound water increases to 1400 mg/g, and the γ_S value increases to 77.6 J/g. This occurs due to the dissolution of acid, both in the substance of phospholipid membranes and in interstitial water clusters. A new maximum appears in the distribution $R(\Delta C)$ at R = 2 nm. The difference in γ_S values is due to the effect of solvation of TFAA molecules by water molecules. Due to the high interaction energy during the freezing process, water can only go from solution to a solid state (hexagonal ice) at very low temperatures, and the difference in interfacial energies determines the solvation energy.

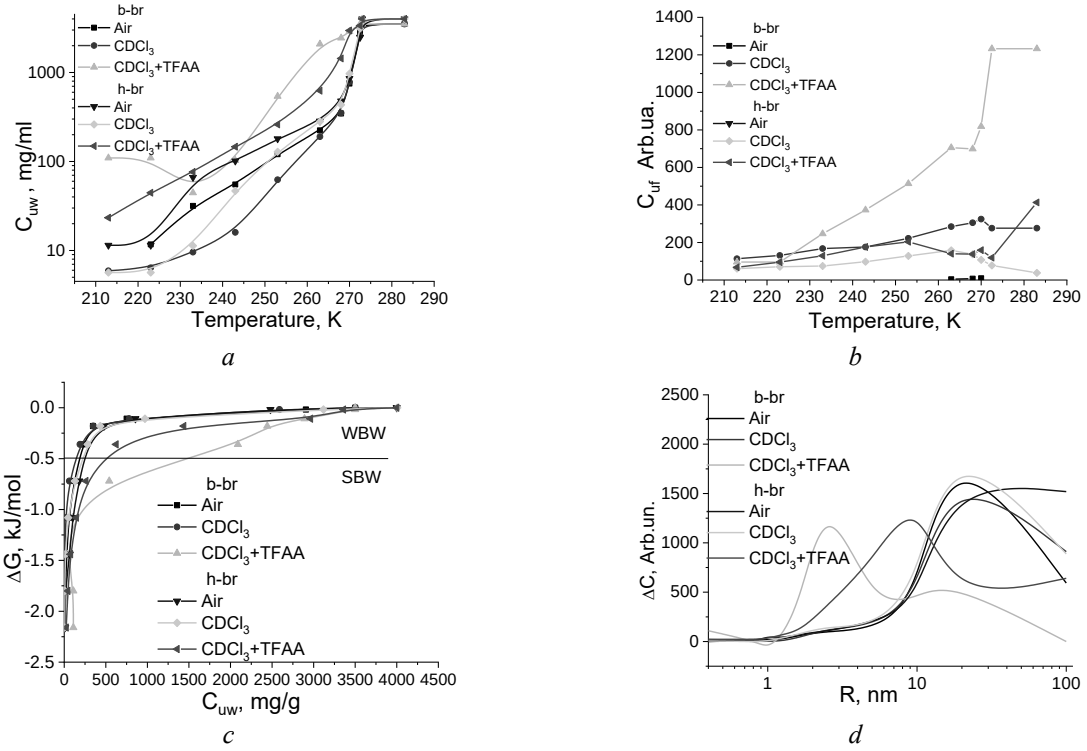

Fig. 4. Temperature dependences of the concentration of non-freezing water (*a*), the contribution of the NMR signal from protons in the region δ_H = 1–2.5 ppm (*b*), changes in the Gibbs free energy from the concentration of non-freezing water (*c*) and distribution along the radii of clusters of interstitial water (*d*)

Table. Characteristics of bound water layers in the nervous tissues of the pig spinal cord and brain

Sample	Environment	C_{uw}^S (mg/g)	C_{uw}^W (mg/g)	ΔG^S (kJ/mol)	γ_S (J/g)
Pig spinal cord	Air	180	3320	–2.16	24
	$CDCl_3$	150	3350	–2.25	19.6
	6$CDCl_3$+1TFAA	1400	2100	–	77.6
Pig brain	Air	225	3775	–2.25	26.8
	$CDCl_3$	210	3790	–2.25	26.1
	6$CDCl_3$+1TFAA	500	3500	–2.25	52.8

Similar effects are also observed for brain tissue, only their magnitude is much smaller. Thus, chloroform had virtually no effect on the binding energy of water in the tissue (Table). The effect of TFAA was also half that for spinal cord tissue. This is probably due to the high water content in the sample and the lower solubility of chloroform in the substance of cell membranes. The radius distribution of interstitial water clusters shows that in the presence of TFAA, the

distribution maximum is at R = 10 nm, *i.e*. the acid concentration in the tissue is significantly less than that in the case of spinal cord tissue.

CONCLUSION

The water in the tissues of the pig's brain and spinal cord is bound. It is part of polyassociates, the radius of which is in the range R = 1–100 nm. During the freezing-thawing process, only the signal of bound water is observed in the ^{1}H NMR spectra, while the protons of biopolymers and phospholipid membranes are not detected in the spectra, what indicates their low molecular mobility. Chloroform can dissolve in the substance of cell membranes, increasing the mobility of aliphatic groups. In this case, the membrane material passes from an ordered to a partially disordered state. This effect is much stronger for spinal cord tissue, which may be due to the lower content of interstitial water.

The obtained research results allow us to visualize more clearly the processes that take place in the nerve cells of living organisms when they are frozen in the presence of organic solvents, which can give a new impetus to the understanding of the mechanisms of their action on functionally differentiated cells.

Вплив температури та добавок органічних речовин на фазові переходи в нервових тканинах свині

Т.В. Крупська, Qiliang Wei, Jinju Zheng, Л.П. Морозова, Weiyou Yang, В.В. Туров

Інститут мікро/нано матеріалів і пристроїв, Технологічний університет Нінбо, місто Нінбо, 315211, Китай
Інститут хімії поверхні ім. О.О. Чуйка Національної академії наук України вул. Генерала Наумова, 17, Київ, 03164, Україна, krupska@ukr.net
Вінницький національний аграрний університет ул. Сонячна, 3, Вінниця, 21008, Україна

Методом низькотемпературної ^{1}H ЯМР-спектроскопії були досліджені при різних температурах тканини спинного та головного мозку свині вихідних зразків, у середовищі хлороформу, у тому числі з добавкою соляної кислоти, та у середовищі суміші хлороформу з трифтороцтовою кислотою (ТФОК) у співвідношенні 6:1.

Було встановлено, що вода в тканинах головного та спинного мозку свині є зв'язаною та входить до складу поліасоціатів, радіус яких знаходиться в діапазоні R = 1–100 нм. У процесі заморожування-відтавання в спектрах ^{1}H ЯМР спостерігається тільки сигнал зв'язаної води, у той час як протони біополімерів та фосфоліпідних мембран у спектрах не фіксуються, що свідчить про їхню малу молекулярну рухливість. Хлороформ розчиняється у речовині клітинних мембран, підвищуючи рухливість аліфатичних груп, у свою чергу, матеріал мембрани перетворюється з упорядкованого в частково розупорядкований стан. Цей ефект значно сильніше проявляється для тканини спинного мозку, що обумовлено меншим вмістом внутрішньотканинної води.

Розраховані показники шарів внутрішньотканинної води: концентрацію сильно- і слабозв'язаної води, максимальне зниження вільної енергії Гіббса в шарі сильнозв'язаної води та міжфазні енергії. Встановлено, що у тканині спинного мозку введення у систему хлороформу супроводжується зменшенням зв'язування води нервовою тканиною (кількість сильнозв'язаної води зменшується від 180 до 250 мг/г), а величина міжфазної енергії знижується від 24 до 19.6 Дж/г. Однак ці зміни слабко впливають на розподіл за радіусами кластерів внутрішньотканинної води. Основний максимум розподілу не змінюється та спостерігається при R = 20 нм. У присутності ТФОК кількість сильнозв'язаної води підвищується до 1400 мг/г, а величина міжфазної енергії до 77.6 Дж/г. Для тканини головного мозку їхня величина виявилася значно меншою. Так, хлороформ практично не вплинув на енергію зв'язування води в тканині, а ефект ТФОК також виявився вдвічі меншим, ніж тканини спинного мозку.

***Ключові слова**: ^{1}H ЯМР-спектроскопія, головний мозок, спинний мозок, фосфоліпідні структури, сильнозв'язана вода, слабозв'язана вода, асоційованість води*

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