[1]I. F. Voloshin, S. V. Medvedev, V. G. Skobov, L. M. Fisher, and A. S. Chernov, Pis'ma Zh. Eksp. Teor. Fix. **23**, 553 (1976) [JETP Lett. **23**, 507 (1976)].
[2]I. F. Voloshin, S. V. Medvedev, V. G. Skobov, L. M. Fisher, and A. S. Chernov, Zh. Eksp. Teor. Fiz. **71**, 1555 (1976) [Sov. Phys. JETP **44**, 814 (1976)].
[3]I. F. Voloshin, V. G. Skobov, L. M Fisher, and A. S. Chernov, Zh. Eksp. Teor. Fiz. **72**, 735 (1977) [Sov. Phys. JETP **45**, 385 (1977)].
[4]I. F. Voloshin, I. A. Matus, V. G. Skobov, L. M. Fisher, and A. S. Chernov, Zh. Eksp. Teor. Fiz. **74**, 753 (1978) [Sov. Phys. JETP **47**, 395 (1978)].
[5]I. F. Voloshin, V. G. Skobov, L. M. Fisher, and A. S. Chernov, Zh. Eksp. Teor. Fiz. **78**, 339 (1980) [Sov. Phys. JETP **51**, 170 (1980)].
[6]D. E. Zherebchevskiĭ and V. P. Naberezhnykh, Fiz. Nizk. Temp. **4**, 467 (1978) [Sov. J. Low Temp. **4**, 229 (1978)].
[7]D. E. Zherebchevskiĭ, V. P. Naberezhnykh, and V. V. Chabanenko, Fiz. Nizk. Temp. **5**, 1035 (1979) [Sov. J. Low Temp. **5**, 489 (1979)].
[8]G. E. H. Reuter and E. H. Sondheimer, Proc. Roy. Soc. **A195**, 336 (1948).
[9]V. P. Silin and A. A. Rukhadze, Electromagnitnye svoĭstva plazmy i plazmapodobnykh sred (Electromagnetic Properties of Plasma and Plasmalike Media), Atomizdat, 1961, p. 116.
[10]G. A. Baraff, Phys. Rev. **B7**, 580 (1973).
[11]G. A. Baraff, J. Math. Phys. **9**, 372 (1968).
[12]G. A. Baraff, Phys. Rev. **B9**, 1103 (1974).
[13]D. É. Zherebchevskiĭ, É. A. Kaner, and V. P. Naberezhnykh, Fiz. Nizk. Temp. **2**, 879 (1976) [Sov. J. Low Temp. **2**, 431 (1976)].
[14]G. A. Baraff, J. Math. Phys. **11**, 1938 (1970).
[15]H. Fisher and Y. H. Kao, Sol. St. Commun. **7**, 275 (1969).

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Precise measurements of NMR shifts

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A new method for precise measurement of NMR shifts is developed. By stabilizing the specimen temperature against an NMR signal having a temperature sensitive shift (this made it possible to keep the specimen temperature constant to within about 0.01 °C), controlling the stability of the NMR line shape, and regulating the frequency of the modulating oscillator using an automatic frequency control circuit with input from the NMR signal, we achieved an accuracy of 8×10^{-5} ppm (0.006 Hz for a spectrometer working frequency of 80 MHz) in measuring shifts for specimens having an external reference standard, and of 2×10^{-5} ppm (0.0015 Hz) for specimens having an internal reference standard. This accuracy exceeds the resolving power of commercial NMR spectrometers by a factor of about 100. Such accuracy in measuring shifts makes it possible to measure magnetic susceptibilities of substances in solution with a sensitivity 100 times that achievable with the known Evans NMR method, to record contact and pseudocontact shifts that are strongly masked by exchange processes, and to investigate weak temperature dependences of chemical shifts and spin-spin interaction constants. This opens up new prospects for investigating the structures of metal-containing macromolecules (enzymes, nucleic acids, etc.) and coordination compounds, and intermolecular and intramolecular interactions. Apparatus for precise measurement of NMR shifts is described.

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In high resolution nuclear magnetic resonance, a considerable part of the information on the structure of the investigated compound is extracted from the shifts of the resonance signals. By the shift we mean the relative position of the resonance line with respect to that of the reference signal on the frequency axis of a plot of the NMR spectrum.

The reason for differences in the values of the NMR shifts for nuclei of the same isotope may be differences in the chemical structure of the investigated substances (the so-called chemical shift), differences in the natures of the van der Waals interactions of the investigated molecules with one another and with solute molecules, and contact and dipole–dipole interactions of the resonating nuclei with paramagnetic centers (contact and pseudocontact shifts). When the reference substance is contained in an isolated microampoule (a so-called "external reference") the geometry of the specimen and the values of the bulk static magnetic susceptibilities of the principal and reference solutions strongly affect the measured shifts.[1,2] The picture is sometimes complicated by chemical exchange of the resonating nuclei between magnetically inequivalent states.[1,3,4] In accordance with what was said above, measurements of NMR signals are used to identify chemical compounds and to establish structural formulas of substances in organic chemistry, to investigate the conformation of complex molecules, to investigate structures of coordination compounds and the dynamics of chemical exchange, and to measure the static magnetic susceptibilities of substances in solution (the Evans method[2]).

The shifts are usually measured in relative units—in parts per million (ppm). The magnitude of the shift in ppm is calculated from the formula

$$\tau=10^6\Delta f/f_0, \qquad (1)$$

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where Δf is the shift in frequency units (i.e. the difference between the resonance frequencies of the investigated and reference signals) and f_0 is the working frequency of the spectrometer. The limiting accuracy of NMR shift measurements is usually assumed to be the resolving power of the NMR spectrometer, which is defined as

$$\rho=(\Delta f_\rho-\Delta f_h)/f_0, \quad (2)$$

where Δf_ρ is the width of a singlet NMR line of a substance having a very small natural line width Δf_h (i.e., long magnetic relaxation times). Thus, for standard proton NMR spectrometers with $f_0=80-100$ MHz we usually have $\Delta f_\rho \sim 0.1-0.2$ Hz, so that $\rho \sim (1-3)\times 10^{-9}$. One usually has $\Delta f_\rho \gg \Delta f_h$ for real spectrometers, Δf_ρ being determined by the residual nonuniformity of the magnetic field near the specimen. It is convenient to express the resolving power in the same units as the shifts; then we have the standard resolving power $\rho_{st} \sim (1-3)\times 10^{-3}$ ppm.

The above mentioned accuracy in measuring NMR shifts is quite adequate for solving problems of structural chemistry, in which the observed changes in the shifts exceed ρ_{st} by tens, hundreds, and thousands of times.[1] However, there are objects that, in principle, are very suitable for study by the method of NMR shifts, but for which this accuracy is inadequate. The current extensive literature on NMR spectroscopy contains no papers in which NMR shifts are measured with an accuracy substantially exceeding the resolving power of the spectrometer for the specific purpose of investigating molecular structure. To be mentioned are only experiments using the double resonance method to determine spin–spin interaction constants and the relative shifts of NMR lines in complex spin-coupled systems,[5] and using spin side band generators to measure the spin–spin interaction constants of well resolved spin doublets.[6,7] High, and even very high accuracy (0.01–0.001 and 10^{-5} Hz, respectively) was achieved in these experiments. However, the conditions for spin coherence and slow nuclear magnetic relaxation greatly limit the range of applicability of double NMR methods. The studies on the use of spin side band generators for precise measurements of NMR shifts have not been developed: one reason for this is doubtless the complexity of a precise frequency analysis of the beats of NMR signals when the NMR spectrum consists of several lines that differ in height and shape.

The limitation of the accuracy in measuring shifts to the resolving power of the NMR spectrometer is not a matter of principle, but has some purely methodological causes: specifically, the same procedures are used to measure the shift of a resonance line as to record an entire spectrum. Thus, the position of the resonance signal on the frequency scale is determined from the position of the peak of the line, and this in itself limits the accuracy of the measurement to a quantity close to ρ_{st}. Measuring in this manner is justified in the case of a multiplet signal (especially when the splitting of the components is not much greater than Δf_ρ), but it is not justified in the case of a narrow isolated singlet. The technique of recording complete spectra does not permit enough values of the shift for statistical processing to be recorded in a reasonable time, since the line must be recorded without distortion, i.e., "slowly" and with adequate filtering of the signal. Inadequate stability of the specimen temperature also makes itself felt in an attempt to record small changes in the shifts. The gas thermostats employed in NMR studies ensure temperature stability of the gas stream bathing the ampoule containing the specimen at the level of 1 °C per hour. When an internal reference is used the observed shift may drift by $(0.3-0.5)\times 10^{-3}$ ppm/deg provided the shifts are only weakly temperature dependent. This drift will be an order of magnitude larger for a specimen with an external reference substance because of the difference between the thermal expansion coefficients of the reference and principal solutions and the resulting change in the difference between the bulk static magnetic susceptibilities [see Eq. (3) below].

It is known, however, that a system for stabilizing the resonance conditions against the NMR dispersion signal from an internal reference substance can ensure a stability of 10^{-11} (10^{-5} ppm) or better for tens of minutes under conditions in which the shape of the resonance line does not change. As we shall see, it is possible in principle to increase the accuracy of the measurements by at least 200 times.

We first encountered the inadequacies of the classical method of measuring shifts in an attempt to detect paramagnetic centers on certain biological macromolecules by measuring the static magnetic susceptibilities of solutions of those molecules, using the Evans method mentioned above.[2,8,9] In general, measurements of the magnetic susceptibilities of substances in solution can provide valuable information on the electron structures of molecules (especially of paramagnetic ones) and is of considerable interest to physicists, chemists, and biochemists.

When measurements are made by the Evans method the outer and inner cylinders of the coaxial ampoule are filled with solutions that differ little in composition and contain several volume percent of the so-called spectator substance, which gives a singlet NMR signal (acetone, dioxane, etc.). The outer solution also contains the investigated substance (ordinarily a paramagnetic material) and the reference substance (TMS, *t*-butyl alcohol, etc.), the signal from which is used to stabilize the resonance conditions of the spectrometer. The ampoule is filled to such an extent that the condition $l/d>10$ is satisfied, where l is the length of the liquid column and d is the outer diameter of the ampoule. The recorded spectrum contains two singlet NMR lines from the spectator substance, one from the inner part of the coaxial specimen and one from the outer part of it. The separation between these lines is determined by the expression

$$\Delta f/f_0=2\pi\Delta\chi/3, \quad (3)$$

in which $\Delta\chi$ is the difference between the static volume magnetic susceptibilities of the solutions in the outer and inner cylinders. This difference contains paramagnetic and diamagnetic components, as well as small diamagnetic corrections.[8] The sensitivity to the shift

of the resonance line, which is determined by the spectrometer resolving power $\rho_{st} = (1-3)\times 10^{-3}$ ppm, corresponds to a concentration sensitivity to the dissolved paramagnetic materials that amounts to $(0.5-1)\times 10^{-3}$ mole/liter of electron spins. When investigating solutions of macromolecules such as enzymes or nucleic acids, it is impossible to raise the concentration significantly above $(1-2)\times 10^{-4}$ mole/liter.

We decided to try to raise the sensitivity of the Evans method to something of the order of 10^{-5} mole/liter of electron spins, i.e., to increase the accuracy in measuring the shift by a factor of 50–100. To do this we improved the method of recording the position of the NMR line on the frequency scale, the system for stabilizing the specimen temperature, the control over the stability of the shape of the resonance signal, and the method of filling the inner and outer cylinders of the coaxial ampoule.

The experimental setup was based on a commercial BS-487C "Tesla" spectrometer with $f_0 = 80$ MHz, working in the standard mode with audio-frequency modulation of the magnetic field. Figure 1 is a block diagram of the apparatus.

The coaxial specimen is prepared as follows: the inner cylinder (a capillary, o.d. 0.8 mm, wall thickness 0.05 mm) is filled with a 20–30% solution of the reference substance. A solution containing 1% of the spectator substance is poured into the outer cylinder (a commercial ampoule, diameter 5 mm, wall thickness 0.5 mm). The NMR signal from a substance whose chemical shift is strongly temperature dependent is used to control the temperature. The solutions were usually made up with heavy water, and then the protons of the ordinary water that is inevitably present as an impurity in the heavy water serve as the temperature sensitive substance. The temperature coefficient of the chemical shift of protons in water is ~0.01 ppm/deg.

The apparatus works as follows (see Fig. 1). The components of the NMR signal, after being amplified in the preamplifier 2, are fed to the synchronous detectors 3–7. At detector 3 (reference oscillator 8) the NMR dispersion signal from the reference substance is separated out and fed to the unit (not shown in the figure) that controls the resonance conditions. At detector 5 (reference oscillator 9) the NMR dispersion signal from the spectator substance is separated out and is fed to the electronic frequency control unit 11 that controls oscillator 8. When the resonance conditions for the spectator substance are not satisfied the signal from detector 5 changes the working frequency of oscillator 8, and this is equivalent to violating the resonance conditions for the reference substance. The control system immediately restores the resonance conditions for the reference substance, and at the same time, those for the spectator substance. Thus, the apparatus includes strong negative feedback as regards the resonance conditions, the loop being closed via the nuclear spin system in the specimen. The transfer constant of the feedback loop is large enough to keep the working frequencies of oscillators 8 and 9 in correspondence with the central points of the NMR dispersion lines of the reference and spectator substances during the time in which measurements are made. At detector 4 the NMR absorption signal from the reference substance is separated out and sent to an automatic correction system that maintains the uniformity of the magnetic field in the Y (vertical) coordinate; this signal is also brought to a signal strength meter (not shown on the figure). At detector 6 the NMR absorption signal from the spectator substance is separated out and fed to a signal strength meter and to the input of an automatic control system that maintains the uniformity of the magnetic field of the quadrature shim of the spectrometer (not shown on the figure). Experience shows that maintaining the signal strengths from detectors 4 and 6 constant within 1–2% is adequate for stabilizing the shapes of the NMR lines of the reference and spectator substances. The frequency meter 12 periodically measures the working frequency φ_8 of oscillator 8. Here $\varphi_8 = \Delta f + 2$ kHz, where Δf is the difference between the resonance frequencies of the spectator and reference substances. The values of φ_8 are accumulated for statistical processing. At detector 7 the NMR dispersion signal from the temperature sensitive substance is separated out and sent to the electronic temperature control unit 13, which regulates the temperature of the gas stream that passes through the thermostat 14 and bathes the ampoule containing the specimen. Experience shows that electronic control of the specimen temperature by reference to the NMR signal from a temperature sensitive substance makes it possible to hold the specimen temperature constant within 0.02 °C.

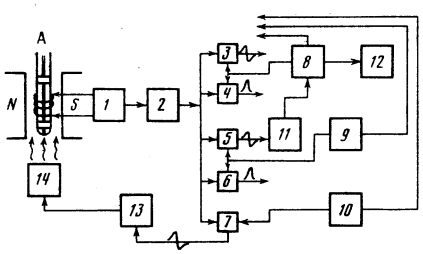

FIG. 1. Block diagram of the apparatus for precise measurement of NMR Shifts. A—coaxial ampoule containing the specimen; 1, 2—spectrometer receiving and amplification channel; 3–7—low-frequency synchronous detectors; 8—variable frequency (2–3 kHz) modulating oscillator; 9—crystal controlled (2 kHz) modulating oscillator; 10—variable frequency (0.1–10 kHz) modulating oscillator; 11—electronic frequency control unit for regulating oscillator 8; 12—type Ch3-35A digital frequency meter; 13—electronic control unit for regulating the gas thermostat; 14—gas thermostat.

With our apparatus it is possible to accumulate up to 120 values (depending on the magnetic relaxation time) of the resonance-frequency difference mentioned above every ten minutes; this ensures effective statistical processing.

To demonstrate the capabilities of the apparatus we performed the following control experiments.

A. We verified the stability and accuracy achieved in reading the difference between the resonance frequencies of the reference and spectator substances. The solutions were made up with heavy water as the solvent.

The inner solution contained 30% of dimethyl sulfoxide CH_3SOCH_3 (the reference substance). The outer solution contained 1% of *t*-butyl alcohol $(CH_3)_3COH$ (the spectator substance), 10^{-3} mole/liter of cystamine, and 10^{-6} mole/liter of copper sulfate (an oxygen absorber). The difference between the resonance frequencies was measured with an accuracy up to 10^{-5} Hz every 30–40 sec for an hour. None of the 90 measured values fell outside a 0.01-Hz interval, the rms deviation from the mean was 0.0024 Hz, and the histogram representing the deviations from the mean was bell shaped and symmetric.

B. We measured the shift of the response frequency of the spectator substance as a function of the concentration of the dissolved paramagnetic salt. The solution in the outer cylinder of the coaxial ampoule contained, in addition to the heavy water solvent and the spectator substance, 0.2 mole/liter potassium phosphate (a buffer system, $pD=7$) and 0.005 mole/liter of ethylene diamine tetraacetic acid (complexon). After measuring the difference between the resonance frequencies of the spectator and reference substances the specimen was dismantled and vanadyl sulfate $VOSO_4$ (a paramagnetic compound having one unpaired electron) was introduced into the outer solution at a concentration of 3×10^{-4} Mole/liter, and the measurement was repeated. After that the specimen was dismantled again, and one-tenth of the volume of the outer solution was withdrawn with a capillary pipet and replaced by the same volume of a solution that differed from the initial solution only in the absence of the paramagnetic compound. The specimen was remounted and the difference between the resonance frequencies was measured again. This sequence of operations was repeated five times (a so-called exponential calibration). The results of these measurements are shown in Fig. 2. The mean deviation between the experimental points and the calculated ones was 0.004 Hz, and the maximum deviation did not exceed 0.008 Hz.

C. We measured the reaction kinetics of oxygen reduction by an oxidation-reduction system of coenzymes of the respiratory chain. In addition to 0.2 mole/liter of a phosphate buffer system ($pD=7$), the outer solution contained 10^{-2} mole/liter of NADH (reduced nicotinamide adenine dinucleotide) and 10^{-5} mole/liter of oxidized FMN (flavin mononucleotide). Before the experiment air was bubbled through the solution to saturate it with oxygen. The difference between the resonance frequencies of the reference and spectator substances was measured every minute or two for 1.5 hr. The specimen temperature was 30°C. The results of the measurements are presented in Fig. 3.

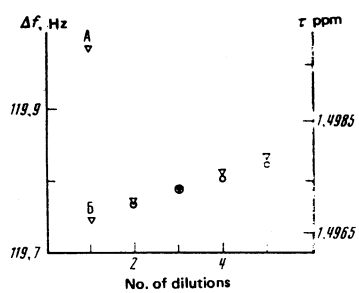

FIG. 2. Calibration against a vanadyl sulfate solution. A—solution with no paramagnetic material, B—solution containing paramagnetic material, ○—calculated points, ▽—experimental points.

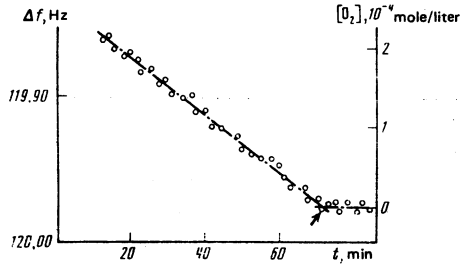

FIG. 3. Time dependence of the difference between the spectator and reference resonance frequencies during reduction of dissolved oxygen by a system of respiratory coenzymes.

The equilibrium concentration of oxygen in dilute aqueous salt solutions is close to 3×10^{-4} mole/liter. The oxygen molecule contains two unpaired electrons in a triplet state. Under the conditions of our experiment, an equilibrium concentration of oxygen may give rise to a paramagnetic shift of the NMR signal from the spectator substance amounting to 0.18 Hz. In case of a large excess of NADH over FMN the oxygen reduction reaction is of zeroth order in oxygen; such a reaction has linear kinetics.[10] The kinetic curve in Fig. 3 is indeed linear, with a sharp transition (marked by an arrow) from a sloping to a horizontal line at the instant when the oxygen was used up. The mean deviation of the points from the regression line was 0.003 Hz. The calculated amount of reduced oxygen was 2.6×10^{-4} mole/liter. The rate constant for the oxidation of NADH calculated from the kinetic curve was 0.7 liter/mole · sec; this agrees with data in the literature[10] and with values obtained by the authors, using spectrophotometry.[11]

D. We measured the temperature dependence of the chemical shift of the methylene protons in ethyl alcohol with respect to the protons of the methyl group, and the spin–spin interaction constants for the methylene and methyl protons. A 0.3% solution of alcohol in heavy water was used. The results of the measurements are presented in Fig. 4.

We note that, in principle, the spin–spin interaction constant for monosubstituted ethanes should be temperature independent.[1] Under the conditions of our experiment (low alcohol concentration and very low *pD*) the alcohol molecule may be regarded as chemically and conformationally stable at moderate temperatures. It is probably the change of the mean number of hydrogen bonds between water and alcohol molecules that is responsible for the observed temperature dependence of the spin–spin interaction constant, as well as for that of the difference between the chemical shifts of methylene protons and methyl protons. We analyzed the possible effect of the partial overlap of the lines of the methylene quadruplet on the measured value of the spin–spin interaction constant and concluded that it is negligibly small.

On the basis of the results of the last experiment, we

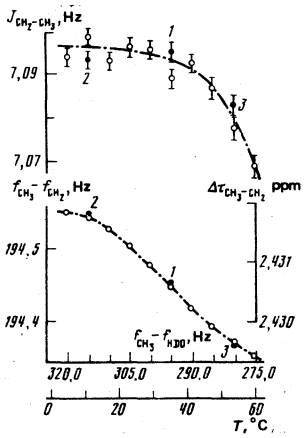

FIG. 4. Temperature dependences of the chemical shift $\Delta\tau_{CH_3-CH_2}$ of the methylene protons in ethyl alcohol with respect to the protons of the methyl group and of the spin-spin interaction constant $J_{CH_2-CH_3}$ for the methylene and methyl protons. The chemical shift was measured between the left-hand large line of the methylene quadruplet and the central line of the methyl triplet. The spin-spin interaction constant was measured as the difference between the resonance frequencies of the large lines of the methylene quadruplet. The black points numbered 1, 2, and 3 were recorded (in that order) during a second run. The difference $f_{CH_3}-f_{HDO}$ between the NMR shifts for the protons in the methyl group of the alcohol and the protons contained in the heavy water as an ordinary water impurity was used to measure the temperature of the specimen and hold it constant.

consider the accuracy achievable in measuring shifts with our apparatus to reach 0.0015 Hz (2×10^{-6} ppm) when an internal reference is used.

Thus, the accuracy achieved in measuring shifts of NMR signals is 0.0015 Hz (2×10^{-6} ppm) when an internal reference is used, and 0.006 Hz (8×10^{-6} ppm) when an external reference is used. This sensitivity enables one to measure very small changes in the static magnetic susceptibility of liquid specimens, e.g., to determine the appearance of paramagnetic centers at concentrations of 10^{-6} mole/liter (for $s=1/2$); this raises the sensitivity of the well-known NMR method of Evans by a factor of 100 to a value of the order of 3.5×10^{-11}, which is close to the sensitivity of the best apparatus for measuring magnetic susceptibilities using a torsion balance in a magnetic field gradient [about 3×10^{-11} (Ref. 12)]. This considerably broadens the magnetometric capabilities of the Evans method. We recall that the Evans method is free of such disadvantages of gravimetric methods as the necessity of freezing the specimen and subjecting it to vacuo, and the necessity of making measurements over a wide range of low temperatures to establish the diamagnetic component of the magnetic susceptibility.

In addition, the accuracy achieved is sufficient for recording contact and pseudocontact shifts that are strongly masked by exchange processes, i.e., for conducting studies of the structures of many coordination compounds and enzymes containing paramagnetic ions—studies that frequently proved to be impossible in the past because of the low solubility of the investigated molecules.

We hope that the proposed method will prove to be useful to investigators interested in the structure of complex molecules, especially in the structure of molecules of biological origin—to biophysicists and biochemists. The new possibilities for studying paramagnetic states in solution may also be of interest to physicists concerned with the magnetic properties of matter.

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[1]J. W. Emsley, J. Feeney, and L. H. Satcliffe, High resolution nuclear magnetic resonance spectroscopy, Pergamon Press, Oxford, New York, 1965-66 (Cited in Russian translation, "Mir," 1968: Vol. 1, pp 66–97, 109, 245, 459; Vol. 2, p. 17).
[2]D. F. Evans, J. Chem. Soc. No. 6, 2003 (1959).
[3]Harden M. McConnell, J. Chem. Phys. 28, 430 (1958).
[4]H. S. G. Gutowsky, D. W. McCall, and C. P. Slichter, J. Chem. Phys. 21, 279 (1953).
[5]R. Freeman and B. Gestblom, J. Chem. Phys. 48, 5008 (1968).
[6]Kenneth W. Gray and Irving Ozier, Phys. Rev. Lett. 26, 161 (1971).
[7]Yu. S. Konstantinov and A. M. Smirnov, Prib. Tekh. Eksp. No. 5, 134 (1972).
[8]Daniel Brault and Michel Rougee, Biochemistry, 13, 4598 (1974).
[9]Able Schejter, Amos Lanir, and Nava Epstein, Arch. Biochem. Biophys. 174, 36 (1976).
[10]Clarence H. Suelter and David E. Metzler, Biochem. Biophys. Acta 44, 23 (1960).
[11]S. E. Bresler, A. M. Kachurin, N. I. Ogurtsova, and V. N. Fomichev, Biokhimiya 41, 426 (1976).
[12]Claude Nicolau (editor), Experimental methods in biophysical chemistry, John Wiley, London, New York, 1973, p. 370.

Translated by E. Brunner