SPIN-ECHO NUCLEAR MAGNETIC RESONANCE EVIDENCE FOR COMPLEXING OF SODIUM IONS IN MUSCLE, BRAIN, AND KIDNEY

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ABSTRACT Na^+ in muscle, brain, and kidney is shown by spin-echo nuclear magnetic resonance (NMR) to consist of two fractions with different NMR parameters. The slow fraction of Na^+ in these tissues has NMR relaxation times T_1 and T_2 of 10–15 $\times$ 10^{-3} sec, which is approximately 4–5 times shorter than for Na^+ in aqueous NaCl solution. The slow fraction may represent Na^+ dissolved in structured tissue water. The fast fraction of tissue Na^+, which is shown to represent approximately 65% of the total tissue Na^+ concentration, has T_2 less than 1 $\times$ 10^{-3} sec, which resembles the values of T_2 observed for Na^+ complexed by synthetic ion-exchange resins. One is drawn to the conclusion that approximately 65% of total Na^+ in muscle, brain, and kidney is complexed by tissue macromolecules.

INTRODUCTION

The classical picture of the cell as a membranous bag containing inorganic ions and proteins dissolved in liquid water is widely accepted. Belief in the classical picture seems to come from its simplicity and from historical precedent. Experimental evidence for its validity is scarce. Important contradictions are contained within the classical picture, the most conspicuous of which is that the ion pumps which must be postulated in order to maintain the observed ion concentration gradients against measured leakages would require 1500–3500% of the total energy produced by the cell (1).

Because of serious deficiencies of the classical theory, a small minority of investigators have taken an entirely different view of the cell. They regard the cell as an organized, nonliquid phase consisting of macromolecules embedded in semicrystalline water. Na^+ and K^+ are believed mostly to be complexed to cell macromolecules and to have low solubility in semicrystalline cell water. A basis for comprehensive theoretical analysis of the minority view of the cell has been developed by Ling (1, 2) using statistical mechanical concepts, under the name of the "association-induction hypothesis." A less comprehensive but simpler treatment of cellular ion complexing

using mass-action methods has been presented by Troshin (3, 4). A simple non-equilibrium thermodynamic treatment of cellular ion leakage phenomena has been derived from the application of solid-state physical concepts by Cope (5–7).

Much progress has occurred in recent years toward experimental proof of the minority view, including evidence for structuring of cell water, and for complexing of Na^+ and K^+. Cell water seems probably to consist of multiple polarized layers (8, 9). Nuclear magnetic resonance (NMR) evidence strongly supports the idea that cell water possesses more structure than liquid water (22, 23). Evidence for Na^+ complexing has come from several sorts of measurements. Equilibrium binding studies of Troshin (3, 4) and Ling (1) showed that the equilibria of Na^+ between cell and solution conformed to the Langmuir adsorption isotherm, with a correction for a minor fraction of Na^+ dissolved in intracellular water. Cation-sensitive intracellular microelectrode measurements (10–13) have been interpreted to indicate that 70% of intracellular Na^+ of muscle was in some form that excluded it from contact with the microelectrode.

NMR analysis of Na^+ has great advantages over other methods for the study of complexing of Na^+ in tissues (14, 15, 6, 16, 17). First, the position of the NMR line of Na^+ is much different from that of all other ions such as H^+, K^+, or Cl^-. Hence, the results of NMR analysis are free from those uncertainties which confuse the interpretation of conductivity and electrode measurements. Second, the width of the steady-state NMR line of Na^+ is dependent upon the chemical and physical environment surrounding the individual Na^+ ion. Therefore, the width of the steady-state NMR line may indicate whether the Na^+ ion is surrounded by an environment like that in free solution, or by some other environment. The same advantages of NMR may be applied to the study of K^+ complexing in biological systems (18, 19).

These properties of the steady-state NMR spectrum have been exploited by Cope (6, 14, 15), Rotunno et al. (16), Ling and Cope (18), and Czeisler et al. (17) to provide strong evidence for complexing of Na^+ in tissues. The steady-state NMR spectrum of muscle (14, 15, 17, 18), brain (15), kidney (15), and frog skin (16) showed 60–75% depressions of peak height compared to the same concentrations of Na^+ in free solution, with little change in line width. A similar picture had been observed previously in ion-exchange resin by Jardetzky and Wertz (20). These authors (20) interpreted Na^+ in wet ion-exchange resin to consist of two fractions: (*a*) a fraction of Na^+ dissolved in water which showed the usual NMR spectrum of Na^+, and (*b*) a fraction of Na^+ complexed by the ion-exchange resin, which markedly shortened the NMR relaxation time to produce a steady-state NMR spectrum of Na^+ that was so broad and low that it was invisible to NMR because it was obscured by instrumental noise. The broadening of the NMR spectrum of Na^+ complexed by ion-exchange resin has been shown directly by Czeisler et al. (17). The steady-state NMR picture observed in tissues was interpreted in the same way to indicate that 60–75% of Na^+ in tissues was broadened to NMR invisibility by complexing with macromolecules, while the second smaller fraction was in free

solution in tissue water, and therefore had its usual steady-state NMR spectrum (6, 14–19).

It was possible that the above two-fraction interpretation might be wrong. The presence of only a single fraction of tissue Na^+ with NMR relaxation times (T_1 and/or T_2) moderately different from Na^+ in liquid water could under some circumstances also give a depression of height with unchanged width of the steady-state NMR spectrum (21). Such a change in T_1 or T_2 of Na^+ might result from the probable structured state of tissue water (22, 23). Errors of interpretation due to instrumental problems might also have been present in the original steady-state NMR studies (6, 14–16, 18). However, a sophisticated study of the instrumental subtleties of the steady-state NMR behavior of muscle Na^+ has just appeared which confirms the two-fraction interpretation (Czeisler et al., 17). The present paper provides additional confirmation of the two-fraction hypothesis by *spin-echo* NMR of Na^+ in muscle, brain, and kidney, and provides direct measurements of NMR relaxation times of both fractions of Na^+ in each of these tissues.

METHODS

A. Spin-Echo NMR

A 2 ml volume of solution or tissue in a glass test tube of 14 mm I.D. was centered in the NMR probe. Sample temperatures were 25° ± 1°C except where otherwise stated. The experiments were performed at the NMR resonance of ^{23}Na at 10×10^6 Hz and 8879 gauss. The magnetic field was produced by either of two different Varian 12-inch diameter electromagnets, each with a gap of 1.75 inches. The first magnet had auxiliary shim coils to increase the homogeneity of magnetic field. Because the second magnet lacked such coils, it had a relatively poor homogeneity. In some situations, the difference in homogeneity necessitated a difference in technique of measurement of T_2, as will be described later in the paper. The NMR probe, in which the sample was placed, had separate transmitter and receiver coils. The measurements used a Model PS-60AW spin-echo NMR spectrometer (Nuclear Magnetic Resonance Specialities, Inc., New Kensington, Pa.), with the addition of a low-noise preamplifier placed close to the probe, and extra RC filtering after the receiver, the phase detected output of which was fed into a D.C. amplifier with a gain of 10 × or 100 ×, which led into an averaging computer (Model TDH-9 of Princeton Applied Research Corp., Princeton, N. J.; or CAT Model 400A of Technical Instruments, Corp., North Haven, Conn.; or Model 5480A of Hewlett-Packard Co., Palo Alto, Calif.). The spectrometer was also modified to put both first and second pulses through the same transmitter channel, so that phase drifts for both pulses were equal. The modifications and accessories listed above provided the high signal-to-noise ratio which was necessary to measure accurately the low concentrations of Na^+ in tissues. In order to plot T_1 and T_2 decay curves more completely, the spectrometer was modified to allow continuous adjustment of the time between pairs of pulses, instead of the stepwise control provided on the standard instrument. An attenuator with 1% accuracy and zero phase shift was included in the receiver to facilitate quantitative comparisons between different Na^+ concentrations. All measurements of time were calibrated against a crystal oscillator, which had an accuracy of 0.001%.

The procedures for spectrometer adjustment were conventional and have been described previously (22). The 90° pulse had a width of approximately 77×10^{-6} sec and a peak-to-peak

height of approximately 460 v. Repetition rate of the pulse pattern was 4 per sec except where stated otherwise. The techniques for measuring free induction decay, T_1, and T_2 were conventional, except for the extensive use of RC filtering and computer averaging, which were necessary to achieve sufficient accuracy in the measurement of the low concentrations of Na^+ in tissues. Free induction decay was measured after a single 90° pulse. Echoes were recorded for a 90°-180° pulse pattern. Then transverse NMR relaxation time (T_2) was measured from the time for the height of the echo to decay to 1/e of a previous value. In some cases, T_2 for Na^+ in tissues or ion-exchange resins was much shorter than the free induction decay which would have been produced by magnetic inhomogeneity. In these cases, T_2 was measured from the decay time of the single exponential decay of the free induction signal. Longitudinal relaxation time (T_1) was measured as described by Hahn (24) and Boehme and Eisner (25). The height of the free induction decay after the second pulse of a 90°-90° pair was measured for various values of pulse separation (τ) and was subtracted from the height of the free induction decay after the first pulse. A plot of the logarithm of these differences vs. τ yielded a straight line, from which the time to decay to 1/e of a previous value was estimated, which provided a value of T_1.

Quantitative analysis of Na^+ concentration from the height of the free induction decay used the following procedure. To measure concentration of Na^+ independently of changes in *shape* of the free induction decay, it is necessary to use the initial height of the curve, i.e., the height at the start of the decay curve immediately after the end of the transmitted pulse. For aqueous solutions of Na^+ with either magnet, and with tissue samples with the low homogeneity magnet, free induction decays were observed to follow a gaussian pattern (Fig. 1), provided that total RC filtering was small enough to avoid excessive prolongation of the transmitted pulse artifact. The initial height was obtained by extrapolation to $t = 0$ of a linear graph of $\log h$ vs. t^2 of the free induction decay curve (t = time after end of 90° pulse, h = height of the curve). For tissue samples in the high homogeneity magnet, the short T_2 converted the free induction decay into a single exponential shape, so that initial height was determined by extrapolation to $t = 0$ of a graph of $\log h$ vs. t. The initial height has the same constant of proportionality to Na^+ concentration regardless of the shape or decay time of the free induction decay curve. This is true in theory, and was proven experimentally for the case of D in D_2O (22), and was confirmed for Na^+ in the presence of paramagnetic ions (see later in the present paper). Therefore, concentrations of Na^+ can be calculated on the assumption of direct proportionality of initial height of the free induction decay to Na^+ concentration (Fig. 3), using a 0.1 N NaCl standard measured immediately before or afterwards and at the same temperature. It was observed that the constant of proportionality between sodium concentration and height of the spin-echo NMR signals varied by less than approximately 10% over a period of weeks, which indicated good stability of the electronic equipment.

B. Chemical and Biological

5 g of each ion-exchange resin in the acid form was treated with 25 ml of 1 N NaOH, which was then decanted. The resin was then washed five times with 25 ml volumes of water. The resin was allowed to settle for 5 min, the last wash water was decanted, and the wet resin was packed up to a 2 ml mark in a test tube for NMR analysis.

Tissue samples were obtained from white male rats (200–300 g body weight) as soon as possible after killing by cervical dislocation, so that all spin-echo NMR measurements were completed within 1 hr after death of the rat, except where stated otherwise. Muscle was cut from the thigh in approximately 0.5 g pieces. The brain was removed intact, except for separation of the cerebellum. Kidneys were cut into quarters. All tissues were blotted to remove

blood and extracellular fluid insofar as possible. Pieces of tissue were then packed up to a 2 ml mark in glass test tube (14 mm I.D.) for NMR measurements. Total Na^+ in all tissue samples was determined by steady-state NMR analysis after ashing to destroy organic molecules which might complex Na^+, using a method described previously, which gave tissue Na^+ analyses agreeing within 4% of results obtained by flame photometry (15). 2 ml samples of 50 mM NaCl solutions showed recoveries averaging 98% after ashing and analysis by the above technique.

I. SPIN-ECHO NMR OF NA^+ IN SIMPLE SYSTEMS

In steady-state NMR spectroscopy, the sample is irradiated continuously with radio-frequency energy. In spin-echo NMR spectroscopy, however, the sample is irradiated with brief pulses of radio-frequency energy, and radio signal emission from the sample is measured following the incident pulses. By this technique, one may measure three different sorts of NMR relaxations or decays of Na^+, which are free induction decay, transverse relaxation time (T_2), and longitudinal relaxation time (T_1). Briefly stated, T_1 measures decay by longitudinal flipping of Na nuclei, T_2 measures the sum of decay due to the flipping plus any dephasing which is random causing loss of phase memory, and free induction is the sum of these two plus additional dephasing for which phase memory exists, due to macroscopic magnetic inhomogeneities. A more comprehensive but elementary discussion of these phenomena was presented in a previous publication (22).

The expected phenomena of spin-echo NMR of Na^+ were observed in aqueous solutions of NaCl. Fig. 1 shows the free induction decay observed after a 90° pulse, and the echo after a 90°-180° pulse pair. Free induction decay shape conformed approximately to a gaussian curve with respect to time (Fig. 2). Echo decay for aqueous NaCl followed a single exponential decay from which T_2 was measured. Decay of the height of the free induction decay after the second pulse of a 90°-90° pattern also conformed to a single exponential in accord with the theory of Hahn (24), and from which T_1 was measured.

A brief study was made of the NMR relaxation times of Na^+ in various aqueous solutions. At a temperature of 20° ± 1°C in aqueous 0.1 N NaCl, Na^+ showed T_2 values of 52, 58, 60, 51, and 59 × 10^{-3} sec (mean = 56 × 10^{-3} sec) and T_1 values of 54, 60, 51, 57, and 53 × 10^{-3} sec (mean = 55 × 10^{-3} sec). These measurements agree well with a previous study of Eisenstadt and Friedman (26), whose data on Na^+ as a function of concentration shows $T_2 = T_1 = 56 \times 10^{-3}$ sec for 0.1 N NaCl. The addition of 0.1 N HCl, 0.1 N KOH, 0.1 M $MgSO_4$, or 0.1 M citric acid did not change T_2 of 0.1 N NaCl significantly. The addition of 0.1 M K_2HPO_4 shortened T_2 by approximately 15%. The addition of 0.1 M $MnCl_2$ or 0.1 M $CuSO_4$ shortened T_2 by approximately 30%. Previously, Jardetzky (27) had found paramagnetic cations to be without significant effect on the steady-state NMR spectrum of Na^+. The shortenings of T_2 by paramagnetic ions observed here are apparently too small to have revealed themselves under the steady-state experimental conditions used by Jardetzky (27).

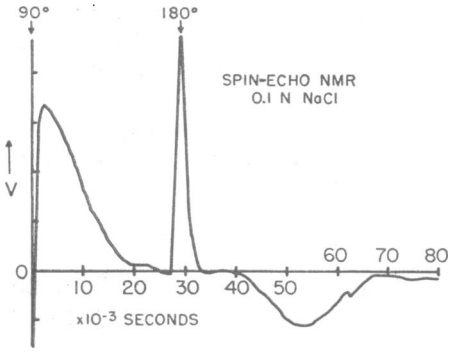

FIGURE 1

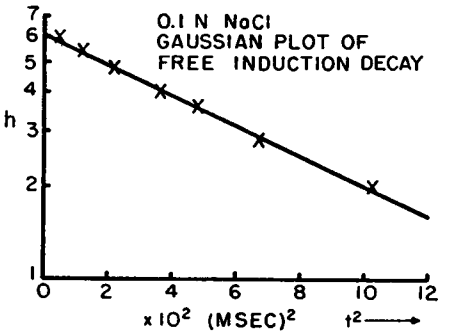

FIGURE 2

FIGURE 1 Free induction decay and echo of aqueous 0.1 N NaCl. V is the voltage output in arbitrary units of the pulsed NMR spectrometer, which is plotted against time. Sample is 2 ml of 0.1 N NaCl at T = 25°C. The initial 90° pulse is followed by emission by the sample of the free induction decay. A 180° pulse is then applied, which is followed on equal time later by emission of an echo. The magnetic field of the high homogeneity magnet deliberately has been made less homogeneous than the best obtainable with this magnet in order to make the free induction decay and echo narrow for ease of observation. RC filtering of approximately 0.75 × 10^{-3} sec is applied to the spectrometer output, and 100 measurements have been averaged to improve signal-to-noise ratio. Repetition rate of the 90°–180° pulse pair is 4 per sec.

FIGURE 2 Gaussian plot of free induction decay of Na^+ in aqueous solution. The high homogeneity magnet was used. On the vertical axis, the log of voltage output (h) of the pulsed NMR spectrometer following a single 90° pulse at a repetition rate of 4 per sec is plotted in arbitrary units against t^2, where t is time after the 90° pulse. The sample is 2 ml of 0.1 N NaCl at 25°C. This data is an average of six measurements. The approximate linearity of this plot indicates conformity to a gaussian curve, which allows a linear extrapolation to $t^2 = 0$ to determine the initial height of the free induction decay.

Initial heights of the free induction decay of various concentrations of Na^+ in NaCl solutions were measured as described in the Methods section. The data plotted in Fig. 3 show that this method of analysis for Na^+ yields a linear standard curve. The initial height of the free induction decay of 0.1 N NaCl was observed not to be significantly changed by the addition of any of the following: 0.1 N HCl, 0.1 N KOH, 0.1 M $MgSO_4$, 0.1 M K_2HPO_4, or 0.1 M citric acid. The addition of paramagnetic ions (0.1 M $MnCl_2$ or 0.1 M $CuSO_4$) also had no significant effect on the initial height of the free induction decay of Na^+, even though these ions shortened T_2 by 30%, as described in the previous paragraph. This confirms for the case of Na the conclusion drawn from previous studies of D in D_2O (22), that the initial height of the free induction decay is a valid measure of Na^+ concentration even when T_2 varies.

A brief study was made of Na^+ in gelatin gels. Incorporation of 0.1 N NaCl into a 5 or 10% gelatin gel was observed to have no effect on the initial height of the free induction decay of Na^+. However, T_2 of Na^+ was shortened by 28 and 45% respectively when 0.1 N NaCl was incorporated into a 5 or 10% gelatin gel. This effect might result from rapid exchange of dissolved Na^+ with a fraction of Na^+

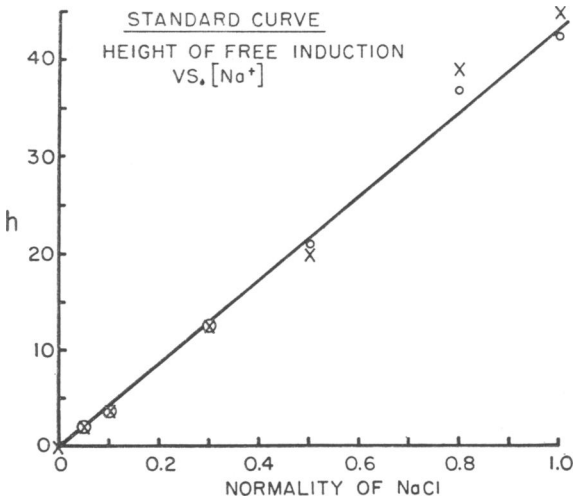

FIGURE 3 Standard curve of initial height of free induction decay vs. Na^+ concentration. On the abcissa is plotted Na^+ concentration for 2 ml volumes of aqueous NaCl solutions at 25°C. On the ordinate, h represents in arbitrary units the initial height of the free induction decay after a single 90° pulse. The two sets of points, depicted by crosses and circles, represent two sets of measurements on the same samples. h was determined by extrapolation of the gaussian decay curve to $t = 0$ as described in the Methods section. Each free induction decay curve was an average of 200 measurements performed at a repetition rate of 4 per sec. RC filtering of 0.75×10^{-3} sec was applied to the output of the pulsed NMR spectrometer. The high homogeneity magnet was used.

complexed by gelatin. However, the present author knows of no studies suggesting complexing of Na^+ by gelatin. Another possible explanation is suggested by the small but real changes in the NMR behavior of water in gelatin gels (22), which suggest that water in gelatin gels has slightly more structure than liquid water. Increased water structure would produce increased electric field gradients, which would be expected to interact with the quadrupole electric moment of the Na nucleus to cause faster NMR relaxation of Na^+, as observed for Na^+ in gelatin.

II. SPIN-ECHO NMR OF NA^+ COMPLEXED BY ION-EXCHANGE RESINS

Jardetzky and Wertz (20) have interpreted *steady-state* NMR measurements of Na^+ in ion-exchange resin preparations to indicate that complexing of Na^+ by the resin produced an NMR spectrum that was so broad and low that it was obscured by instrumental noise. A marked broadening of the steady-state NMR spectrum of Na^+ complexed by ion-exchange resin was observed directly by Czeisler et al. (17). In the present study, packed, wet Dowex 50 resin in the Na^+ form was analyzed by pulsed NMR. For Na^+ in the resin, the free induction decay following a single 90° pulse was much shorter than that of Na^+ in aqueous solution and was a simple exponential decay instead of the gaussian curve which was seen with aqueous solu-

TABLE I

T_2 AND T_1 OF Na^+ COMPLEXED BY ION-EXCHANGE RESINS

Resin name	Resin structure	T_2($\times 10^{-3}$ sec)		T_1($\times 10^{-3}$ sec)	
		Data	Mean	Data	Mean
Dowex 50 (×8)	Polystyrene-SO_3Na	1.4, 1.3, 1.4, 1.3, 1.4, 1.3	1.35	2.75, 2.2	2.5
Amberlite IRC-50	Acrylic-COONa	0.33, 0.36	0.34		
Amberlite IR-100	Polyphenol-CH_2SO_3Na	0.24, 0.23	0.24		
Aqueous solution of 1 N NaCl (ref. 26)			56.00		56.0

RC filtering of 0.02×10^{-3} sec time constant was used for the Dowex 50 measurements. Filtering of 0.05×10^{-3} sec was used for the other resins.

tions. For Na^+ in Dowex 50 subjected to a 90°-180° pulse pattern, echoes could not be seen unless the pulse interval was shortened to approximately the time constant of the free induction decay, so that the echoes overlapped the free induction decay. Therefore, it was clear that the short free induction decay observed for Na^+ in wet Dowex 50 must be determined by T_2. T_2 measured from free induction decays of Na^+ in wet Dowex 50 averaged 1.35×10^{-3} sec (Table I), compared with a T_2 value of 55×10^{-3} sec for Na^+ in aqueous 1 N NaCl solution. By the 90°-90° pulse technique, T_1 for Na^+ in Dowex 50 was observed to average 2.5×10^{-3} sec (Table I) compared with 55×10^{-3} sec for aqueous 1 N NaCl solution. T_2 measured for Na^+ in packed wet preparations of two other ion-exchange resins (Amberlite IRC-50 and IR-100) showed even greater shortenings to mean values of 0.35×10^{-3} and 0.25×10^{-3} sec respectively (Table I). These measurements indicate that complexing by ion-exchange resins shortens T_2 of Na^+ by 40–200 times. Fast NMR relaxation is indeed what one would expect for a Na atom whose quadrupole electric moment interacts with the large electric field gradients present at a charged complexing site. The observed shortening of NMR relaxation time is consistent with previous evidence for marked broadening of the steady-state NMR line of complexed Na^+ (17, 20).

III. SPIN-ECHO NMR OF Na^+ IN MUSCLE, BRAIN, AND KIDNEY

Preliminary study of Na^+ in fresh brain or kidney by pulsed NMR using a single 90° pulse with a filtering time constant 0.1×10^{-3} sec yielded a free induction decay curve which consisted of a sum of two different components: (A) an initial component which decayed as a single exponential with a decay time of approximately 1×10^{-3} sec, superimposed on (B) a slower component which had a gaussian shape, decaying over 3–4 $\times 10^{-3}$ sec, which was the decay produced by inhomogeneities of the field of the magnet used, and was identical with the free induction

decay observed with aqueous NaCl solutions. This indicated that tissue Na^+ consisted of two distinct fractions. The task was then undertaken to measure the NMR relaxation times of each of the two fractions independent of the other, and to determine the concentrations of the two fractions.

First, the *slow* fraction of tissue Na^+ was studied. T_2 of the slow fraction was determined from the decay of the height of the echo as a function of time after a 90°-180° pulse pair. Only times longer than approximately 3×10^{-3} sec were used, in order that signal emission from the fast fraction would no longer be of significant magnitude. Echo decays obtained from muscle, brain, and kidney showed a single exponential pattern (Fig. 4), from which T_2 was measured. T_2 for the slow fraction of Na^+ in muscle, brain, and kidney showed mean values in the range of $9–14 \times 10^{-3}$ sec (Table II), which compares with a value of 56×10^{-3} sec for aqueous 0.1 N NaCl solution. T_1 for the slow fraction of tissue Na^+ was determined from the measurement of height of the second pulse of a 90°-90° pulse pair as a function of time at times longer than 3×10^{-3} sec which yielded a single exponential curve (Fig. 5). T_1 for the slow fraction of Na^+ in muscle and brain showed values resembling those measured for T_2 (Table II), which is moderately shorter than T_1 of aqueous NaCl.

The next problem was to determine the relative concentrations of the slow and fast fractions of Na^+ in tissues. This was accomplished by a measurement of the concentration of the slow fraction in a way which minimized errors due to the presence of the fast fraction. Subtraction of the concentration of the slow fraction

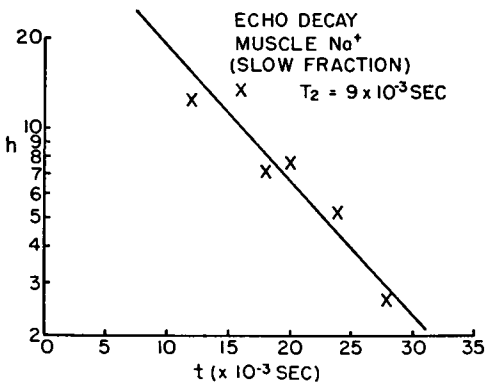

FIGURE 4 Echo decay for muscle Na^+ (slow fraction). Sample is 2 ml of fresh rat muscle at 26°C. Height (h) of the echo following a 90°–180° pulse pattern was measured as a function of the time (t) between the echo and the 90° pulse. h is expressed in arbitrary units. h on a log scale is plotted against t. From the straight line drawn through the data, T_2 is estimated to be 9×10^{-3} sec, which is the time for h to decay to 1/e of a previous value. Each data point shown here represents an average of 4800 measurements, performed at a rate of 16 per sec, with RC filtering of 1×10^{-3} sec time constant applied to the detector output. The low homogeneity magnet was used.

TABLE II

T_1 AND T_2 OF SLOW FRACTION OF NA^+ IN TISSUES

Specimen	T_2($\times 10^{-3}$ sec)		T_1($\times 10^{-3}$ sec)	
	Data	Mean	Data	Mean
Aqueous 0.1 N NaCl	52, 58, 60, 51, 59	56	54, 60, 51, 57, 53	55
" " " (ref. 26)		56		56
Rat muscle	9, 16, 26, 11, 7	14	12, 10, 12, 12	12
Rat brain	11, 10, 9, 11, 11	10	14, 25, 12, 11	15
Rat kidney	8, 8, 10, 12, 8, 8	9		

This data was obtained under the experimental conditions given in Table III.

TABLE III

EXPERIMENTAL CONDITIONS FOR DATA OF TABLE II

Specimen and measurement	RC filtering ($\times 10^{-3}$ sec)	No. of repetitions averaged	Repetition rate
			repetitions/sec
0.1 N NaCl—T_2	1	240	4
" " T_1	1	200	4
Muscle—T_2	1	4800	16
" T_1	1	4800	16
Brain—T_2	1.5	200	4
" T_1	1	1920	16
Kidney—T_2	1.5	400	4

Temperature of samples was maintained between 25° and 30°C. All measurements on tissues were completed within 1 hr after death of the rat, except those on muscle which sometimes required as long as 2 hr. Kidney and brain T_2 measurements used a high homogeneity magnet, so that T_2 was shorter than the free induction decay of a single 90° pulse and was measured from the latter. 0.1 N NaCl was measured with the high homogeneity magnet but used decay of echoes. Muscle T_2 values were obtained from echo decays with the low homogeneity magnet. All other measurements used the low homogeneity magnet. An exception is the last value of T_2 for muscle in Table II, which was obtained from the free induction decay with the high homogeneity magnet, used RC filtering of 1.5×10^{-3} sec, and is an average of 2230 repetitions of a single 90° pulse at a rate of 11 repetitions per sec. Each value of T_2 or T_1 for tissue in Table II was obtained on tissue from a different rat.

of tissue Na^+ from total tissue Na^+ then yielded the concentration of the fast fraction.

The concentration of the slow fraction of tissue Na^+ could in principle have been determined from the free induction curve following a single 90° pulse, which yielded the two-component curve described in the first paragraph of this section. This method would involve extrapolation of the slower, the gaussian, portion of the

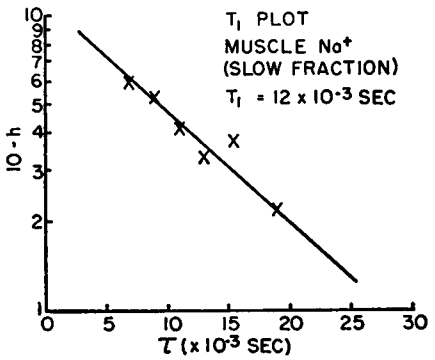

FIGURE 5 T_1 plot for muscle Na^+ (slow fraction). Sample is 2 ml of fresh rat muscle at 26°C. Height (h) of the free induction decay of the *second* pulse of a 90°–90° pulse pattern was measured as a function of time (τ) between pulses. h is expressed in arbitrary units. Height of the free induction decay of the first pulse was measured equal to 10.0. ($10-h$) on a log scale is plotted against τ. From the straight line drawn through the data points, T_1 is estimated to be 12×10^{-3} sec, which is the time for ($10-h$) to decay to 1/e of a previous value. Each data point shown here represents an average of 4800 measurements, performed at a rate of 16 per sec, with RC filtering of 1×10^{-3} sec time constant applied to the detector output. The low homogeneity magnet was used.

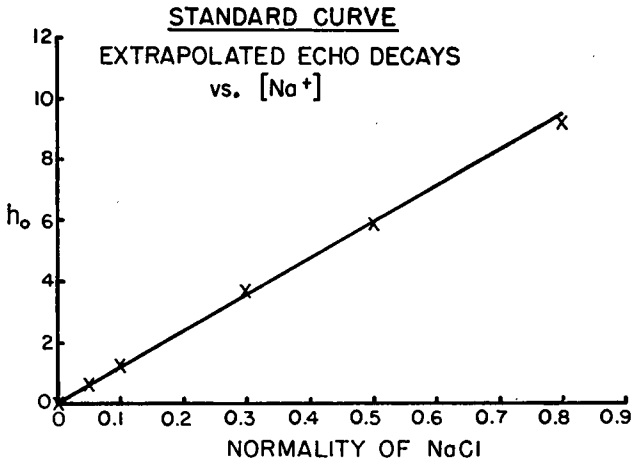

FIGURE 6 Standard curve of initial height of extrapolated echo decays vs. Na^+ concentration. On the abcissa is plotted Na^+ concentration in 2 ml volumes of aqueous NaCl solutions at 25°C. On the ordinate, h_0 represents the height of the echo decay curve extrapolated on a semilog plot to $t = 0$. Each echo is an average of 200 measurements with a 90°-180° pulse pattern at a repetition rate of 4 per sec. RC filtering of 0.5×10^{-3} sec was used. The low homogeneity magnet was used.

curve back to $t = 0$, to yield the initial height of the free induction decay of the slow fraction, which could be compared with the same measure for a 0.1 N NaCl standard, to calculate the concentration of Na^+ in the slow fraction. This method proved inaccurate because slight errors in adjustment of magnetic field to NMR resonance

caused large errors in shape of the slow portion of the free induction curve, so that gaussian extrapolations to $t = 0$ showed large variability. This difficulty was eliminated by plotting echo decay curves of the slow fraction for times longer than 3×10^{-3} sec after a 90°-180° pulse pattern, and then extrapolating to $t = 0$ the

TABLE IV

CONCENTRATIONS OF SLOW AND FAST FRACTIONS OF TISSUE NA^+

Rat No.	Type of tissue	Total Na^+	Slow fraction	Fast fraction	Per cent fast faction
		mmoles/ liter	*mmoles/ liter*	*mmoles/ liter*	%
1	Muscle	27	11	16	59
2	"	26	9	17	66
3	"	23	11	12	52
4	"	24	10	14	58
5	"	25	9	16	64
6	"	36	11	25	70
Mean	"				62
7	Brain	43	14	29	67
8	"	50	17	33	66
9	"	55	19	36	66
10	"	43	13	30	70
11	"	45	16	29	65
12	"	50	17	33	66
Mean	"				67
13	Kidney	59	24	35	59
14	"	64	20	44	70
15	"	65	20	45	70
16	"	66	22	44	68
17	"	55	19	36	66
Mean	"				67

Total tissue Na^+ concentrations were measured by steady-state NMR after ashing (15). Concentration of the slow fraction (i.e. the fraction of tissue Na^+ with a long ($10–15 \times 10^{-3}$ *sec*) NMR relaxation time) was measured by semilog extrapolation of the echo decay curve to $t = 0$ to yield an initial height from which $[Na^+]$ was determined by comparison with a similar measurement on an aqueous NaCl standard. Concentration of the fast fraction (i.e. the fraction of tissue Na^+ with $T_2 \cong 1 \times 10^{-3}$ sec) was calculated by subtraction of the concentration of the slow fraction from total Na^+. The fast fraction was also computed as per cent of total tissue Na^+. All concentrations are reported as per unit volume of fresh tissue. Echoes were measured after a 90°-180° pulse pattern at a repetition rate of 4 per sec with RC filtering of 0.5×10^{-3} sec on the detector output. Each echo-decay curve for tissue or NaCl standard was based on four echo heights of different pulse spacing longer than 3×10^{-3} sec. Each echo recording was an average of 700, 300, and 200 measurements for muscle, brain, or kidney respectively.

resulting single exponential decay curve. Thus, one obtained the initial height (h_0) of the signal emitted by the slow fraction of tissue Na^+ without errors due to signal emission from the fast fraction. Since h_0 is theoretically and experimentally (Fig. 6) proportional to Na^+ concentration, the concentration of the slow fraction of tissue Na^+ was calculated by comparison of h_0 of the tissue sample with the same measurement on a 0.1 N NaCl standard solution obtained immediately afterwards. To test experimentally the theoretical expectation that concentration of Na^+ measured in this way would be independent of NMR relaxation time, h_0 measured in 0.1 N NaCl was compared with values obtained for 0.1 N NaCl plus 0.5 M $CuSO_4$ plus 15% gelatin, which shortened T_2 to approximately 10×10^{-3} sec. Values of h_0 obtained for the Cu-gelatin solution were 3.9, 3.5, 4.3, 3.7, and 3.1 (mean = 3.7) which were similar to the values observed for pure 0.1 N NaCl solution (3.9, 3.5, 3.6, 3.7, and 3.7 [mean = 3.7]). The same arbitrary units are used for both sets of measurements.

The relative concentrations of slow and fast fractions of Na^+ measured in rat muscle, brain, and kidney are presented in Table IV, which shows that most Na^+ (60–70%) in these tissues belongs to the fast fraction.

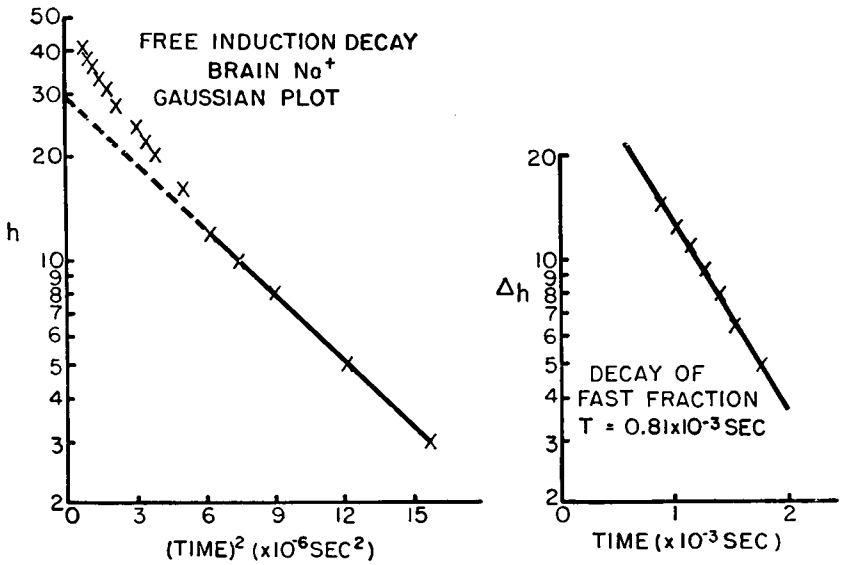

FIGURE 7 Measurement of T_2 of fast fraction of brain Na^+. Left curve is gaussian plot of free induction decay of 2 ml of rat brain after a single 90° pulse with RC filtering = 0.05×10^{-3} sec and averaged 3720 times. h represents in arbitrary units the voltage emitted by the sample. The linear lower portion of the curve representing the magnetic inhomogeneity decay of the slow fraction of brain Na^+ is extrapolated by the dotted line to $t = 0$, which reveals the presence of an additional fast fraction of brain Na^+ at short times. The heights of the fast fraction (Δh) were determined by subtracting the heights of the dotted extrapolation from the heights of the measured curve at the same times. The curve on the right shows a semilog plot of the free induction decay of the fast fraction (Δh) as a function of time, which is linear, and from which T_2 of the fast fraction of brain Na^+ was measured.

Finally, T_2 of the fast fraction of tissue Na^+ was measured. Using the free induction decay after a single 90° pulse with only minimal RC filtering, the gaussian decay curve of the slow fraction of tissue Na^+ was subtracted from the total curve to leave the free induction decay of the fast fraction of tissue Na^+, which showed single exponential decay from which T_2 was measured (Fig. 7). This procedure was appropriate because T_2 of the slow fraction was significantly *longer* than the decay time due to magnetic inhomogeneity, while T_2 of the fast fraction was significantly *shorter* than the inhomogeneity decay. Details of this procedure are given in the captions of Fig. 7 and Table V.

Table V shows that T_2 of the fast fraction of Na^+ in muscle, brain, and kidney has values in the region of 0.5–1.0 $\times$ 10^{-3} sec, which resemble the values recorded for Na^+ complexed by synthetic ion-exchange resins (Table I), but differ markedly from the $T_2 = 56 \times 10^{-3}$ sec observed for Na^+ in aqueous 0.1 N NaCl solutions.

We may summarize these observations by stating that the NMR behavior of Na^+ is similar in muscle, brain, and kidney. 60–70% of tissue Na^+ shows a fast NMR relaxation time resembling those measured for Na^+ complexed by ion-exchange resins. Therefore, it seems reasonable to surmise that 60–70% of Na^+ in tissue is complexed by tissue macromolecules. The remainder of tissue Na^+ (the slow fraction) has NMR relaxation times only moderately faster than Na^+ in aqueous solu-

TABLE V

T_2 OF FAST FRACTION OF TISSUE Na^+

Rat No.	T_2 ($\times$ 10^{-3} sec)		
	Muscle	Brain	Kidney
18	1.06	0.81	0.81
19	1.00	0.62	0.81
20	0.81	0.62	0.48
21	0.75	0.94	0.95
Mean	0.90	0.75	0.76

Samples are 2 ml of rat tissue within 1 hr postmortem at 25° ± 1°C. RC filtering on spectrometer is 0.05 $\times$ 10^{-3} sec. Free induction decays were measured after a single 90° pulse, repeated 32 times per sec, averaged on a 10 $\times$ 10^{-3} sec sweep. Number of repetitions averaged to give the free induction curve for each tissue are as follows: kidney, 1860; brain, 3720; muscle, 7440. Gaussian plot (log h vs. t^2) of each free induction curve was made. The linear portion of the plot at later times was linearly extrapolated back to $t = 0$, and was subtracted from the heights of the earlier portion of the measured curve (Fig. 7). The logarithm of difference height (Δh) was plotted against t to yield a straight line, from which T_2 of the fast fraction of Na^+ was calculated. When this procedure was used with a sample of aqueous 0.05 N NaCl solution, no fast fraction of Na^+ was detectable.

tion. Since evidence exists that cell water possesses more structure than liquid water (22, 23), it seems likely that the slow fraction represents Na^+ dissolved in structured water of tissue. More structure may be expected to produce higher electric field gradients, which may be expected to interact with the quadrupole electric moment of Na to produce faster NMR relaxation than observed for Na^+ in liquid water (22). Alternatively, the slow fraction might represent Na^+ complexed by a type of site different from that which complexes the Na^+ of the fast fraction.

Exchange between the Na^+ of the fast and slow fractions must be relatively slow. Otherwise, the T_2 decay curves of the two fractions would merge into a single exponential curve which would account for all of tissue Na^+.

These *spin-echo* NMR measurements of Na^+ in tissues conform closely to the interpretations previously derived from *steady-state* NMR studies of tissue Na^+ (14–18). The spin-echo NMR data agree well with other evidence of Ling (1, 2) and Troshin (3, 4) that most cellular Na^+ is complexed, and that cell water is structured. The spin-echo data is *not* consistent with the classical hypothesis that cell Na^+ is mostly free in solution in liquid water.

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REFERENCES

1. LING, G. N. 1969. *Int. Rev. Cytol.* **26**:1.
2. LING, G. N. 1960. A Physical Theory of the Living State. Blaisdell Publishing Company, New York.
3. TROSHIN, A. S. Problems of Cell Permeability. Original Russian edition published in Moscow. 1956. German translation published by Gustav Fischer Verlag, Jena. 1958. Revised English edition published by Pergamon Press Ltd., London. 1966.
4. TROSHIN, A. S. 1961. *In* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. Academy Press Ltd., London.
5. COPE, F. W. 1965. *Bull. Math. Biophys.* **27**:99.
6. COPE, F. W. 1967. *Bull. Math. Biophys.* **29**:691.
7. COPE, F. W. 1970. *Adv. Biol. Med. Phys.* In press.
8. LING, G. N. 1965. *Ann. N.Y. Acad. Sci.* **125**:401.
9. COPE, F. W. 1967. *Bull. Math. Biophys.* **29**:583.
10. LEV, A. A. 1964. *Nature (London).* **201**:1132.
11. LEV, A. A. 1964. *Biofizika.* **9**:686.
12. HINKE, J. A. M. 1959. *Nature (London).* **184**:1257.
13. MCLAUGHLIN, S. G. A., and J. A. M. HINKE. 1966. *Can. J. Physiol. Pharmacol.* **44**:837.
14. COPE, F. W. 1965. *Proc. Nat. Acad. Sci. U.S.A.* **54**:225.
15. COPE, F. W. 1967. *J. Gen. Physiol.* **50**:1353.
16. ROTUNNO, C. A., V. KOWALEWSKI, and M. CEREIJIDO. 1967. *Biochim. Biophys. Acta.* **135**:170.
17. CZEISLER, J. L., O. G. FRITZ, and T. J. SWIFT. 1970. *Biophys. J.* **10**:260.
18. LING, G. N., and F. W. COPE. 1969. *Science (Washington).* **163**:1335.
19. COPE, F. W., and R. DAMADIAN. 1970. *Nature (London).* In press.

20. JARDETZKY, O., and J. E. WERTZ. 1960. *J. Amer. Chem. Soc.* **82**:318.
21. POPLE, J. A., W. G. SCHNEIDER, and H. J. BERNSTEIN. 1959. High Resolution Nuclear Magnetic Resonance. McGraw-Hill Book Company, New York.
22. COPE, F. W. 1969. *Biophys. J.* **9**:303.
23. HAZLEWOOD, C. F., B. L. NICHOLS, and N. F. CHAMBERLAIN. 1969. *Nature (London).* **222**:747.
24. HAHN, E. L. 1950. *Phys. Rev.* **80**:580.
25. BOEHME, H., and M. EISNER. 1967. *J. Chem. Phys.* **46**:4242.
26. EISENSTADT, M., and H. L. FRIEDMAN. 1966. *J. Chem. Phys.* **44**:1407.
27. JARDETZKY, O. 1956. A Study of Interactions of Aqueous Sodium Ion by Nuclear Spin Resonance. Ph.D. Thesis. University of Minnesota, Minneapolis.