NMR Evidence for Complexing of Na^+ in Muscle, Kidney, and Brain, and by Actomyosin. The Relation of Cellular Complexing of Na^+ to Water Structure and to Transport Kinetics

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ABSTRACT The nuclear magnetic resonance (NMR) spectrum of Na^+ is suitable for qualitative and quantitative analysis of Na^+ in tissues. The width of the NMR spectrum is dependent upon the environment surrounding the individual Na^+ ion. NMR spectra of fresh muscle compared with spectra of the same samples after ashing show that approximately 70% of total muscle Na^+ gives no detectable NMR spectrum. This is probably due to complexation of Na^+ with macromolecules, which causes the NMR spectrum to be broadened beyond detection. A similar effect has been observed when Na^+ interacts with ion exchange resin. NMR also indicates that about 60% of Na^+ of kidney and brain is complexed. Destruction of cell structure of muscle by homogenization little alters the per cent complexing of Na^+. NMR studies show that Na^+ is complexed by actomyosin, which may be the molecular site of complexation of some Na^+ in muscle. The same studies indicate that the solubility of Na^+ in the interstitial water of actomyosin gel is markedly reduced compared with its solubility in liquid water, which suggests that the water in the gel is organized into an icelike state by the nearby actomyosin molecules. If a major fraction of intracellular Na^+ exists in a complexed state, then major revisions in most theoretical treatments of equilibria, diffusion, and transport of cellular Na^+ become appropriate.

INTRODUCTION

Most investigators have assumed that the Na^+ of the cell was largely in free solution in intracellular water. The opposite conclusion was drawn from the application of a kinetic theory of Cope (1) to Van der Kloot's data (2) on Na^+ leakage from muscle. On other grounds, Nasonov (3), Troshin (4, 5), Ling

(6–12), Shaw and Simon (13), Simon et al. (14), and Simon (15) previously had deduced that a large fraction of intracellular Na^+ existed in a complexed state. To test the prediction of Cope's kinetic theory, and because a knowledge of the extent of complexing of intracellular Na^+ is of fundamental importance for the derivation of theories of ion transport and nerve conduction, a new experimental approach to Na^+ complexing was sought. Nuclear magnetic resonance (NMR) proved to have the sensitivity, specificity, and clarity of interpretation necessary for the determination of Na^+ complexing in tissue. Preliminary NMR studies indicated that 70–75 % of frog muscle Na^+ existed in a complexed state (16). By further exploitation of the unique capabilities of NMR analysis, much additional information on complexing of tissue Na^+ has been obtained, as will be described on the following pages.

In both past and present, most investigators have considered the cell to consist essentially of a membranous sac containing water in which are dissolved electrolytes in free solution. Additional postulates regarding membrane permeabilities to ions and regarding the existence of ion pumps in the membrane are generally employed to reconcile the predictions of this model of the cell with experimental data. However, the use of these ancillary postulates leads to serious contradictions with experimental data, as reviewed by Nasonov (3), Troshin (4), and Ling (10, 11).

The contradictions seem to resolve themselves if one assumes that a major fraction of intracellular ion is complexed to macromolecules, and that this complexing plays a major role in the regulation of ionic equilibria and transport. Nevertheless, the hypothesis of cellular ion complexation has failed to win wide acceptance, especially in the case of Na^+. The barrier to acceptance seems not to have been evidence against cellular Na^+ complexing, but rather to the indirect nature of the experimental evidence favoring it.

Prior to the use of NMR, the most direct evidence for Na^+ complexing in tissues consisted of the equilibrium binding studies of Troshin (4, 5) and Ling (12), and of cation-sensitive microelectrode studies (17, 18, 54, 55). The equilibrium binding studies showed that equilibria of Na^+ between cell and solution conformed to the Langmuir absorption isotherm, with a correction for a minor fraction of Na^+ dissolved in intracellular water. The microelectrode studies showed that 70 % of intracellular Na^+ of muscle was in some form that excluded it from contact with the microelectrode. Both of these findings were easy to explain from the hypothesis of Na^+ complexing by cellular macromolecules, but were difficult to explain if cellular Na^+ was assumed to be mostly in free solution.

The author believed that the question of intracellular ion complexing was of great importance, and therefore searched for more direct experimental methods, which led to the development of an NMR method of analysis for complexing of tissue Na^+. Preliminary NMR analyses indicated that 70–75 %

of muscle Na^+ existed in a complexed state (16). NMR analysis of muscle Na^+ has been exploited more extensively in the present study to provide evidence regarding the molecular sites of complexing, and regarding the possibility that complexing of Na^+ is conditioned by an icelike state of cytoplasmic water. Further experiments indicate that kidney and brain resemble muscle in Na^+ complexing properties. In addition, the relation of cellular ion complexing to ion equilibria, diffusion, and transport are discussed. The hypothesis of ion complexation suggests analogies of ion transport in cells to electron transport in semiconductor solids, from which kinetic equations of a fundamentally new form have been derived for ion transport.

METHODS

A. *Preparation of Muscle and Method of NMR Analysis for Na^+*

A large bullfrog (*Rana catesbeiana*) was killed by decapitation, the muscle was cut off in fairly large pieces from the upper portion of the hind leg, and was blotted to remove blood and extracellular fluid insofar as possible. Pieces of muscle then were packed tightly up to an 8 ml mark in a Pyrex test tube that had been coated with petroleum jelly (Vaseline) to prevent possible complexing of Na^+ with the glass wall of the tube. The NMR spectra of Na^+ were obtained in a conventional manner on a Varian (Varian Associates, Palo Alto, Cal.) wide-line NMR spectrometer with a setting of approximately 8900 gauss modulated sinusoidally at 80 cycles per sec, and with a radio frequency field of 10 Mc. Two to four repetitions of the NMR spectrum were recorded, and the peak heights of the spectra were averaged to reduce errors due to instrumental noise. The NMR spectrum of a standard solution of 0.1 NaCl in a tube also lined with petroleum jelly was recorded before and after each set of muscle measurements. The standard contained also 0.1 N KCl, corresponding approximately to the cellular concentration of K^+, because careful measurement showed that 0.1 N K^+ changed the shape of the NMR spectrum of Na^+ slightly, resulting in a change in peak height of about 5%. These observations are consistent with conductivity measurements (19) and with other NMR studies (20) which indicated small but significant interactions between pairs of mixed inorganic cations in aqueous solutions. Instead of the above primary standard, a secondary standard of 0.1 N NaCl was often used. All Na^+ concentrations reported in this paper are corrected back to the values that would have been obtained with the primary standard. Total Na^+ was determined on the same muscle sample by NMR analysis after ashing followed by dilution in 10 ml of 0.1 N HCl. Ashing was carried out in a 30 ml platinum crucible for 12–15 hr at 700°C, preceded by drying under a heat lamp. Porcelain crucibles could not be used because they gave off significant amounts of Na^+ when treated with HCl. Quartz crucibles could not be used because they absorbed Na^+ from the samples during ashing.

B. *Muscle Homogenate Experiments*

From the upper hind leg of a large bullfrog, approximately 12 g of muscle were excised, weighed, and cut with scissors into small pieces (approximate volume of

one piece equal to 2–10 mm^3), which were dropped immediately into 25 ml of the homogenizing solution. Homogenization was carried out for 10 min in a rotating blade homogenizer (Virtis Co. Inc., Gardiner, N. Y. model 23, speed setting = 50, using a 50 ml flask #4285-K04). Next, an additional 25 ml of the homogenizing solution was added to the homogenate and mixed by homogenization for 30 sec. Microscopic examination of such an homogenate showed it to contain a network of fibers of subcellular size (presumably the actomyosin fibrils of Szent-Gyorgyi (21)) mixed with occasional small clumps of segments of muscle cells and with occasional collagen fibers. The homogenate was then centrifuged at 95,000 *g* for 30 min. The clear supernatant was decanted thoroughly, leaving a precipitate with a volume of approximately 7 ml. The entire precipitate was packed loosely (so that it was distributed evenly over an 8 ml volume) in a Pyrex test tube lined with petroleum jelly. The measured concentrations of Na^+ were corrected for this dilution. These procedures were performed at 0–5°C. The tube containing the precipitate was then placed in an ice bath, from which it was removed only 1–2 min before insertion into the probe of the NMR spectrometer, which was at 25°C. Although the heights of the NMR spectra of Na^+ in the muscle precipitates increased gradually (by a maximum of 10%) as the samples warmed up, analytical variations due to temperature differences were minimized by maintaining the time delays before and during the NMR analysis approximately constant. After completion of the NMR measurement on the fresh precipitate, the sample was dried, ashed, and reanalyzed by NMR to determine total Na^+ by the same technique that was used for intact frog muscle.

C. *Actomyosin Experiments*

Actomyosin was prepared by alkaline extraction of muscle by a method approximating that of Szent-Gyorgyi (21). The psoas muscles of an adult white male New Zealand rabbit (approximately 20 g of muscle) were cut into pieces weighing 0.5–1.0 g which were homogenized in a Waring Blendor for 2 min with 90 ml of 0.6 N KCl, 0.01 M Na_2CO_3, 0.04 M $NaHCO_3$, pH 9. All preparative procedures were carried out at 0–5°C. The homogenate was allowed to stand for 16–20 hr, and was then diluted with 120 ml of 0.6 N KCl and was filtered through surgical gauze. The filtrate was diluted to approximately 0.1 N KCl by the addition of 1.2 liter of water. Crude actomyosin then precipitated during a 30 min period of quiet standing, and was separated by centrifugation at 1300 *g* for 20 min.

To purify the crude actomyosin while simultaneously replacing complexed K^+ by Na^+, the protein was reprecipitated from NaCl solution as follows. The crude actomyosin precipitate was dissolved in 100 ml of 0.6 N NaCl, 0.01 M Na_2CO_3, 0.04 M $NaHCO_3$, pH 9, and was allowed to stand for 30 min to replace complexed K^+ by Na^+. The solution was then diluted to approximately 0.1 N NaCl by the addition of 500 ml of water and was allowed to stand for 30 min to precipitate the actomyosin, which was separated by centrifugation at 1300 *g* for 20 min. The actomyosin precipitate was diluted with 50 ml of 0.1 N NaCl and was neutralized to pH 7.0 with HCl. The suspension was centrifuged at 5000 *g* for 15 min, and the precipitate was centrifuged again at 95,000 *g* for 30 min. The final compact precipitate was then analyzed for free Na^+ by NMR as described in the Muscle Homogenate part of the

Methods section. The supernatant was also analyzed by NMR, and was found to contain an average of 0.11 N free Na^+ and no detectable complexed Na^+. The precipitate was then transferred to a preweighed platinum crucible, and the wet precipitate was weighed. While still in the crucible, the precipitate was lyophilized for approximately 15 hr, and then dried to constant weight at 100°C in a vacuum oven, which required about 3 hr. The sample was again weighed. The sample was then ashed by the same techniques used for frog muscle as described previously and the ashed sample was weighed. The ashed sample was then dissolved in 10 ml of 0.1 N HCl and analyzed for Na^+ by NMR to give total Na^+ as previously described. The water content of the precipitate was calculated to be the difference between wet weight and dry weight. The protein content of the precipitate was calculated as dry weight minus the weight of ash, which is based on the assumption that the concentration of nonprotein organic impurities is relatively low.

D. *Freezing Experiments*

The general plan of these experiments was to freeze rapidly NaCl or Na citrate solution in a small plastic tube by immersion in liquid nitrogen, and then to record repeatedly the NMR spectrum of Na^+ as a function of temperature during slow rewarming. The experiment with NaCl used a 0.4 N solution. The experiment with Na citrate used a 0.5 M solution of citric acid, which had been neutralized with NaOH to pH 6.1, resulting in a citrate solution which contained approximately 1.45 N Na. To make the distribution of ions within the ice as uniform as possible, the freezing was done as rapidly as possible. An empty celluloid test tube (8 mm diameter) was partially immersed in liquid nitrogen, and then 2.7 ml of solution was pipetted slowly into the tube, so that each increment was completely frozen before the next increment was added. To maintain a rate of rewarming slow enough to permit repeated recording of NMR spectra and of temperature and to maintain uniformity of temperature throughout the sample, the sample tube was placed within a helical plastic coil through which flowed N_2 gas, the temperature of which was controlled. The plastic coil was small enough so that the coil and sample tube fitted within the cavity of the probe of the NMR spectrometer. The N_2 gas was cooled by passage through a copper coil in liquid nitrogen, followed by partial rewarming with an electric heater, the temperature of which was controlled. The temperature of the sample was measured by a thermocouple which had been frozen in place in the approximate center of the sample tube.

RESULTS AND DISCUSSION

A. *Accuracy and Interpretation of Na^+ Analysis by NMR, with Application to Na^+ Complexing in Frog Muscle*

Nuclear magnetic resonance (NMR) as a method of analysis for Na^+ has several characteristics which make it useful for the study of the state of Na^+ in tissues. First, the position of the NMR line of Na^+ is far different from that of all other elements found in the cell, so that Na^+ can be distinguished clearly from 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 studies. Second, provided that concentrations of anions capable of complexing Na^+ are kept low, the height of the NMR resonance line of Na^+ is directly proportional to Na^+ concentration, so that NMR can be used for the quantitative analysis of Na^+. Third, the width of the NMR line of Na^+ is dependent upon the chemical and physical environment surrounding the individual Na^+ ion. Therefore, the width of the NMR line may indicate whether the Na^+ ion is surrounded by an environment like that in free solution, or by some other environment. In the following paragraphs, these properties of NMR analysis for Na^+ are explored in more detail, and are applied to the evaluation of Na^+ complexing in frog muscle.

Systematic studies of the NMR spectra of aqueous solutions of Na^+ in the presence of various anions were carried out by Jardetzky (22) and by Jardetzky and Wertz (23–26). They showed that the NMR spectrum of Na^+ in aqueous solution consists of a single line with a width of about 32 milligauss (26), and that the peak height of the NMR spectrum of Na^+ is proportional to Na^+ concentration, when concentrations of anions capable of complexing Na^+ are kept low (25).

The first experiment of the present study was the measurement of peak height of the NMR spectrum of Na^+ vs. concentration of aqueous NaCl, which was selected because the data of Jardetzky and Wertz (25) had indicated that Cl^- did not complex with Na^+. The resultant standard curve (plotted in Fig. 1) shows good linearity and a direct proportionality between Na^+ concentration and the peak height of the NMR spectrum over a range of 0.004 N to 0.1 N.

Next, the adequacy of NMR analysis after ashing as a method for the determination of total Na^+ of muscle was tested. A mixture of 0.5 ml of 0.4 N NaCl plus 1 ml of 1 N KCl was placed in a platinum crucible and was carried through the drying, ashing, and NMR analysis procedure that was used for determination of total Na^+ of muscle as described in the Methods section. These quantities of Na^+ and K^+ duplicate approximately those found in 8 ml of muscle. Six such studies yielded recoveries of 94, 93, 89, 93, 107, and 96 % (mean 95 %), which indicated that this analytical procedure was reasonably accurate and reasonably reproducible. Analyses by NMR after ashing for total Na^+ in muscle samples of six different frogs yielded values of 27.3, 33.0, 29.6, 26.2, 25.1, and 29.4 (mean 28.5) millimolar in terms of volume of fresh muscle. This mean value of 28.5 mM for total muscle Na^+ agrees well with the mean value of 27.4 mM for collected data of other investigators using different methods (6, p. 217) (computed on the basis of specific gravity = 1.1 for muscle). Thus, the accuracy of NMR analysis after ashing as a method for determination of total tissue Na^+ is confirmed.

Broadening of the NMR line of *hydrogen* or of *chloride* as a criterion of com-

plexation or adsorption has been used by a number of investigators. Broadening of the NMR resonance of H in penicillin was employed as a criterion for the complexing of penicillin by serum albumin (27). Broadening of the NMR resonance line of protons of water was observed when water was adsorbed on starch or egg albumin (28), on carbon or cellulose (29, 30), or on silica gel (31). In addition, the NMR spectrum of Cl^- was broadened by complexation with Hg^{++}, and the broadening was markedly increased when the Hg^{++} was

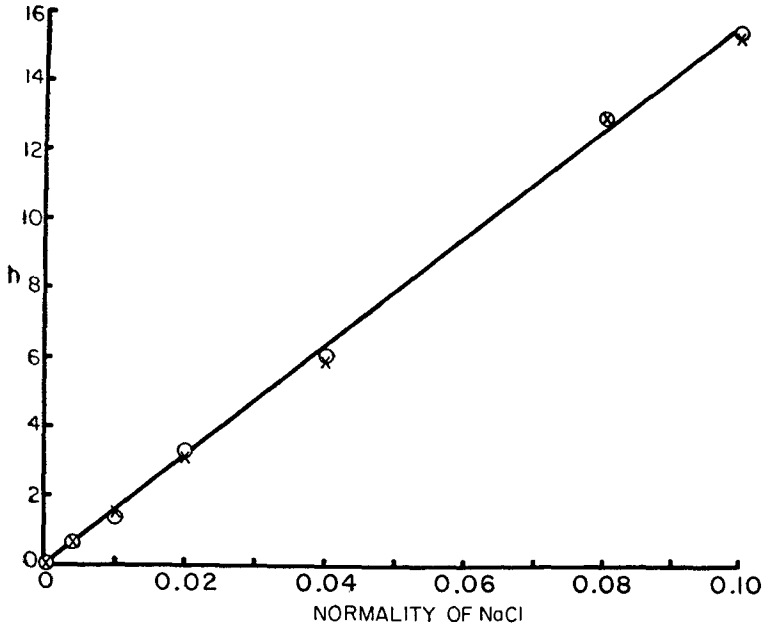

FIGURE 1. Standard curve for the quantitative analysis of aqueous Na^+ by NMR. On the abscissa are plotted the normalities of the NaCl solution as determined by weight and dilution. On the ordinate, h represents the peak-to-peak height of the NMR spectrum measured for the sample, divided by the voltage gain of the output amplifier of the NMR spectrometer. The two sets of points represent two sets of recordings of NMR spectra for the same samples.

coupled to a protein (32). This leads to the concept that complexing of Na^+ in tissue might be detected from broadening of the NMR resonance of Na^+.

Pertinent to this possibility are the experiments of Jardetzky and Wertz (25). They showed that when certain anions are present in solutions of Na^+, the NMR spectrum of Na^+ is broadened and the peak height is lowered, presumably due to complex formation of Na^+ with anion. Ion exchange resin apparently complexes Na^+ so tightly that the NMR spectrum of Na^+ is broadened so greatly that it becomes invisible to NMR (25). This data suggests that one might obtain information about the state of complexation of Na^+ in muscle by comparison of the line width and height of the NMR spec-

trum of Na^+ in fresh muscle with those of a known concentration of aqueous NaCl.

To study the state of Na^+ in intact muscle, NMR spectra of Na^+ were obtained on frog muscle as soon as possible after the death of the frog and at frequent intervals during the succeeding 6–7 hr, at 25°C. The NMR spectrum of Na^+ observed in frog muscle was approximately similar in shape and position to the spectrum of aqueous NaCl, although line broadening of 20 % or

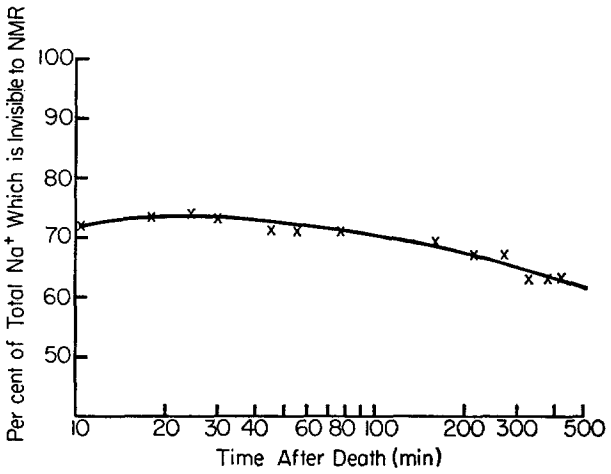

FIGURE 2. Time course of NMR-invisible Na^+ of frog muscle. Time is plotted on a logarithmic scale. This is an average of data from six muscle samples from six different frogs, computed so as to give equal weight to each individual muscle sample. Curves for individual muscle samples were plotted using points representing averages of peak heights of sets of four successive NMR spectra. Some parameters of the individual curves are given in Table I. To compute the average curve of the six samples, time intervals were selected so that at least one point on each of the six individual curves fell within the interval, and a mean value for that interval for that curve was computed. Then a mean of the values of all six curves for each time interval was computed, thus yielding a mean time curve giving equal weight to all six individual curves. The mean concentration of total Na^+ for the six muscle samples is 28.5 mM.

less could not be ruled out because of the interference of instrumental noise. Concentrations of Na^+ that were visible to NMR in fresh muscle were calculated on the assumption that the peak height of the NMR spectrum of Na^+ of muscle was proportional to Na^+ concentration, using a NaCl standard as described in the Methods section. Concentrations of *total* Na^+ in the same samples were determined by NMR analysis after ashing. These studies showed that the concentration of Na^+ that was visible to NMR in fresh muscle was much smaller than the total Na^+ that was measurable after ashing. In other words, much of the Na^+ in fresh muscle was invisible to NMR. The size of the NMR-invisible fraction of muscle Na^+ was calculated by subtraction of

NMR-visible Na^+ of the fresh muscle sample from total Na^+ as determined on the ashed muscle.

The NMR-invisible fraction of muscle Na^+ as a function of time after decapitation of the frog is shown in Fig. 2. The size of the NMR-invisible fraction varies in a biphasic manner as a function of time due to postmortem changes in the muscle. At 10 min after death, an average of 72 % of muscle Na^+ is invisible to NMR. By 30–40 min after death, NMR-invisible Na^+ has increased to approximately 74 % of total Na^+. Thereafter, NMR-invisible Na^+

TABLE I

PARAMETERS OF TIME CURVES OF Na^+ COMPLEXING FOR INDIVIDUAL FROG MUSCLE SAMPLES

		NMR-invisible Na^+ as % of total muscle Na^+ at various times after decapitation of frog		
Frog No.	Total muscle Na^+	10 min	Maximum at 20–60 min	7 hr
	mM	%	%	%
1	27.3	72	75	68
2	33.0	66	68	56
3	29.6	75	79	62
4	26.2	78	78	67
5	25.1	60	64	54
6	29.4	78	78	71
Mean	28.5	72	74	63

The parameters in the last three columns were estimated from individual frog muscle curves, from which the average curve shown in Fig. 2 was calculated. Values of total muscle Na^+ represent concentrations of Na^+ *per unit volume of fresh muscle* as determined by NMR analysis after ashing. Some possible reasons for the observed variation between individual muscle samples are discussed in the text.

decreased slowly but steadily to a mean value of 63 % at the end of 7 hr. An extrapolation of the data of Fig. 2 to zero time may yield a valid estimate of NMR-invisible Na^+ in muscle of the *living* frog. All six curves on the muscle from six different frogs show the same general biphasic pattern evident in the average curve of Fig. 2, although quantitative difference between muscle samples are present. These differences are indicated in Table I by some parameters of the curves for the individual samples. Some of this variability may be the result of differences in the completeness with which extracellular fluid, blood, and lymph were removed from the muscle samples by blotting prior to the NMR measurements. Additional variability may be due to variation between frogs with respect to the state of nutrition. Decisions regarding the relative influences of these or other factors cannot be made from the data now available.

The most obvious explanation for the NMR-invisibility of the major fraction of muscle Na^+ is that the spectrum of the NMR-invisible Na^+ is broadened beyond detection due to complexing with macromolecules. This is the interpretation used by Jardetzky and Wertz (25) to explain the disappearance of the Na^+ spectrum due to complexing by ion exchange resins.

It was thought desirable to explore also the possibility that the disappearance of the NMR spectrum of Na^+ of muscle might have been due to a chemical shift, rather than to broadening. A chemical shift is a displacement of the position of an NMR spectrum along the H axis, which occurs when the local magnetic field experienced by the individual nucleus is different from the magnetic field applied by the investigator, due to summation of fields generated by orbital electrons with the field that is applied. The magnitude of the chemical shift is often a function of the type of compound in which the atom is situated. Two types of possible chemical shifting of the Na^+ spectrum in frog muscle were explored experimentally. First, it was demonstrated that the NMR-*visible* spectra of Na^+ in frog muscle occurred at the same position along the H axis as the spectrum of Na^+ in the standard solution of 0.1 N NaCl, to an accuracy of perhaps 3–4 ppm. Therefore, the NMR-visible fraction of Na^+ in frog muscle was not chemically shifted to any significant extent. Second, the possibility was explored that the *invisibility* to NMR of a major fraction of Na^+ of frog muscle might have resulted from a shift of the NMR spectrum of that fraction to a different position along the H axis. This hypothesis was tested using the muscle of frog No. 5 (Table I) at 350–400 min after decapitation of the frog, when 45–50 % of the total muscle Na^+ could not be accounted for by the muscle Na^+ spectrum at the position of Na^+ in the NaCl standard. Magnetic field strength in the muscle was slowly varied 900 milligauss above and then below the center of the spectrum of Na^+ in the NaCl standard, but no additional NMR spectrum could be detected. Therefore, no evidence could be found that the NMR-invisible Na^+ of muscle was lost due to a chemical shift over a range of 100 ppm above, or 100 ppm below the position of Na^+ in free solution. The possibility of a very large chemical shift still remains, but if found, would imply that the NMR-invisible fraction of Na^+ in muscle was in some form other than in free solution.

The NMR evidence that 65–80 % of muscle Na^+ exists in a complexed form correlates well with the cation-sensitive microelectrode studies of Lev (17, 18) on individual muscle fibers, which indicated that 70 % of intracellular Na^+ was in some state that excluded it from contact with the microelectrode. Similar microelectrode results were obtained by Hinke (54) and McLaughlin and Hinke (55). The NMR evidence supports the theoretical and experimental work of Troshin (4, 5), Ling (6–12), and Simon (13–15), and the kinetic theory of Cope (1, 53).

B. *Na^+ Complexing by Homogenates of Muscle*

The experiments of the last section indicated that approximately 70 % of the Na^+ in intact muscle exists in a complexed state. It will now be shown that the same is true when muscle cell structure is destroyed by homogenization. It will also be shown that the equilibrium of Na^+ complexed by muscle homogenates with the Na^+ in surrounding solution is fairly rapid.

Homogenization was used to destroy the cellular and subcellular structure of muscle as completely as possible while causing minimum damage to cellular macromolecules. Destruction of cellular and subcellular structure appeared

TABLE II

COMPLEXING OF Na^+ BY PRECIPITATED HOMOGENATES OF FROG MUSCLE

Exp. No.	1	2	3	4	5	Mean	6	7	8	Mean
Concentration of NaCl added to homogenizing solution	←——— 0.12 M ———→						←——— 0 M ———→			
Concentrations in muscle precipitate										
Total Na^+ (mM)	99	102	99	99	89	97				
Free Na^+ (mM)	32	35	26	34	24	30	0	0	0	0
Complexed Na^+ (mM)	67	67	73	65	65	67	7	0	0	2
% complexing	68	65	74	66	74	69				

Experiments 1–5 used muscle from five different frogs. Three different frogs were used for experiments 6–8.

excellent upon microscopic examination. By NMR was measured the Na^+ complexed by homogenized muscle while it was in equilibrium with a solution containing a concentration of Na^+ approximating that in plasma. The homogenizing solution contained 0.12 M Na^+, 3 mM Mg^{++}, and 3 mM Ca^{++}. The muscle homogenate was centrifuged at 95,000 *g* to produce a precipitate, which was analyzed by NMR for free and complexed Na^+. The volume of the precipitate approximated 60 % of the initial volume of the intact muscle. The experimental techniques are described in detail in the Methods section.

The results (Table II, experiments 1–5) indicate that substantial quantities of Na^+ are complexed by homogenized muscle. Per cent complexation of Na^+ ranged from 65–74 % with a mean of 69 %, which may be compared with a mean of 72 % for intact frog muscle at 10 min after the death of the frog (Table I).

The concentrations of total Na^+ in these preparations of homogenized muscle are greater than in intact muscle by a factor of approximately three-

fold. Part of this increase is likely due to an approximate doubling of the concentration of complexing macromolecules in the precipitated homogenates compared with the intact muscle, since the final volume of the precipitaet averaged 7 ml compared with 12 ml for the intact muscle. Preliminary experiments suggest that other factors which may influence the concentration of complexed Na^+ in muscle homogenates include the concentrations of Mg^{++}, Ca^{++}, and ATP in the homogenizing solution, and the season of the year at which the frogs were caught. For the experiments in Table II, spring frogs were used. The experiments reported in Table II show that when conditions are constant and close to physiological, Na^+ complexing by homogenized muscle is substantial and reproducible. However, these experiments are not intended to define the role of cofactors in Na^+ complexing.

The foregoing experiments dealt with the equilibrium of free Na^+ in the supernatant with complexed Na^+ in the muscle precipitate, but gave no information regarding the rapidity of sluggishness of attainment of equilibrium. That the equilibrium state is approached fairly rapidly is indicated by experiments 6–8 of Table II. They show that when the homogenizing solution contains no Na^+, then very little complexed Na^+ is contained in the precipitated muscle homogenate. Hence, one may infer that a significant degree of release of complexed Na^+ from muscle precipitate occurred during the hour or so that the precipitate was in contact with the homogenizing solution.

C. *Na^+ Complexing by Actomyosin*

In the last section, homogenates of muscle were shown to complex Na^+. The next step was to study complexing of Na^+ by purified preparations of the molecular constituents of muscle. In this section, it will be demonstrated that actomyosin, which comprises approximately 40–60 % of muscle protein (33), complexes substantial quantities of Na^+.

Although many proteins are unable to complex Na^+, several proteins do have a capacity to complex significant quantities of Na^+. Complexation of Na^+ by β-lactoglobulin (34, 35), casein (36), zein (34), fibrinogen (34), and myosin (37) has been demonstrated by Na electrode techniques. In this section, Lewis and Saroff's (37) demonstration of Na^+ complexing by myosin is extended to actomyosin using the NMR method.

Actomyosin was prepared by alkaline KCl extraction of rabbit muscle by the method of Szent-Gyorgyi (21) as described in detail in the Methods section. The actomyosin was then equilibrated with aqueous NaCl to replace complexed K^+ by Na^+, and was centrifuged at 95,000 *g* for 30 min to prepare a precipitate, which was analyzed for free and complexed Na^+ by NMR (Table III), and was also analyzed for protein and for water (Table IV).

Since the free (NMR-visible) Na^+ of the precipitate is presumably dissolved

TABLE III

COMPLEXING OF Na^+ BY ACTOMYOSIN

Exp. No.	Na^+ concn. per g of precipitate			Free Na^+ per liter of H_2O of precipitate	Complexed Na^+ per g protein of precipitate
	Total Na^+	Free Na^+	Complexed Na^+		
	μmoles/g	*μmoles/g*	*μmoles/g*	*mM*	*μmoles/g*
1	98	48	50	57	330
2	96	43	53	50	340
3	94	44	50	52	334
4	93	45	48	53	353
5	96	55	41	64	304
6	85	56	29	65	200
Mean	94	49	45	57	310

Experiments 1–6 of Tables III and IV were performed on muscle samples from six different rabbits. The calculations in the last two columns in this table make use of the analyses of the precipitates for protein and water that are given in Table IV.

in the water of the precipitate, the concentration of free Na^+ per liter of water of the precipitate was computed, and was found to have a mean value of 57 mM (Table III). The supernatants were also analyzed by NMR and were found to contain approximately 110 mM free Na^+ and no detectable complexed Na^+. Hence, the concentration of free Na^+ in the water of the precipitate is only about 50 % of the Na^+ concentration observed in the supernatant. Pertinent to this finding are previous measurements of solubilities of small molecules in the water of hydration of protein cyrstals. Hydrated crystals of hemoglobin (38, 39), chymotrypsin (39), and β-lactoglobulin (39, 40) have been studied. The concentrations of sucrose and ammonium sulfate in the water of hydration of these protein crystals were found by these investigators to vary from 50–70 % of the concentrations present in the solution outside the crystals, depending upon the experimental conditions.

TABLE IV

COMPOSITION OF ACTOMYOSIN PRECIPITATES

Exp. No.	Composition per g of actomyosin precipitates		
	Protein	Water	Ash
	g	*g*	*g*
1	0.154	0.841	0.005
2	0.157	0.838	0.005
3	0.150	0.845	0.005
4	0.136	0.858	0.006
5	0.135	0.860	0.005
6	0.135	0.860	0.005
Mean	0.145	0.850	0.005

These studies of protein crystals were interpreted (38–40) to mean that some or all of the water of hydration is organized by the protein into a "bound" state, resulting in a decreased affinity of this water for dissolved small molecules. Additional evidence that proteins can organize nearby water into a state that is different from liquid water includes proton NMR studies of hydrated collagen (45–47), and the finding that proteins absorb water in accord with the Bradley isotherm, which implies that water adjacent to proteins probably exists as multiple polarized layers (9). The present author suggests that a similar organization of water into a bound or icelike state is responsible for the finding that the concentration of free Na^+ in the water of actomyosin precipitates is much lower than in the supernatants with which the precipitates are in equilibrium. This implies that actomyosin should have a similar organizing effect on the water in the living muscle cell, which should cause the concentration of free Na^+ in intracellular water to be much lower than the concentration of Na^+ in extracellular water or plasma with which the cell is in equilibrium. The postulate of water adsorption by cellular proteins can explain a considerable volume of cell hydration data (52).

NMR analysis indicates that approximately 45 μmoles of Na^+ are complexed per g of wet actomyosin precipitate (Table III). This was compared with values measured with the Na electrode by Lewis and Saroff (37). Lewis and Saroff (37) deduced from their data that one molecule of myosin contains approximately 50 sites capable of complexing Na^+, K^+, or H^+. Actin itself failed to complex any K^+, but when actin was combined with myosin in a weight ratio of 1:5, the binding capacity of the myosin was reduced to approximately 20 molecules of cation per molecule of myosin (37). Let us assume that the actomyosin preparation used in the NMR studies contains actin and myosin in the preferred ratio (21) of 1:2.5 by weight, and that the molecular weight of myosin is 420,000 (37). Then, we may calculate that Lewis and Saroff's finding of 20 molecules of complexed cation per molecule of myosin in the presence of actin implies that 34 μmoles of Na^+ should be complexed by 1 g of actomyosin. By comparison, the present NMR study gives a mean value of 310 μmoles of Na^+ complexed per g of actomyosin (Table III).

The 10-fold greater complexing of Na^+ observed in the present study seems likely to be related to the fact that Lewis and Saroff studied the protein in free solution (1–2.5 % protein) while the present investigation was concerned with the gel state (approximately 15 % protein; see Table IV). In the gel state, two factors occur which are likely to produce marked enhancement of complexing compared with that in free solution. The first is concerned with the icelike organization of water in the gel, the evidence for which was presented in a previous paragraph of this section. It will be demonstrated in section E of Results and Discussion that the organization of water into ice markedly enhances complexing of Na^+ by anions which have only slight com-

plexing ability when in free solution. Hence, it seems likely that an icelike state of water in the actomyosin gel stimulates complexing of Na^+ by protein sites which show little complexing in free solution. The second factor likely to stimulate complexing by the gel may be called the aggregation effect. This has been discussed by Ling (6, p. 17), who considers it relevant to ion complexing in living cells. The aggregation enhancement of complexing of cations is of large magnitude and is easily observed when detergent molecules are caused to aggregate to form micelles (41, 42). The author has discussed this phenomenon with several experts on the physical chemistry of detergents who freely conceded the existence of the effect, but could offer no explanations regarding its cause. It is reasonable to expect that aggregation of actomyosin molecules into a gel might also enhance cation complexing.

To what part of the actomyosin molecule is the Na^+ complexed? Lewis and Saroff (37) and Saroff (43) believe cations are complexed by the formation of chelates with carboxyl-imidazole pairs and carboxyl-amino pairs on the myosin molecule when it is in free solution. Enough free carboxyl groups are available on the myosin molecule to account for the additional quantity of Na^+ complexed by actomyosin gel. Under the assumptions used in the penultimate paragraph, the titration data of Mihalyi (44) leads to an estimate of 1310 μmoles of free carboxyl groups per g of actomyosin, which is more than enough to account for the 310 μmoles of complexed Na^+ observed by NMR. Additional complexing sites may be available on the actin molecule, since Mihalyi (44) reported actin to have a larger acid binding capacity than myosin.

D. *Na^+ Complexing in Rat Muscle and in Rabbit Kidney and Brain*

In this section, it is shown that the per cent complexing of Na^+ in rat muscle, rabbit kidney, and rabbit brain is similar to that in frog muscle.

First, rat muscle was compared with frog muscle. Muscle of the hind legs of three adult white male rats was analyzed by NMR for Na^+ at about 20 min after the death of the rats and again after ashing. Total Na^+ was 28.6, 28.1, and 29.2 mM, with 65, 72, and 66 % complexing respectively for the three muscle samples. By comparison with the data in Table I, it is evident that both per cent complexing of Na^+ soon after death and total Na^+ are quite similar in the muscle of rat and frog. In another experiment, the postmortem time course of Na^+ complexing in a sample of rat muscle was measured. It was found to be essentially flat for 7 hr postmortem (Fig. 3), and did not show the biphasic time curve seen in frog muscle (Fig. 2). This might reflect a species difference or a nutritional difference, since the rat was well fed but the frogs had not been fed for several weeks while in captivity.

Na^+ complexing was also measured in rabbit kidney and brain by the same technique used for frog and rat muscle. The data depicted in Fig. 3 show that per cent Na^+ complexing in kidney and brain soon after death of the

animal had values only slightly less than for muscle. In kidney, the later time course of Na^+ complexing showed a slow increase over 7 hr postmortem (Fig. 3). In brain, the trend of Na^+ complexing was a slow decline (Fig. 3). The flatness of these curves suggests that extrapolations to zero time are probably valid, indicating that in the *living* animal, 60–70 % of total Na^+ of kidney and brain (as well as of muscle) probably exists in a complexed form. NMR analyses on two additional specimens of kidney from two other rabbits at about 20

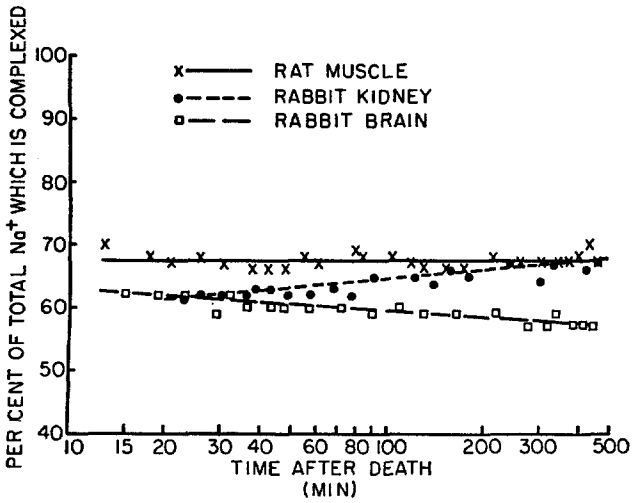

FIGURE 3. Time course of Na^+ complexing in rat muscle, rabbit kidney, and rabbit brain. Time is plotted on a logarithmic scale. Total tissue Na^+ concentrations measured in these experiments were 33 mM in rat muscle, 100 mM in rabbit kidney, and 58 mM in rabbit brain, expressed as concentration of Na^+ per unit volume of tissue. Muscle was obtained from the hind legs of a 259 g male white rat. Kidney and brain were obtained from adult New Zealand white male rabbits. The NMR analyses were made on pieces of tissue after blotting using the same techniques as with intact frog muscle, as described in the Methods section. Most of the light-colored tissue in the central portion of the kidney was excised before NMR analysis.

min postmortem revealed 58 and 64 % complexing of Na^+ with total Na^+ concentrations of 88 and 85 mM. Similar measurements on two other specimens of brain from additional rabbits revealed 60 and 62 % Na^+ complexing with total Na^+ concentrations of 52 and 58 mM respectively.

E. *Enhancement of Na^+ Complexing by Freezing*

It was suggested in section C of Results and Discussion that intracellular proteins might organize intracellular water into an icelike state. It will now be shown that such an icelike state of intracellular water might enhance the complexation of Na^+ by intracellular anions. This provides one possible reason

why proteins in cells or in gels might be expected to have greater ability to complex Na^+ than the same proteins in free solution.

It will be demonstrated that when water becomes organized into ice, then Na^+ is forced to complex markedly with anions for which it has but little affinity when in liquid water. This phenomenon has been demonstrated in a model system, using a simple organic anion in aqueous solution. Citrate was

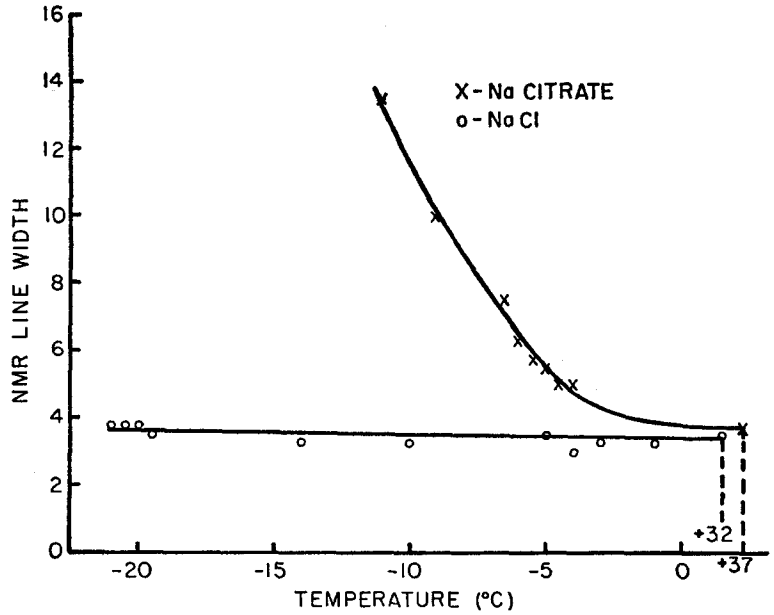

FIGURE 4. NMR line widths of Na^+ during thawing of Na citrate and of NaCl solutions. Solutions were frozen very rapidly by immersion in liquid nitrogen and rewarmed gradually while NMR spectra of Na^+ were recorded as a function of sample temperature. Line widths of NMR spectra of Na^+ are given in arbitrary units of magnetic field strength measured between the two peaks of the NMR derivative spectrum. At temperatures below $-11°C$ for Na citrate and below $-21°C$ for NaCl, spectra were not detectable, presumably due to extreme line broadening due to incorporation of Na^+ into the crystal lattice of ice.

chosen as the anion because it had been shown by NMR (25) and by titration (48) to be able to complex slightly with Na^+ in aqueous solution. The experiment was done by fast freezing of a sodium citrate solution in liquid nitrogen, and then observing the NMR spectrum of Na^+ as a function of temperature during slow rewarming. At temperatures below $-11°C$, no NMR spectrum was visible. At $-11°C$, a wide, low spectrum of Na^+ appeared and became gradually narrower as the temperature increased (Fig. 4). To prove that citrate was involved in this line broadening in the frozen state, a control experiment was run using NaCl instead of sodium citrate. Unlike citrate, Cl^-

has never been shown (to the author's knowledge) to have any capacity to complex Na^+. From the time that the Na^+ spectrum first appeared at a temperature of about $-21°C$ until the temperature rose to $+35°C$, the line width of the Na^+ spectrum of aqueous NaCl remained essentially constant (Fig. 4). Hence, in the absence of an anion able to complex Na^+, freezing did not broaden the NMR spectrum of Na^+.

These results suggest that organization of water by freezing enhances the complexing of Na^+ by citrate, which suggests that an icelike state of intracellular water due to the proximity of macromolecules would facilitate complexing of Na^+ by cellular anions. When dissolved in liquid water, the same anions might have only slight tendencies to complex Na^+.

SOME IMPLICATIONS OF ION COMPLEXING FOR ION EQUILIBRIA, DIFFUSION, AND TRANSPORT

A. *Introduction*

This section describes some of the theoretical implications of the concept that most Na^+ in the cell is complexed. The hypothesis of ion complexation allows intracellular ion concentrations to be maintained at the observed levels without the consumption of energy. At *equilibrium*, the general form of the equations of ion diffusion across the cell wall remains similar whether or not ion complexing is postulated, although the meanings of the rate constants change. However, for *nonequilibrium* ion transport (when net ion movements across the cell wall occur), the hypothesis of ion complexing leads to kinetic equations of fundamentally new form, and analogies of ion transport in cells to electron transport in semiconductor solids become appropriate.

The equilibrium case and the nonequilibrium case are considered separately below.

B. *The Equilibrium Case*

Let us first consider the situation where the concentrations of ions inside the cell are in equilibrium with those outside the cell, so that both intracellular and extracellular ion concentrations remain constant over time.

If intracellular ions are assumed *free* in intracellular water, it is necessary to assume that intracellular ion concentration is maintained by an equilibrium between a leak and a pump in the cell wall. Energy is necessary to maintain this equilibrium, since energy is necessary to operate the pump. Ling (9, 11, 51) has calculated that the metabolism of the cell does not produce enough energy to operate the postulated ion pumps at rates which are fast enough to maintain the intracellular ion concentrations. Hence, the leak-pump theory is thermodynamically impossible. However, if intracellular ions are assumed to be complexed to macromolecules, then the maintenance of equilibrium

intracellular ion concentrations depends on the relative affinities of complexing sites for different ions. In this case, no ion pumps need be postulated, so that consumption of energy is *not* necessary to maintain equilibrium intracellular ion concentrations. The ion complexation hypothesis is further supported by equilibrium ion binding studies in cells. Troshin (4, 5) has shown experimentally that intracellular vs. extracellular ion concentration curves of Na^+ (and also of various other ions) conform to the Langmuir adsorption isotherm, with a correction for a minor fraction of Na^+ dissolved in intracellular water. These observations are most easily explained by the hypothesis that most intracellular ions exist in a complexed state. The smallness of the dissolved fraction of Na^+ is probably caused by the icelike state of intracellular water induced by nearby protein molecules, as discussed in section C of Results and Discussion.

If intracellular ions are assumed to be free in intracellular water, then differences in rates of diffusion of different fractions of intracellular ions are usually explained by the hypothesis of compartmentalization of intracellular ions by membranous barriers. However, if intracellular ions are assumed to be mostly complexed by macromolecules, then differences in rates of diffusion of different fractions of intracellular ions may be attributed to differences in dissociation rates of ions complexed by different sites. The minor fraction of ion which is free in cell water should have a still different rate of diffusion. Regardless of whether intracellular ions are complexed or free, one may predict from mass action theory that measurements of the diffusion rate of radioactive ion from the cell under conditions of equilibrium concentrations may be described by sums of exponential functions of time, which describe numerous experimental observations.

C. *The Nonequilibrium Case*

In this section, we shall deal with the situation where *net* movements of ions occur across the cell wall, so that intracellular ion concentrations change with time.

If intracellular ions are assumed *free* in liquid intracellular water, then we may again use a mass action approach, dealing with sums of forward and backward diffusion rates of ions from compartments enclosed by membranes. One may then predict that ion concentrations should be describable by sums of exponential functions of time, or by more complicated functions. From the same postulate, using a nonequilibrium thermodynamic method, one may consider a current of ions to be driven across an activation energy barrier in the cell membrane by the difference in chemical potentials of free ions on the two sides of the membrane. Such an approach yields kinetic equations like those derived from the simple mass action approach. More complicated mass

action methods based on leaks, pumps, and ion carriers in the membrane have also been used.

If the major fraction of intracellular Na^+ exists in a *complexed* state, and if intracellular water exists in an icelike state, then a major change in point of view seems warranted. Instead of regarding the cell as essentially a semipermeable bag containing an aqueous solution of ions, it seems more appropriate to consider the cell as an organized, nonliquid phase, consisting of macromolecules embedded in an icelike matrix. Intracellular Na^+ may then be supposed to locate itself mostly in complexed form upon sites on the macromolecules, but to be capable of mobility by hopping from site to site through the icelike matrix in which the Na^+ is only sparingly soluble. Such a picture leads to the concept that the conduction and potential of Na^+ in the cell might conform to mathematical laws analogous to those governing electrons in semiconductor solids. It has already been indicated that protons in ice are conducted by a mechanism like that responsible for electron conduction in semiconductors (49, 50). Such concepts lead one to regard the cell surface (the plasma membrane) not as a membrane in the classical sense, but rather as an interface or junction between two dissimilar materials, which like a semiconductor junction may be expected to have conductive properties different from either of the two materials which it separates. From such considerations, a quantitative theory of nonequilibrium ion transport has been developed, which predicts that ion leakage from cells should conform to the Elovich equation, which describes considerable experimental data (1, 53).

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