NUCLEAR MAGNETIC RESONANCE OF SODIUM-23 LINOLEATE–WATER

BASIS FOR AN ALTERNATIVE INTERPRETATION OF SODIUM-23 SPECTRA WITHIN CELLS

M. SHPORER *and* MORTIMER M. CIVAN

From the Departments of Isotope Research and Polymer Research, the Weizmann Institute of Science, Rehovot, Israel, and the Departments of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114, and Harvard Medical School, Boston, Massachusetts 02115. Dr. Civan's permanent address is the Laboratory of Renal Biophysics, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114.

ABSTRACT The ^{23}Na spectrum from liquid crystals of sodium linoleate in water has been studied by nuclear magnetic resonance (NMR) techniques. The integrated intensity of the visible central spectral line was 34–39% of the intensity of a reference sample containing an equal quantity and concentration of ^{23}Na nuclei. Since satellite signals were clearly demonstrable, the effect reflected a nuclear quadrupolar interaction rather than a splitting of the ^{23}Na into two populations of bound and free nuclei. It is proposed that a similar quadrupolar effect may be the basis for the apparent binding of the ^{23}Na observed in biological systems.

INTRODUCTION

The electrolyte composition of biological cells differs distinctly from that of the medium bathing the cell exterior. Characteristically, the intracellular concentration of Na^+ of most cells is considerably lower, and the intracellular concentration of K^+ considerably greater, than in the extracellular fluid. The basis for this phenomenon has been under study for many years. Experimental results have suggested that the altered intracellular composition arises primarily from either (*a*) metabolically dependent transport properties of the plasma membrane (membrane hypothesis) (1–4), or (*b*) differential binding properties of sites within the cell cytoplasm (association-induction hypothesis) (5).

The first hypothesis has been strongly supported by studies of squid giant axon and red blood cell ghosts. The cytoplasm may be extruded from the nerve (6, 7), the residual intracellular contents cleaned out with proteases (8) or cysteine (9), and the cellular interior perfused with solutions of well defined composition. When

the electrolyte composition of the perfusate approximates that of the cytoplasm extruded, the electrical properties of the perfused preparation also approximate those of intact preparation. Similarly, erythrocyte ghosts depleted of some 90% of their hemoglobin can accumulate K^+ (10) and actively extrude Na^+ (11). To the extent that generalization from one cell type to others is justified, the cell membrane appears to constitute a selective ionic barrier with the capacity to actively transport Na^+ and K^+.

A variety of arguments has been adduced in support of the second hypothesis (5, 12–14). Under certain experimental conditions, some fraction of the intracellular cations may be bound somewhere within the cell (15); however, the magnitude and relationship of this binding to maintenance of concentration gradients between cell and external fluid, and to transepithelial transport have remained obscure. Therefore, the association-induction hypothesis has not been widely accepted. This position must now be reexamined after the recent series of publications (16–25) purporting to demonstrate by the techniques of NMR that Na is largely bound within the cell.

Biological tissue may be packed into a test tube and the NMR spectrum of its

TABLE I

REPORTED APPARENT PERCENTAGE BINDING OF ^{23}Na IN BIOLOGICAL TISSUES, ESTIMATED FROM NMR STUDIES OF ^{23}Na

Tissue	Apparent binding	Reference
	%	
Frog muscle	63–72	Cope (17)
	58–65	Ling and Cope (22)
	37	Martinez et al. (23)
	55–63	Czeisler et al. (21)
Rat muscle	65–72	Cope (17)
	62	Cope (19)
Homogenated frog muscle	65–74	Cope (17)
Rabbit muscle actomyosin	48	Cope (17)
Frog skin	57–64	Rotunno et al. (25)
	44	Reisin et al. (24)
Rat testicle	24	Reisin et al. (24)
Rabbit kidney	58–70	Cope (17)
Rat kidney	67	Cope (19)
Rabbit brain	60–70	Cope (17)
Rat brain	67	Cope (19)
Rabbit myelinated nerve	56	Cope (20)
Frog liver	66	Martinez et al. (23)

^{23}Na content measured. The tissue may then be ashed, solvent added to restore the volume, and the NMR signal reexamined. Cope (17) was the first to observe that the intensity of the ^{23}Na signal before ashing of several biological preparations was some 30–40% of the intensity measured after ashing. Since, under certain circumstances (26–29), the integrated intensity of the spectral line is a direct measure of the ^{23}Na ions in solution, Cope reasoned that the NMR data defined two populations of Na nuclei. One population, comprising 60–70% of the total, was hypothesized to be bound, and thought to be characterized by a transverse relaxation time of $T_2 = 1$ msec (19). The second population of Na ions, comprising 30–40% of the total, was considered to be free, characterized by a T_2 of 10–15 msec (19), and thought to be solely responsible for the NMR-visible spectrum.

Cope's basic observation has now been confirmed for a wide variety of biological tissues (Table I) by a number of investigators who have drawn similar conclusions from their data.

In the present report, we would like to suggest an alternative possible interpretation. The NMR data may arise not from different populations of Na ions, but rather from different energy transitions reflecting all the Na nuclei. In order to provide an example of this alternative mechanism, data are presented from a model system, where only some 34–39% of the ^{23}Na signal is observable, but which does not, in fact, reflect splitting into two populations of bound NMR-invisible and free NMR-visible sodium ions.

METHODS

All NMR measurements were performed with a DP60 Varian Spectrometer (Varian, Palo Alto, Calif.) using a fixed frequency unit (V-4311) operating at 8.13 MHz. In order to detect satellite signals, the derivative of the spectrum was recorded using the wide line mode of operation and applying a field modulation of 20 Hz.

Intensity measurements were performed by directly recording the absorption signal in the high resolution mode. In order to stabilize the base line, a modulation frequency of 1980 Hz (a value much larger than the line width) was applied, and the receiver output was phase detected by the NMR V-3521 integrator unit; the side bands obtained by this technique served also for chart calibration.

Linoleic acid, practical grade, was obtained from The British Drug Houses, Poole, England; in order to determine the approximate fatty acid concentration, a sample was mixed with a large volume of distilled water, a known amount of NaOH added in excess, and the solution back-titrated against pH with HCl. The experimental samples were prepared by thoroughly mixing equimolar amounts of linoleic acid and NaOH to form 1.986 g sodium linoleate in a total sample weight of 8.075 g. Glycerol was added to each reference sample of NaOH solution in order to equalize the volume with that of the experimental sample, and to provide a comparable line width by increasing the viscosity of the reference solution.

The signals from the soap and reference samples were recorded using the same instrumental settings. For purposes of data reduction, the product of the height and width at half-height was taken as a measure of the relative integrated intensity for each absorption signal.

Care was taken to ascertain that the intensity of the radio frequency signal was below saturation.

RESULTS

Fig. 1 presents representative absorption spectra of ^{23}Na obtained, on the left, from an experimental sample of sodium linoleate in water, and on the right, from a control sample of NaOH and glycerol. The width of the central spectral line of the experimental sample is 70 Hz. The integrated intensity of the mesophase is, however, only 39% of that for the reference solution, despite the fact that both test tubes contained identical numbers of Na nuclei in identical volumes. On the basis of the means of five such measurements from each of two different experiments containing different samples of material, the central line constituted 39 and 34%, respectively, of the total integrated intensity. The satellite signals are not visible using the relatively insensitive absorption mode.

Fig. 2 presents a recording of the ^{23}Na spectrum taken from a similar experimental sample, but using the more sensitive derivative mode. In addition to the central line, two satellite signals are easily distinguished; the separation corresponding to the peak satellite absorption lines is 11.1 kHz. As anticipated, no satellite lines were visible in the control reference sample. The shape of the satellite lines is characteristic of the powder pattern for nuclei with a spin number of 3/2 arising from a first-order quadrupolar interaction (30), and is not characteristic of the macroscopic alignment usually noted for liquid crystals (31). A powder pattern spectrum has also been noted for ^{35}Cl and ^{37}Cl in liquid crystals, on the basis of second-order quadrupolar nteractions (32).

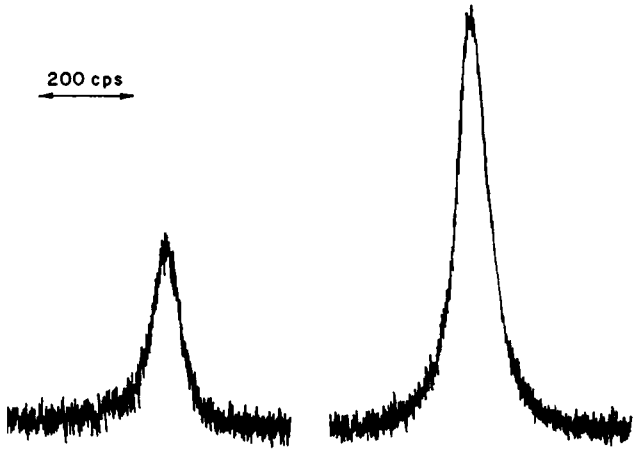

FIGURE 1 Absorption spectrum of ^{23}Na obtained from sodium linoleate in water (left) and from a reference sample of NaOH in water and glycerol (right). The integrated intensity of the visible experimental signal is 39% of that for the reference signal, although both samples contained the same concentration and quantity of ^{23}Na nuclei.

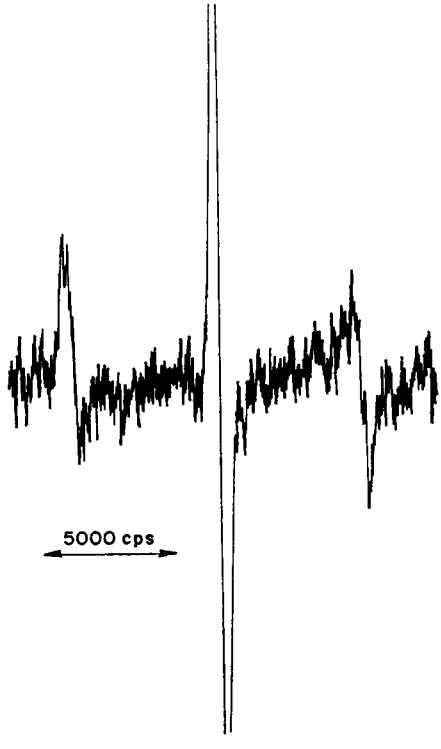

FIGURE 2 Derivative of the ^{23}Na signal obtained from sodium linoleate in water. In addition to the central signal, satellite lines are easily distinguished.

DISCUSSION

In analogy with sodium linolenate under similar conditions (33), the liquid crystals of the current study are likely to have been in the median phase, presumably in the form of a hexagonal array of rods, with the hydrocarbon chains facing inwards, and the polar groups outwards in contact with the surrounding water (34). The integrated intensity of the sharp central ^{23}Na signal from the sodium linoleate proved to be only some 34–39% of the integrated intensity observed for a control solution of NaOH, containing the same number and concentration of ^{23}Na nuclei, but with glycerol in place of fatty acid. One might be tempted to apply the same approach taken in recent NMR studies of biological systems, concluding that 34–39% of the Na nuclei was free to present an observable signal, wheras binding of the remaining nuclei broadened the ^{23}Na signal beyond detection; however, in the present study, satellite lines on each side of the central signal were clearly demonstrable. These lines are quite inexplicable within the framework of separate distinct populations of free and bound Na nuclei, but are qualitatively and quantitatively in agreement with the concept of a first-order quadrupolar interaction.

Since the ^{23}Na nucleus possesses a spin quantum number (I) of 3/2, the nucleus

may exist in one of four different energy states characterized by the magnetic quantum numbers $m = 3/2, 1/2, -1/2$, and $-3/2$. In the event of interaction of the nuclear magnetic moment solely with the magnetic field, the three permitted transitions in energy from $m = 3/2, 1/2$, and $-1/2$ to $m' = 1/2, -1/2$, and $-3/2$, respectively, are all characterized by the same decrement in energy (ΔE); however, ^{23}Na has a quadrupole moment. In the presence of an asymmetrical electrical environment, the three energy transitions will be characterized by different values of ΔE, and two of the three spectral lines will be shifted equally about the central signal by a first-order change in frequency (ν_m), where:

$$\nu_m = (\nu_Q)\ (m - 1/2)\ (3\mu^2 - 1)/(2), \text{ and} \tag{1}$$

$$\nu_Q = (e^2qQ/h)\ (3)/(4I^2 - 2I). \tag{2}$$

μ is the cosine of the angle between the gradient of the electrical field and the constant applied magnetic field, and (e^2qQ/h) is a quadrupole-coupling constant. For nuclei such as ^{23}Na with a spin number of 3/2, the intensity of the central line would be expected to be 40% of the total integrated intensity (30), very close to the 34–39% which was actually observed.

As may be appreciated from equation 1, the frequency shift ν_m depends upon μ. Since, in a powdered solid sample, the electric field gradients are randomly oriented, the separation ($\Delta\nu_m$) between the maxima of the two satellite lines of ^{23}Na may be obtained from equations 1 and 2 to be $(e^2qQ/2h)$.

The data presented clearly arise from a nuclear quadrupolar effect, an effect not appreciated in the presence of rapid molecular tumbling in the isotropic homogeneous liquid media usually employed, where rapid tumbling is defined by the condition $\omega_0\tau \ll 1$ (ω_0 is the Larmor angular frequency and τ is the correlation time); under these conditions, the three spectral lines converge to a single line characterized by a single relaxation time T_2.

The physical basis for the quadrupolar effect observed is less clear, possibly an immobilization of all the ^{23}Na nuclei, or an "ordering effect" induced by the liquid crystalline stucture.

The former possibility seems unlikely from a consideration of the magnitude of the quadrupolar interaction. Since $\Delta\nu_m$ was measured to be 11.1 kHz, the quadrupolar coupling constant should be 22.2 kHz. This value is an order of magnitude smaller than the constants calculated for a variety of sodium complexes (35), with the possible exception of soluble RNA, whose binding activity has yet to be rigorously defined.

It is more likely that the quadrupolar effect underlying the current data arises from an ordering parameter characterizing the liquid crystals (32, 36). This ordering effect may reflect either or both of two possible phenomena. First, rapid exchange may exist between a pool of free Na nuclei and a fraction of bound nuclei of indeterminate size, which may be very small; the central spectral line is then a weighted

average of the ½ to −½ transitions of all the free and bound Na ions, and similarly, the satellite signals are weighted averages of the remaining two permitted transitions of all the bound plus free ^{23}Na nuclei. Second, an additional possible basis for the ordering effect would be that the Na nuclei are in anisotropic domains, producing ordering of the electric field gradients.

It should be emphasized that these possible ordering effects are entirely distinct in principle from the model of two slowly exchanging populations of free and bound Na nuclei which has been widely applied to biological systems. Ordering, as used here, refers to a population of Na ions *all* of which contribute to the central visible spectral line, and all of which are characterized by the same NMR parameters, which might reflect an averaging process; the fraction of Na nuclei bound in a thermodynamic sense is not specified, and may be very small.

Although the NMR spectrum of ^{23}Na linoleate in water reflects different energy transitions and not different populations of sodium nuclei, the precise basis for the spectral properties of ^{23}Na in biological systems is less clear.

Satellite signals of ^{23}Na have not been reported for biological tissue. This does not preclude the possibility, however, that different energy transitions underlie the NMR properties of intracellular Na, for at least two reasons.

First, the intracellular [Na] is characteristically low, so that the signal-to-noise ratio is decidedly unfavorable. Furthermore, as may be appreciated from equation 1, ν_m is a continuous function of μ, so that the satellite signal is distributed on both sides of the central spectral line. ν_m is also directly proportional to (e^2qQ/h). For systems with a large quadrupolar coupling constant, the range of distribution of ν_m will be proportionately larger. Since the total integrated intensity of the satellite signals remains equal to 60% of the total ^{23}Na signal, their peak heights must decline with increasingly large quadrupolar interaction. Therefore, in systems with a large value of (e^2qQ/h), the satellite signals may be indistinguishable from the background noise, even where there is clear evidence (32) that such signals must be present.

Second, Hubbard (37) has pointed out that, on theoretical grounds, two distinct ^{23}Na signals may arise in isotropic liquids characterized by $(\omega_0\tau) \geq 1$, and need not arise solely from quasi-solids. Experimental evidence supporting this theoretical possibility has been provided by electron spin resonance (ESR) studies of Cr^{3+} in water and water-glycerol mixtures (38). The electronic ground state of Cr^{3+} is characterized by an electron spin quantum number of 3/2 (analogous to the nuclear spin number of sodium), and is affected by a zero field splitting (analogous to the quadrupolar splitting of Na). The mathematical formalisms of ESR (39) and NMR (37) are similar, but experimental demonstration of this concept of ESR is facilitated by the fact that the magnetic moment of the electron is orders of magnitude greater than that of the nucleus. It is resonable to assume that a similar situation would prevail even if a fraction of the Na nuclei, characterized by $\omega_0\tau \geq 1$, were in rapid exchange with the bulk of the Na ions characterized by fast tumbling.

Therefore, on the basis of the data and considerations presented above, the ^{23}Na spectra of biological cells might equally well arise from splitting of the intracellular Na nuclei into two populations, binding of the larger Na fraction rendering its signal invisible (hypothesis 1), or a quadrupolar effect with the Na nucleus obscuring 60% of the spectral intensity of a single NMR population of Na nuclei (hypothesis 2).

The data of Table I do suggest, however, that the second is the more likely possibility. The visible ^{23}Na signal obtained from almost all of the tissues studied has been close to 40% of that anticipated on the basis of the total Na content. It seems remarkable that tissues as disparate as muscle, kidney, frog skin, liver, brain, and nerve should all bind Na to approximately the same extent, although the protein and phospholipid contents of the tissues are very different. It is all the more remarkable that even Dowex 50 Resin (Dow Chemical Co., Midland, Mich.) has been reported to apparently bind 59% of the total K content (40); since K also possesses a spin number of 3/2, the same considerations concerning quadrupolar interaction apply to this nucleus, as well.

Within the framework of hypothesis 1 above, the remarkable similarity of the estimates of the apparent fractional binding is inexplicable other than as a fortuitous coincidence; however, this is directly predictable from the basic formulation of hypothesis 2.

It should be noted that three of the reported values of Table I deviate appreciably from the predicted 60% apparent binding. The data for rat testicle and frog skin (24) include large spectral contributions from ^{23}Na in the extracellular space. The basis for the low value obtained for frog muscle by Martinez et al. (23) is less clear; their data do not agree, furthermore, with the much higher values obtained by other investigators for frog muscle (17, 21, 22), frog muscle homogenates (17), and rat muscle (19).

In summary, only 40% of the central ^{23}Na signal is visible in Na linoleate in water under the conditions of the current study. This phenomenon seems to arise from a quadrupolar effect on the ^{23}Na spectral line. We submit that a similar mechanism also provides an alternative interpretation of the ^{23}Na data obtained from biological tissues, and suggest that it is the more likely of the two hypotheses thus far advanced. The precise basis of the quadrupolar effect is not clear and requires further experimental study.

We are grateful to Professors Z. Luz and S. Alexander for helpful discussions.

Dr. Civan was a Public Health Service Special Fellow (National Heart and Lung Institute grant 2 FO3 HE 18528-03) during the period of this study, and is currently an Established Investigator of the American Heart Association.

The study was supported in part by grant HE-06664 from the National Heart Institute.

Received for publication 14 June 1971.

REFERENCES

1. DEAN, R. B. 1941. *Biol. Symp.* **3**: 331.
2. KROGH, A. 1946. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **133**:140.
3. USSING, H. H. 1949. *Physiol. Rev.* **29**:127.
4. HODGKIN, A. L. 1951. *Biol. Rev. (Cambridge).* **26**:339.
5. LING, G. N. 1962. A Physical Theory of the Living State: The Association-Induction Hypothesis. Blaisdell Publishing Co., Waltham, Mass.
6. BAKER, P. F., A. L. HODGKIN, and T. I. SHAW. 1961. *Nature (London).* **190**:885.
7. OIKAWA, T., C. S. SPYROPOULOS, I. TASAKI, and T. TEORELL. 1961. *Acta Physiol. Scand.* **52**:195.
8. TASAKI, I., I. SINGER, and T. TAKENAKA. 1965. *J. Gen. Physiol.* **48**:1095.
9. HUNEEUS-COX, F., H. L. FERNANDEZ, and B. H. Smith. 1966. *Biophys. J.* **6**: 675.
10. GARDOS, G. 1954. *Acta Physiol. Acad. Sci. Hung.* **6**:191.
11. HOFFMAN, J. F. 1962. *J. Gen. Physiol.* **45**:837.
12. SHAW, F. H., and S. E. SIMON. 1955. *Aust. J. Exp. Biol. Med. Sci.* **33**:153.
13. COPE, F. W. 1965. *Bull. Math. Biophys.* **27**:99.
14. TROSHIN, A. S. 1966. Problems of Cell Permeability. Pergamon Press, Ltd., Oxford, England.
15. FENICHEL, I. R., and S. B. HOROWITZ. 1969. *In* Biological Membranes. R. M. Dowben, editor. J. and A. Churchill, Ltd., London. 177.
16. COPE, F. W. 1965. *Proc. Natl. Acad. Sci. U.S.A.* **54**:225.
17. COPE, F. W. 1967 *a. J. Gen. Physiol.* **50**:1353.
18. COPE, F. W. 1967 *b. Bull. Math. Biophys.* **29**:691.
19. Cope, F. W. 1970 *a. Biophys. J.* **10**:843.
20. COPE, F. W. 1970 *b. Physiol. Chem. Phys.* **2**:545.
21. CZEISLER, J. L., O. G. GRITZ, JR., and T. J. SWIFT. 1970. *Biophys. J.* **10**:260.
22. LING, G. N., and F. W. COPE. 1969. *Science (Washington).* **163**: 1335.
23. MARTINEZ, D., A. A. SILVIDI, and R. M. STOKES. 1969. *Biophys. J.* **9**:1256.
24. REISIN, I. L., C. A. ROTUNNO, L. CORCHS, V. KOWALEWSKI, and M. CEREIJIDO. 1970. *Physiol. Chem. Phys.* **2**:171.
25. ROTUNNO, C. A., V. KOWALEWSKI, and M. CEREIJIDO. 1967. *Biochim. Biophys. Acta.* **135**:170.
26. JARDETSKY, O., and J. E. WERTZ. 1956 *a. Arch. Biochem. Biophys.* **65**:569.
27. JARDETSKY, O., and J. E. WERTZ. 1956 *b. Amer. J. Physiol.* **187**:608.
28. JARDETSKY, O., and J. E. WERTZ. 1960. *J. Amer. Chem. Soc.* **82**:318.
29. WERTZ, J. E., and O. JARDETSKY. 1956. *J. Chem. Phys.* **25**:357.
30. ABRAGAM, A. 1961. The Principles of Nuclear Magnetism. Clarendon Press, Oxford, England.
31. FUNG, B. M., M. J. GERACE, and L. S. GERACE. 1970. *J. Phys. Chem.* **74**:83.
32. LINDBLOM, G., H. WENNERSTROM, and B. LINDMAN. 1971. *Chem. Phys. Lett.* **8**:489.
33. HUSSON, F. 1961. *C. R. Seances Soc. Biol. Filiales.* **253**:2948.
34. LUZZATI, V. 1968. *In* Biological Membranes. D. Chapman, editor. Academic Press, Inc., New York. 71.
35. HAYNES, D. H., B. C. PRESSMAN, and A. KOWALSKY. 1971. *Biochemistry.* **10**:852.
36. BUCKINGHAM, A. D., and K. A. MCLAUCHLAN. 1967. *In* Progress in NMR Spectroscopy. J. W. Emsley, J. Feeney, and L. H. Sutcliffe, editors. Pergamon Press, Ltd., Oxford, England. **2**:63.
37. HUBBARD, P. S. 1970. *J. Chem. Phys.* **53**:985.
38. LEVANON, H., S. CHARBINSKY, and Z. LUZ. 1970. *J. Chem. Phys.* **53**:3056.
39. RUBINSTEIN, M., A. BARAM, AND Z. LUZ. 1971. *Mol. Phys.* **20**:67.
40. COPE, F. W., and R. DAMADIAN. 1970. *Nature (London).* **228**:76.