A Nuclear Magnetic Resonance Study of $^{23}Na^+$ Complexing by Ionophores*

Duncan H. Haynes,† Berton C. Pressman,‡ and Arthur Kowalsky§

ABSTRACT: The complexing of ionophores which function as mobile carriers of alkali cations in membranes has been studied with ^{23}Na nuclear magnetic resonance spectroscopy. The width of the ^{23}Na resonance is a function not only of the rotational correlation time of the Na^+ but also of the coupling constant between the electric field gradient at the nucleus and the quadrupole moment of the nucleus and thus gives information about the symmetry of the electromagnetic environment of the cation. The values of the coupling constant for the 1:1 ionophore–cation complexes (observed in methanol) ranged from 0.47 to 1.64 MHz. These low values indicate a very symmetric electric field around the complexed ^{23}Na nucleus and a very small amount of covalent interaction between the cation and the ligand oxygens. The order of increasing coupling constants (monactin < enniatin B < valinomycin < monensin < cyclohexyl ether < nigericin) is in good qualitative agreement with the order of increasing field gradient predicted from consideration of space-filling models and published crystallographic structures. A good correlation was observed between the ^{23}Na resonance positions of the 1:1 complexes and log stability constants indicating that electronic interaction of the oxygen atoms of the ionophore and the cation is the principal determinant of the energy of complex formation. Fast exchange between free and complexed Na^+ was shown to occur with $k_{off} > 10^2$ sec^{-1} for all ionophores studied except monensin. This is consistent with the mobile carrier mechanism of ionophore action and also with the measured turnover number of 200 sec^{-1} for valinomycin-induced mitochondrial K^+ transport.

Ionophores such as valinomycin have been the subject of extensive physical chemical examination directed toward explaining the remarkable capacity of these small molecules to render biological and artificial membranes permeable to alkali cations (Pioda *et al.*, 1967; Pressman *et al.*, 1967; Pressman and Haynes, 1969, Eisenman *et al.*, 1969). The study of ionophores is of particular interest because they have some of the dynamic properties attributed to membrane-bound carriers or permease molecules and they are also possible models for monovalent cation activation of certain enzymes. The mechanism underlying their biological activity is particularly susceptible to investigation by physical techniques since they are of low molecular weight, of known primary and stereochemical structure, are available in pure form in reasonable quantities, and are stable in a variety of solvents. In contrast, studies of natural carriers are more formidable. The structural complexity of the natural membrane-bound carriers complicates the problems of isolation, purification, stability, and evaluation of activity.

Despite the simplicity of ionophore molecules there has been some controversy about their fundamental mode of action. The early suggestion that ionophores may create hydrophilic pores or channels (Mueller and Rudin, 1967) was subscribed to by several investigators (Andreoli *et al.*, 1967; Chappel and Crofts, 1965) until strong evidence was presented that ionophores act as mobile carriers (Pressman *et al.*, 1967). It was demonstrated that ionophores can extract cations from water into nonpolar solvents and transport cations across considerable distances in bulk phases (Pressman *et al.*, 1967).

The concentration dependence of the extraction indicated no cooperative interaction between ionophore molecules. Nuclear magnetic resonance experiments also showed no evidence for intermolecular cooperation during K^+ complexing by valinomycin and provided evidence for conformational changes slightly different for each complexed cation (Haynes *et al.*, 1969). Subsequently Ovchinnikov *et al.* (1969) combining information from infrared and nuclear magnetic resonance spectroscopy arrived at a conformation for the valinomycin–K^+ complex in solution which agreed closely with that determined independently by X-ray crystallography (Pinkerton *et al.*, 1969).

Proton magnetic resonance has also been used to study the kinetics of association and dissociation of valinomycin and K^+ as a function of solvent (Haynes *et al.*, 1969). These reactions were found to be immeasurably slow in $CDCl_3$ but rapid in the more polar 80% CH_3OH–20% $CDCl_3$. The dissociation rate in CH_3OH–$CDCl_3$ was found to be within an order of magnitude of the turnover number for valinomycin-catalyzed mitochondrial K^+ transport (Pressman *et al.*, 1967). These observations mitigate against the channel mechanism which would require shuttling of K^+ between ionophore molecules even in the nonpolar region of membranes and provide additional support for the mobile carrier mechanism. The present investigation extends the previous observation of the fast exchange between valinomycin and K^+ in polar solvents to various ionophores and Na^+.

Previous nuclear magnetic resonance studies of ionophores

* From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the Papanicolaou Cancer Research Institute, Miami, Florida 33136. *Received August 19, 1970.* This work was supported by National Institutes of Health Grants 5T01-GM-00894 and R01-AM-13966.

† Supported in part by a grant from Eli Lilly and Co., Indianapolis, Ind., and by National Defense Education Act Title IV Fellowship 66-4826. Present address: Max-Planck Institut, für Physikalische Chemie, Göttingen 3400, Germany.

‡ Career Development awardee supported by Grant K3-GM-3626 from the U. S. Public Health Service. Present address: Department of Pharmacology, School of Medicine, University of Miami, Fla.

§ Supported in part by Career Development Award K3-GM-3212 from the U. S. Public Health Service. Present address: Department of Biophysics, Albert Einstein College of Medicine, Bronx, N. Y.

have been restricted to observing the proton spectrum of ionophores and their complexes. The present paper extends these nuclear magnetic resonance studies to the effect of complexing on the cation, $^{23}Na^+$, itself. The ^{23}Na nucleus possesses a quadrupole moment (Pople *et al.*, 1959; Abragam, 1961) and its magnetic resonance absorptions will be characterized by two parameters; position, depending on the shielding of the nucleus by surrounding electrons, atoms and groups; and width, depending on the coupling of the nuclear quadrupole with electric field gradients at the nucleus and on the correlation time for reorientation of these field gradients with respect to the nucleus. In this paper variations in the width of the resonance are correlated with changes in the symmetry of the electric field about the cation. Changes in the position of the ^{23}Na resonance are interpreted in terms of the interaction between the cation and the complexing oxygen atoms of the ionophores. Finally, lower limits of the kinetic constants for complexing and dissociation have been estimated by analysis of the variation of the ^{23}Na chemical shift with the degree of complexing.

To date there have been very few studies of ^{23}Na magnetic resonance in biological systems. Jardetzky and Wertz (1960) and James and Noggle (1969b) investigated the complexing of ^{23}Na by biologically important compounds and James and Noggle (1969a) have studied the complexing of ^{23}Na by RNA. The state of Na^+ in isolated frog muscle and liver has also been investigated by magnetic resonance (Cope, 1967; Rotunno *et al.*, 1967).

Materials and Methods

Materials. Valinomycin, nigericin, and monensin were obtained from Eli Lilly and Co., Indianapolis, Ind.; enniatin B from Hoffman-La Roche, Basle; monactin from CIBA, Basle; cyclohexyl ether[1] (Pedersen, 1967) from E. I. du Pont de Nemours and Co. These compounds, except for monensin and nigericin, were found to have less than 10^{-4} mole % of contaminating paramagnetic ferric or manganous ion, as determined by atomic absorption spectroscopy. The monensin sample was obtained as the 1:1 Na^+ salt and was checked for Na^+ content by atomic absorption spectroscopy. The nigericin sample was received as a mixture of the Na^+ and K^+ complexes. It was converted into the 1:1 Na^+ complex by exhaustive washing of a toluene solution of the complex with 1 M NaCl buffered at pH 7 with Tricine.

Magnetic Resonance. ^{23}Na magnetic resonance spectra were observed with a Varian DA-60 spectrometer using the variable-frequency rf unit at 15.9 MHz. Audiofrequency field molulation at 209 Hz was used for side-band detection of absorption signals. Resonance positions (δ) are expressed in parts per million relative to an external standard of 2.0 M NaCl. Upfield shifts are designated by $\delta > 0$. Corrections for bulk magnetic susceptibility differences were ignored since calculations showed that the correction was always less than 3 Hz and within experimental error.

Measurements of line widths at half-height ($\Delta\nu_{1/2}$) and position were taken on at least four spectra for each sample. The average value and standard error are reported. Care was taken to ensure that saturation and distortion of the line shape by the modulating field did not occur. Widths and positions of resonances of the various complexes were determined by one of two procedures; (a) when the width was less than 100 Hz, by direct observation of the resonance of the 1:1 Na–ionophore complex, and (b) when the width was greater than 100 Hz, by extrapolation of titration data (see below) to the 1:1 complex. In those cases where both procedures could be employed, good agreement between them was obtained.

Titrations. NaCNS, 100 mM in CH_3OH, was progressively complexed by adding small aliquots of solutions of the ionophores (valinomycin, monactin, enniatin B, or the cyclohexyl ether) and changes in resonance widths and positions were observed. The volume of the solution after the titration did not differ from the original by more than 5%. In the titrations of monensin and nigericin the Na^+ ionophore complex at a concentration of about 100 mM was progressively titrated with excess NaCNS.

Viscosity Measurements. The viscosities of the ionophore and ionophore complex solutions were determined with an Ostwald viscosimeter which had a flow time for CH_3OH of about 160 sec at 21°.

Results

The spectrum of the ^{23}Na resonance of 100 mM NaCNS in CH_3OH at 21° is shown in Figure 1. Line-shape analysis indicated a Lorentzian form with $\Delta\nu_{1/2} = 35$ Hz. This figure also shows the spectrum of the same solution after addition of 30 mM valinomycin. Addition of each of the ionophores studied similarly broadened the resonance and shifted it. The direction and the extent of the shift were dependent on the ionophore, its total concentration and the strength of the binding.

Careful titrations of 100 mM NaCNS with ionophores in which the final ionophore concentrations were between 10 and 100 mM showed that the width and position of the ^{23}Na resonances were linear functions of the fractions of Na^+ in the bound form and obeyed eq 1 and 2. The subscripts Na

$$\delta_{obsd} = P_{Na}\delta_{Na} + P_{NaI}\delta_{NaI} \quad (1)$$

$$\Delta\nu_{1/2,obsd} = P_{Na}\Delta\nu_{1/2,Na} + P_{NaI}\Delta\nu_{1/2,NaI} \quad (2)$$

and NaI indicate solvated Na^+ and its ionophore complex,[2] respectively, and P represents the fraction of a given component present in solution. Compliance of the experimental data with eq 1 and 2 indicates that the free Na^+ was exchanging rapidly with the bound Na^+ such that $1/\tau > (\delta_{NaI} - \delta_{Na})\nu_0$, where τ is the lifetime and ν_0 is the observing frequency. The value of $(\delta_{NaI} - \delta_{Na})\nu_0$ varied between 20 and 200 sec^{-1} depending on the added ionophore. Figure 2 shows the results of a titration of 100 mM NaCNS in methanol with the cyclohexyl ether. At the end point, $\Delta\nu_{1/2,NaI} = 215$ Hz and $\delta_{NaI} - \delta_{Na} = +15.2$ ppm.

Table I shows the effect of complexing with the ionophores on the width and position of the ^{23}Na resonances. All of the ionophores except valinomycin and monensin shift the resonance upfield relative to the CH_3OH solvated cation. All of the ionophores broadened the resonances to between 68 and 300 cps. Substitution of the solvent $CHCl_3$ for CH_3OH broad-

[1] The abbreviations used are: cyclohexyl ether, 2,5,8,15,18,21-hexaoxatricyclo[20.4.0.09.14]hexacosane; K_s, stability constant; HEEDTA, N^1-(2-hydroxyethyl)ethylenediamine-N,N,N^1-triacetic acid; NTA, nitrilotriacetic acid.

[2] The symbol "I" denotes the ionophore irrespective of the ionophore charge. Of the ionophores studied, monensin and nigericin have a negative charge at pH 7. All other are uncharged. To avoid confusion the net charge of the complex is omitted in the equation.

[3] Unpublished observation.

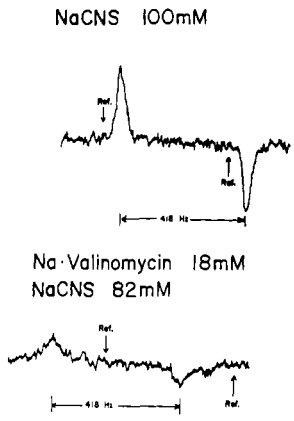


FIGURE 1: ^{23}Na magnetic resonance spectrum in absence and presence of valinomycin. The position of the 2 M NaCl reference resonance is indicated by the arrows.

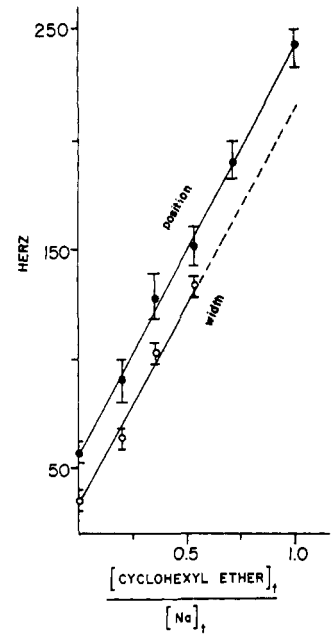


FIGURE 2: Titration of $^{23}Na^+$ with cyclohexyl ether: width and position. Increasing amounts of the cyclohexyl ether were added to a sample of 100 mM NaCNS in methanol. Since K_a for the complex is of the order of 10^3 M^{-1} essentially all of the added ionophore was complexed with Na^+. In this case the width of the resonance of the 1:1 complex was obtained by extrapolation as indicated by the dashed line.

ened the resonances still further, but gave a detectable change in chemical shift only in the case of monactin.

Control experiments were carried out to see if the solvent, the ionic strength or the concentration of the salt could affect the position or width of the ^{23}Na resonances. Resonances of the following solutions had the same chemical shift and width values within the experimental error: (1) 50 mM NaCNS in CH_3OH, (2) 50 mM NaCNS in 80% CH_3OH–20% $CHCl_3$ (v/v), (3) 800 mM NaCNS in 80% CH_3OH–20% $CHCl_3$ (v/v), and (4) 50 mM NaCNS + 100 mM KCNS in 80% CH_3OH–20% $CHCl_3$(v/v). This suggests that the effect of ionic strength is not significant in these experiments, and that ion pairing of Na^+ and CNS^-, if it occurred, did not change the width or position significantly.

Specific viscosities were measured at concentrations limited by the amount of ionophore available. For monactin and valinomycin, the specific viscosity was independent of concentration, consistent with the absence of ionophore aggregation. Although this is a relatively insensitive means of detecting aggregation of small molecules, the results are in agreement with the nuclear magnetic resonance evidence of lack of cooperative interaction of these ionophores in solution (Haynes *et al.*, 1969). For the purpose of calculating the viscosities of the solutions upon which the actual ^{23}Na nuclear magnetic resonance spectra were taken, the specific viscosity was assumed to be constant for the other ionophores as well.

Discussion

Quadrupole Coupling Constants. The relaxation of the ^{23}Na nucleus ($I = {}^3/_2$) is dominated by the interation of the nuclear quadrupole moment, eQ, with the electric field gradient at the nucleus (eq). The line width at half-height is given by

$$\Delta\nu_{1/2} = \frac{2\pi}{5}\left(\frac{e^2qQ}{h}\right)^2\tau_c \qquad (3)$$

where the quantity e^2qQ/h is the quadrupole coupling constant, h is Planck's constant, and τ_c is the correlation time which characterizes fluctuations of the electric field gradient. This expression is valid in the limit $\tau_c \ll 2\pi\nu_0^{-1}$ (Abragam, 1961). Thus the resonance can be broadened by either an increase in the electric field gradient or an increase in the correlation time for reorientation of the field gradient with respect to the external magnetic field. The reorientation of the electric field gradient could be accomplished by three means: (1) a change in the geometry of the ionophore complex and hence the field gradient; (2) changes in the electric field gradient produced by transient states during the processes of complexing and dissociation; and (3) rotation of the entire complex (*i.e.*, reorientation of the field gradient) with respect to the stationary magnetic field.

The first possibility is difficult to assess but it does not seem likely. Rearrangements of the complex extensively affecting the electric field gradient, if they occurred, would imply a loose binding of the Na^+ by the electronegative oxygens. The ionophores with smaller ring size (cyclohexyl ether and enniatin B) have fewer degrees of freedom for changes in oxygen configuration than the larger ring compounds. They might, however, be capable of changing the conformation of their side chains without affecting the oxygen configuration, but this would be a weak long-range effect. The ionophores with larger ring size (monactin, valinomycin) have a larger number of degrees of freedom for reorientation of both the complexing oxygens and the other atoms. The observed widths (Table I) for the resonances of these four ionophore complexes shows that this first explanation is inadequate to describe the results.

The second possibility can be eliminated since the observed resonance shifts and width were direct linear functions of the fraction of complexed cation, indicating that the ion is exchanging rapidly between free and complexed species and that the resonance observed is a weighted average of these two forms. If the complexing and dissociation processes themselves provided the mechanism for relaxation, then the exchange rate would determine the resonance width and the composite line shape would be a complex function, not only of the rate, but also of the populations of the two states. It also follows that if the dissociation rates were actually comparable to ν_0, the transition states must then be extremely

TABLE I: Widths and Positions of the Sodium Resonance of Ionophore–Sodium Complexes.

Ionophore	Solvent	$\Delta\nu_{1/2}$ (Hz)	δ (ppm)
Valinomycin	CH_3OH	215 ± 50[a]	−10.7 ± 2.5[a]
Monensin	CH_3OH	105 ± 20	2.7 ± 1.2
	CH_3OH	35.2 ± 0.8	3.5 ± 0.2
Nigericin	CH_3OH	330 ± 20[a]	4.4 ± 0.6[a]
Enniatin B	CH_3OH	68 ± 3	7.1 ± 0.2
Monactin	CH_3OH	89 ± 5	12.8 ± 0.2
Cyclohexyl ether	CH_3OH	215 ± 40	15.2 ± 0.6[a]
Monensin	$CDCl_3$	>300[b]	[c]
Nigericin	$CDCl_3$	≫400[b]	[c]
Cyclohexyl ether	$CDCl_3$	>250[b]	15.6 ± 5.0
Monactin	$CDCl_3$	221 ± 4	17.8 ± 1.1

[a] Obtained by extrapolation as described in Methods section. In the case of valinomycin and enniatin B, it was necessary to use the published stability constant (see legend of Figure 5) to determine [NaI] as a function of $[I]_t$. [b] Calculated using the maximum signal height and the area of the unbroadened or partially broadened resonance. [c] Resonance signal was severely broadened making exact determination of the position impossible even at the highest radiofrequency power available.

TABLE II: Calculated ^{23}Na Quadrupole Coupling Constants.

Ionophore	Solvent	$\bar{r}$ (Å)	$\tau_c \times 10^{10}$ (sec)	e^2qQ/h (MHz)
Monactin	CH_3OH	8.0[a]	4.0	0.5
Enniatin B	CH_3OH	5.0	1.0	0.7
Valinomycin	CH_3OH	6.0	2.5	0.8
	CH_3OH	3.7	0.3	0.9
Monensin	CH_3OH	5.0	1.0	0.9
Cyclohexyl ether	CH_3OH	6.0	1.4	1.1
Nigericin	CH_3OH	5.0	1.0	1.6
Monactin	$CHCl_3$	8.0[a]	3.8	0.7
Cyclohexyl ether	$CHCl_3$	6.0	1.4	>1.2
Monensin	$CHCl_3$	5.0	0.9	>1.6
Nigericin	$CHCl_3$	5.0	0.9	>1.9

[a] Denotes a value based on X-ray crystallography (*cf.* legend of Figure 3).

electronically symmetrical, *i.e.*, the substitution of complexing oxygen atoms of the ionophore into the first hydration shell of the cation must be done in such a fashion that the symmetry of the electronic distribution about the cation is not affected.

Thus, the relaxation process is dominated most likely by the third mechanism, in which the correlation time of the ^{23}Na nucleus is determined by the rotational diffusion time of the complex.

A knowledge of the rotational correlation time of the ionophore complexes would then allow calculation of the quadrupole coupling constant and permit comparison of the latter to the constant for solvated Na^+. This would provide information on changes of the electric field gradient at the nucleus which result from complexation. The rotational correlation time for a sphere may be estimated by the expression

$$\tau_c = \frac{4\pi\eta r^3}{3kT} \tag{4}$$

where r is the radius of the sphere and η is the viscosity (Bloembergen *et al.*, 1948).

The use of this equation in the present case must be qualified. It gives approximate values of the correlation time and it is limited to spherical shapes. However, these complexes are similar in structure, size, and physical characteristics, *i.e.*, all of them contain a central metal ion, a hydrophilic interior and a lyophilic exterior, and the equation, while not giving accurate values should give the relative ordering of times in the correct order of magnitude.

The error introduced by approximating the shapes of the complexes as spheres can be evaluated from the following considerations.

Shimizu (1962) calculated relative correlation times and the mean correlation time for rotation about the two axes of an ellipsoid of revolution and found that when the axial ratio is 2 or less the values are close to those of spheres of equivalent volume. Consideration of space-filling molecular models shows that the cyclohexyl ether complex is best approximated as a prolate ellipsoid of axial ratio 2 while the other complexes are best approximated as oblate ellipsoids of axial ratio between 1.0 and 1.5. The relative rotational relaxation times about the two axes of an ellipsoid of revolution, when calculated for the cyclohexyl ether complex (Perrin, 1936) agreed within 30% with that calculated for a sphere whose radius is the average of the two axes of the ellipsoid. The error in the calculation of τ_c would then be estimated to be ±30% giving an uncertainty in the calculated quadrupole coupling constant of ±15%.

Table II lists the average molecular radii of the 1:1 complexes, the estimated correlation times as calculated from viscosity data and the calculated quadrupole coupling constants. The radii of the complexes were calculated from the X-ray crystallographic data available and from measurements of molecular models in which the liganding oxygens were in van der Waals contact with the complexed cation. For purposes of comparison the coupling constant of the Na^+ solvated by CH_3OH was calculated on the assumption that the first solvation shell is fixed in relation to the cation and that rotation of the solvated ion is the only mechanism giving rise to the electric field fluctuations. Since quadrupole coupling constants depend on the symmetry of the electric field at the nucleus, Table II shows that the enniatin B and monactin complexes have a more symmetric distribution of ligand dipoles about the cation than the first solvation shell of CH_3OH, while the valinomycin, cyclohexyl ether, monensin, and nigericin complexes have a less symmetric arrangement. It might be expected that CH_3OH would furnish the most symmetric field at the cation nucleus. However, the assumption that the tumbling unit extends only to the first solvation shell may not be strictly valid. In this case τ_c would be larger and the coupling constant smaller.

Table III compares the quadrupole coupling constants for various Na^+ complexes taken from the literature. It is note-

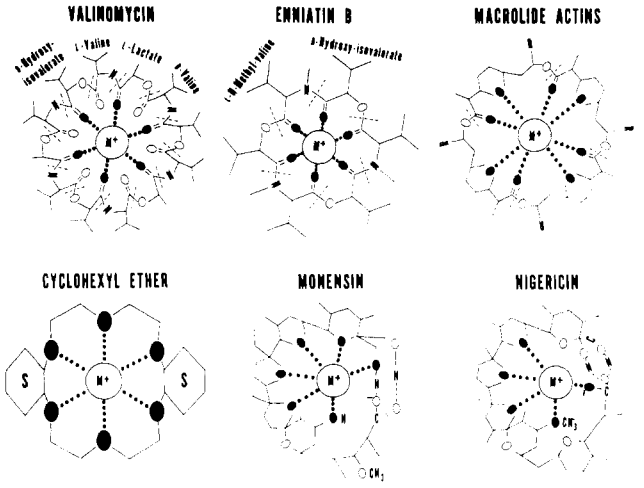


FIGURE 3: The primary structures of the ionophores studied. The oxygen atoms which have been shown by X-ray crystallographic analysis (nonactin, Kilbourn *et al.*, 1967; enniatin B, Dobler *et al.*, 1969; valinomycin, Pinkerton *et al.*, 1969; monensin, Agtarap *et al.*, 1967; nigericin, Steinrauf *et al.*, 1968) or by studies with molecular models, to be within van der Waals contact with the complexed cation are denoted by dark circles. In nonactin, monactin, dinactin, and trinactin, 0, 1, 2, and 3 methyls progressively replace protons at the groups designated "R".

worthy that the crystalline compounds, which have a relatively high degree of symmetry and whose bonds have only a very small amount of covalent character, have coupling constants comparable to those of the ionophore complexes, whereas the nitriloacetate complexes have significantly higher quadrupole coupling constants. Thus the environment of Na^+ in the ionophore complexes is much closer to the environment of Na^+ in crystalline salts than to the environment in aminocarboxylic acid complexes. Those complexes with lower coupling constants presumably have bonds with high ionic character. Since a degree of high symmetry in the configuration of complexing oxygens is a sufficient (but not necessary) condition for low electric field gradients at the center of the array, an inverse correlation between degree of symmetry as determined from the preferred or expected structure in solution and quadrupole coupling constant would be predicted.

The primary structures of the ionophore complexes are shown in Figure 3. Two of the antibiotics, monensin and nigericin, are similar in structure; complexing is achieved by ether, hydroxyl and carboxyl groups and there is no possible symmetry. Since the other ionophores and their complexes do have elements of symmetry, the monensin and nigericin complexes would be expected to have the largest coupling constants. Despite the apparent symmetry of its formal structure the cyclohexyl ether complex also has a large coupling constant but this can be explained (see below).

Nigericin. The nigericin complex in solution appears to have the lowest degree of symmetry. The X-ray data of Steinrauf *et al.* (1968) on the Ag^+ complex shows that the cation is complexed by five oxygen atoms. The distances from the cation vary between 2.25 and 2.70 Å (L. K. Steinrauf, personal communication). However, unlike the case of monensin, the charged carboxylate group is in van der Waals contact with the cation. The reasonable assumption can be made that the silver complex has the same conformation as the sodium complex since the two complexes have isomorphic crystal structures (Steinrauf *et al.*, 1968). Thus the resulting high electric field gradient would be expected to make the quadrupole coupling contant significantly larger than that of any of the other ionophores, as was observed.

TABLE III: Quadrupole Coupling Constants for $^{23}Na^+$ in Various Environments.

Material	Solvent	Quadrupole Coupling Constant (MHz)
RNA	H_2O	0.049–0.49[a]
Crystalline $NaNO_3$		0.334[b]
Crystalline $NaClO_3$		0.779[b]
Crystalline $NaBrO_3$		0.842[b]
CH_3OH	CH_3OH	0.870
Ionophores	CH_3OH	0.47–1.64
$HEEDTA^{3-}$	H_2O	~4[c]
NTA^{3-}	H_2O	~4[c]

[a] James and Noggle (1969a). [b] Gutowsky and Williams (1957) and Bernheim and Gutowsky (1960). [c] James and Noggle (1969b).

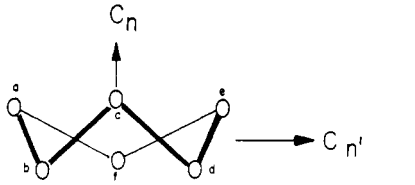
Monensin. The X-ray structure of the monensin complex with Ag^+ was determined by Agtarap *et al.* (1967), and by Pinkerton and Steinrauf (1970), who showed that the cation was surrounded by six oxygen atoms which vary from 2.4 to 2.7 Å from the center of the cation. A negatively charged carboxyl group 3.8 away was hydrogen bonded to two hydroxyls.

Crystals of the sodium complex of monensin are very similar to, although not isomorphic with, crystals of the potassium and silver complexes (Pinkerton and Steinrauf, 1970). This suggests that for monensin also the conformation of the antibiotic is the same in all these complexes.

Although monensin is very similar to nigericin in structure, the carboxylate group in the monensin complex is not within bonding distance of the central metal ion. In nigericin, on the other hand, the carboxylate oxygen is in van der Waals contact with the cation. Thus the single negative charge of the complexed monensin molecule is at a greater distance from the cation than in the nigericin complex and its contribution to the field gradient would be less. Furthermore, the coordination number of nigericin is five and of monensin is six which is also consistent with a larger field gradient for nigericin.

Cyclohexyl Ether. The cyclohexyl ether has six ether oxygen atoms which can be divided into two groups: (1) a group of two which are bonded only to ethyl groups, and (2) a group of four which are bonded to both ethyl and cyclohexyl groups. Models of this molecule show that the most effective contact between these oxygens and an alkali cation can be achieved when the molecule is folded into the puckered "crown" conformation. Since the two types of oxygen are not equivalent, the complex as depicted in Figure 4 could have only a twofold axis of symmetry (C_2) (Cotton, 1963) although it could have three vertical planes of symmetry (σ_v). To be weighed against these possibilities is the unknown cis–tran isomerization of the cyclohexyl rings and the possibility of ion pairing in solution.

Recent X-ray studies (Truter and Bright, 1970) have shown that the 18-crown-6-dibenzo ether complex with K^+ and Rb^+ exists, in the crystal, as an ion pair, with the anion in van der Waals contact with the cation on one side of the ring plane.



Valinomycin a=c=e b=d=f n= 3, n'= 1
Enniatin B a=c=e b=d=f n= 3, n'= 1
Cyclohexyl Ether a=b=d=e c=f n= 1, n'=2

FIGURE 4: The configuration of complexing oxygen atoms expected for the valinomycin-type ionophores. The figure represents schematically the possible spatial relationship of the complexing oxygen atoms in the backbones of valinomycin, enniatin, and the cyclohexyl ether. The solid lines represent the backbone of the molecule. The symmetry properties are listed in the figure. The cyclohexyl ether also has three planes of symmetry (σ) which include the C_n axis. Nonactin, which is not indicated in this figure has two twofold axes of symmetry (S_2).

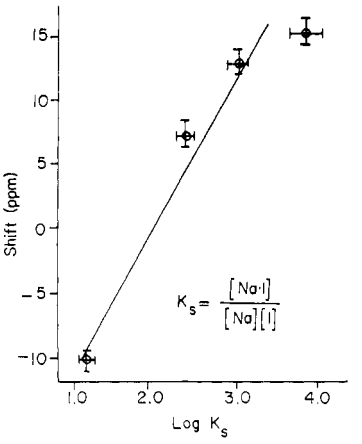


FIGURE 5: Correlation between the resonance positions of the ^{23}NaI complexes and log K_s in methanol. The resonance positions were taken from Table I. The following K_s values were used: monactin, $K_s = 1.1 \pm 0.1 \times 10^3$ (Pioda *et al.*, 1967); cyclohexyl ether, isomer Ia, 1.2×10^4 (Frensdorff, 1971) valinomycin, 14 ± 0.4 (Haynes *et al.*, 1969); 12 ± 17 (sic) (Wipf *et al.*, 1968); enniatin B, $2.4 \pm 0.5 \times 10^2$ (Wipf *et al.*, 1968).

Such ion pairing may occur with the cyclohexyl ether complex. For this, a conformation similar to that of the dibenzo ether which has no cis–trans forms would be required. The high value of the quadrupole coupling constant of the cyclohexyl ether complex could then be explained by the consequent large field gradients. It should be noted that models of enniatin B which has a small coupling constant show that the CNS^- could come into van der Waals contact with the complexed cation. However, the X-ray crystallographic data of Dobler *et al.* (1969) indicate that such close ion pairing does not occur between CNS^- and the K^+ enniatin B complex in the solid state. Thus the large quadrupole coupling constant of the cyclohexyl ether complexes, comparable in magnitude to those of the nigericin and monensin complexes, can be explained by asymmetric ion pairing.

Enniatin B. The proton nuclear magnetic resonance work and calculations of Shemyakin *et al.* (1969) and the x-ray crystallographic evidence of Dobler *et al.* (1969) indicate that the complex itself, as well as its carbonyl oxygens has a threefold axis of symmetry (C_3). As shown in Figures 3 and 4, the difference between the sets of amide carbonyl oxygens, a, c, and e, and ester carbonyl oxygens, b, d, and f, lies in the optical configuration of the residues. This would produce only a small field gradient along the C_n axis, and the complex would be expected to have a smaller coupling constant than the complexes of monensin and nigericin.

Valinomycin. The conformation of the K^+–valinomycin complex has been shown by Pinkerton *et al.* (1969) to be cylindrical with the six amide N—H and C=O groups involved in intramolecular hydrogen bonds *ca.* 3.0 Å from the cation and the six ester C=O groups involved in ion dipole interactions with the cation 2.8 Å away. This is almost exactly the same conformation as was reported for the K^+ complex in organic solvents independently by Ivanov *et al.* (1969). The K^+ complex depicted in Figure 4 has a threefold axis of symmetry (C_3) and its two types of ester carbonyl oxygens are placed in a configuration similar to that of enniatin B. Therefore a small field gradient, similar to that of enniatin B, would be expected which would result in a small coupling constant.

Although the hydrogen-bonded amide carbonyls were not thought by Ivanov *et al.* (1969) to be involved in interactions with the cation, the X-ray data of Pinkerton *et al.* (1969) show that the amide oxygens are only 3.0 Å from the center of the cation as compared to 2.8 Å for the ester carbonyl oxygens. Asymmetries in the placement of the amide groups in addition to the possible asymmetry along the C_n axis, would be sufficient to explain why the quadrupole coupling constant is greater than that of enniatin B.

Monactin. Kilbourn *et al.* (1967) have shown by X-ray crystallography that the K^+ complex of nonactin, a close analog of monactin, has a tetrahedrally symmetric placement of carbonyl oxygens, and a complimentary tetrahedral placement of ether oxygens. These two sets constitute a cubic array of oxygen atoms around the central cation. The molecule thus has a twofold alternating (S_2) axis of symmetry. If the structure of the Na^+ complex in solution were similar to the K^+ complex in the crystalline state, then a very low quadrupole coupling constant would be expected, as was observed.

Chemical Shift. Complexation with all the ionophores except valinomycin and monensin shifted the ^{23}Na resonance upfield with respect to ^{23}Na in CH_3OH. There is a good correlation between the chemical shift of the complex and the free energy for the complex formation in the same solvent as is shown in Figure 5. The correlation extends over a range of 27 ppm and has the form

$$\delta_{NaI} = \delta_0 + m \log K_s \qquad (5)$$

where δ_0 and m are constants and K_s is the stability constant for the complex. Since the total free energy of complex formation is presumably the sum of free-energy terms corresponding to a conformation change (Haynes *et al.*, 1969), dehydration of the cation (Haynes *et al.*, 1969), and an electronic interaction, this correlation suggests that the strength of complex formation is determined primarily by the electronic interaction between the sodium and the oxygen atoms of the ionophore.

Saika and Slichter (1954) formulated the screening constant, σ, which determines the position of the resonance, as the sum of various diamagnetic (shielding) and paramagnetic (deshielding) contributions. σ_d is the diamagnetic component,

$$\sigma = \sigma_p + \sigma_d + \sigma_o \qquad (6)$$

σ_p is the paramagnetic component, and σ_o represents the

contribution from other atoms in the molecule. For nuclei of higher atomic number such as ^{19}F and ^{31}P the paramagnetic term dominates. Studies of ^{23}Na chemical shifts in crystals and in solution indicate that this is true also for this nucleus. Two alternative interactions which determine the magnitudes of σ_p and σ_d for sodium have been proposed.

In a study of ^{23}Na resonances in different solvents, Bloor and Kidd (1968) found a correlation between the pK_a of the solvent and the chemical shift of the cation; the shifts were lower in ether solvents (good Lewis bases) than in ketone or amide solvents (poor Lewis bases) by about 10 ppm. This correlation was attributed to a small amount of covalent interaction between solvent and cation. Overlap between the s and p orbitals of the solvent molecules in the first hydration shell and the outer p orbitals of the sodium would permit partial electron transfer to the cation. A calculation showed that if complete transfer of an electron occurred, σ_d would change by +10 ppm and σ_p would change by −270 ppm.

This electron transfer mechanism would offer, through covalent interaction, an additional means of stabilization of the complex. However, it does not account satisfactorily for the ionophore–complex shifts. Examination of the structures of the complexes in Figure 3 shows that carboxyl, carbonyl, hydroxyl, and ether oxygens are utilized as ligands. The shift for the cyclohexyl ether complex is 25 ppm higher than that of the valinomycin complex, although the ether oxygens of this compound are better Lewis bases than the carbonyl oxygens of valinomycin. Furthermore, monensin and nigericin, which have the highest Lewis basicity (by virtue of their carboxylate groups) have only intermediate chemical shifts. Thus the Lewis base character of the ligand oxygens in the ionophore is not the primary determinant of the strength of interaction with the cation.

A second type of paramagnetic interaction was proposed by Kondo and Yamashita (1959), who suggested that the chemical shift of cations and anions in alkali halide crystals arises from the overlap repulsive forces between the closed shell of the ions. These forces cause excitation of electrons to higher states, the result again being a decrease in the shielding of the nucleus. The calculated shift of $^{87}Rb(H_2O)_6^+$ relative to the unhydrated ion (Ikenberry and Das, 1965), obtained with this theory, was in good agreement with an experimentally derived value (Baron, 1963). Deverell and Richards (1966) studied the resonances of alkali metal ions in solution and by an extension of Kondo and Yamashita's treatment, interpreted the chemical shift as arising from similar interactions between cation and anion or, in dilute solution, between cation and solvent.

If overlap repulsive interactions were operative in these ionophore complexes the shift would be determined by the geometry of the complex and the tightness of the fit of the cation in the ionophore cage. The X-ray data have shown variations in the distances between the cation and the ligand oxygen atoms of the different complexes but these variations are small (2.45–2.80 Å) and no definite trend can be discerned. It is noteworthy that the cation–oxygen center-to-center distances in the ionophore complexes are greater than the sum of the crystallographic radii (2.13 Å for K^+, 1.93–2.05 Å for Ag^+, Lange, 1961), indicating that covalent interaction would not be expected. Less orbital repulsion might occur in the ionophore complexes than in the first CH_3OH solvation shell where there would be less steric restraint. This is consistent with the observation that the resonance positions of most of the ionophore complexes are shifted upfield relative to the solvent.

The fact that the quadrupole coupling constants in these complexes are similar in magnitude to those of the crystalline Na salts and that the chemical shift of the ^{23}Na resonance in these salts appears to arise from orbital repulsion, suggests that this interaction is present in the ionophore complexes. Thus the stability of the complexes would seem to arise from ion dipole interactions (plus ion–ion electrostatic attraction in the cases of nigericin and monensin), and the complexes are not further stabilized by covalent interaction.

Although changes in the paramagnetic term, σ_p, determine the observed variation in σ, the contribution of σ_d and σ_o would seem to be small and essentially not variable. Through the series of complexes σ_d itself would remain essentially constant, but the contribution from the other atoms in the molecule, σ_o, could vary. There are six to eight oxygens surrounding the complexed Na^+ and the summation of their effect might be appreciable, but since the calculation for the paramagnetic shift of hydrated $Rb(H_2O)_6^+$ agrees well with experiment, this correction appears to be small.

A second possible component of σ_o would arise from the magnetic anisotropy of neighboring atoms or groups (notably the amide or carbonyl group). The effect can be either diamagnetic or paramagnetic depending on the orientation of the group or atom with respect to the cation. However, since the maximum paramagnetic effect of carbonyl groups on neighboring protons is a shift of about −2 ppm, this does not seem to be a major factor. The magnetic anisotropy of the cyclohexyl ether oxygen bonds would be expected to be much less than that of a carboxyl group.

It is appropriate to point out that the data on the ionophore complexes might constitute a very good basis for a theoretical evaluation of the factors determining sodium resonance shifts since these complexes probably have more rigid, symmetric, and predictable geometries than other sodium complexes found in solution.

Exchange Kinetics. When a given nucleus exchanges between two sites, i and j, with different chemical shifts it is possible to deduce information about the residence times at i and j, τ_i and τ_j, respectively, by comparing the widths or positions of the resonance under the conditions of exchange to those observed in absence of exchange (Pople *et al.*, 1959). For the case of equal populations of the sites and small $\Delta\nu_{1/2}$ values for the resonances, a single fused resonance peak is obtained for the condition

$$\tau_i = \tau_j = \frac{\sqrt{2}}{\pi\nu_0(\delta_i^0 - \delta_j^0)} \tag{7}$$

where δ_i^0 and δ_j^0 represent the positions of the resonances of sites i and j, respectively, in the absence of exchange. This expression was used with monactin at the point in the titration, where $P_{NaI} = P_{Na}$, to calculate the lower limit for k_{off} for the reaction

$$Na^+ + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} NaI \tag{8}$$

where

$$k_{off} = -\frac{\frac{d[NaI]}{dt}}{[NaI]} = \frac{1}{\tau_{NaI}} \tag{9}$$

TABLE IV: Calculated Limits of the Rate Constants k_{off} and k_{on} in Methanol.

Ionophore	$\nu_0(\delta_i^0 - \delta_j^0)$ (cps)	P_{NaI}	$1/\tau$ (sec^{-1})	k_{off} (sec^{-1})	k_{on} (M^{-1} sec^{-1})
Cyclohexyl ether[a]	187	0.18	$>1.17 \times 10^3$	$>9.6 \times 10^2$	$>4.6 \times 10^6$
Enniatin B	58	0.15	$>3.64 \times 10^2$	$>3.1 \times 10^2$	$>7.5 \times 10^4$
Valinomycin	225	0.18	$>1.41 \times 10^3$	$>1.2 \times 10^3$	$>1.7 \times 10^4$
Monactin[b]	168	0.50	$>1.0 \times 10^3$	$>1.0 \times 10^3$	$>1.1 \times 10^6$

[a] k_{on} calculated from K_s of I_B isomer (a cis-trans isomer). [b] Value of k_{off} calculated from eq 7.

Since eq 7 requires that the populations of the two sites be approximately equal, which was not true with some of the systems in the present study, eq 10 (modified from Johnson, 1965) for the position of the dominant i resonance was used. δ_i

$$\delta_i - \delta_i^0 = P_j \frac{(\delta_i^0 - \delta_j^0)}{P_i} \left[\frac{1}{1 + 4\pi^2\nu_0^2(\delta_i^0 - \delta_j^0)^2\tau^2} \right] \quad (10)$$

is the position of the i resonance in the presence of exchange, P_i and P_j are the fractional populations of the two sites, and τ is given by

$$\frac{1}{\tau} = \frac{1}{\tau_i} + \frac{1}{\tau_j} \quad (11)$$

Equations 10 and 1 are equivalent in the limit of fast exchange ($\tau^{-1} \gg 2\pi\nu_0(\delta_i^0 - \delta_j^0)$ and $P_i \gg P_j$. In the case of slow or intermediate exchange rates, eq 10, predicts that the shift of the i resonance due to exchange of i with the small j population will be less than that given by eq 1 by the factor $1/[1 + 4\pi^2\nu_0^2(\delta_i^0 - \delta_j^0)^2\tau^2]$.

In the titrations of 100 mM NaCNS with ionophores, no significant deviation of the titration curve from the linear relationship of eq 1 was observed, indicating large values of $1/\tau$. However, the lower limits of $1/\tau_{NaI}$ (*i.e.*, k_{off}) were established by making use of the maximum error in the determination of the change in δ_{Na} with P_{NaI} as shown in Figure 2 for small P_{NaI} values. Analysis of the titration data for $P_{NaI} \simeq 0.1$ or 0.2 showed that the value of $\delta_{Na} - \delta_{Na}^0$ could not be less than half the value predicted by eq 1, establishing a limit, $1/\tau > 2\pi\nu_0(\delta_i^0 - \delta_j^0)$. The lower limits of k_{off} were calculated from this value by means of eq 11 and 10 and the value of P_{NaI}. The results are given in Table IV for all ionophores except monensin and nigericin which exhibited $\delta_i^0 - \delta_j^0$ values too small for this procedure to be applicable.

The calculated high rates of exchange in the polar solvent CH_3OH are consistent with a prior observation of rapid exchange for valinomycin and nonactin with K^+ in the polar solvent 80% methanol–20% $CDCl_3$ by use of ^1H nuclear magnetic resonance (Haynes *et al.*, 1969; Pressman and Haynes, 1969). The results support the carrier theory of ionophore action (Pressman *et al.*, 1967; Pressman and Haynes, 1969).

The following picture of ionophore action emerges from this study and from previous studies. The ionophores appear to function as mobile carriers in biological membranes by virtue of their extreme lipophilicity and their high complexing affinity. For the valinomycin-type ionophores, complex formation is achieved by the ionophore surrounding the cation with an extremely symmetrical array of electronegative oxygen atoms. The strength of complex formation appears to lie in the electronic interaction (ion–dipole and ion–ion) between the cation and the complexing oxygens. The complexing and dissociation reactions have high rate constants, consistent with the rapid turnover demanded by the carrier mechanism of action.

Acknowledgments

The authors acknowledge many helpful discussions with Drs. George Reed and John S. Leigh. They also wish to thank D. Mildred Cohn and Dr. Leigh for their assistance in carrying out this investigation.

References

Abragam, A. (1961), Principles of Nuclear Magnetism, London, Oxford University.

Andreoli, T., Tieffenberg, M., and Tosteson, D. (1967), *J. Gen. Physiol. 50*, 2527.

Agtarap, A., Chamberlin, J. W., Pinkerton, M., and Steinrauf, L. (1967), *J. Amer. Chem. Soc. 89*, 5737.

Baron, R. (1963), *J. Chem. Phys. 38*, 173.

Bernheim, R. A., and Gutowsky, H. S. (1960), *J. Chem. Phys. 32*, 1072.

Bloembergen, N., Purcell, E. N., and Pound, R. V. (1948), *Phys. Rev. 73*, 679.

Bloor, E. G., and Kidd, R. G. (1968), *Can. J. Chem. 46*, 3425.

Chappell, J. B., and Crofts, A. R. (1965), *Biochem. J. 95*, 393.

Cotton, F. A. (1963), Chemical Applications of Group Theory, New York, N. Y., Interscience.

Cope, F. W. (1967), *J. Gen. Physiol. 50*, 1353.

Deverell, C., and Richards, R. E. (1966), *Mol. Phys. 10*, 551.

Dobler, M., Dunitz, J. D., Krajewski, J. (1969), *J. Mol. Biol. 42*, 603.

Eisenman, G., Ciani, S., and Szabo, G. (1969), *J. Membrane Biol. 1*, 346.

Frensdorf, H. K. (1971), *J. Amer. Chem. Soc. 93* (in press).

Gutowsky, H. S., and Williams, G. A. (1957), *Phys. Rev. 105*, 464.

Haynes, D. H., Kowalsky, A. and Pressman, B. C. (1969), *J. Biol. Chem. 244*, 502.

Ikenberry, D., and Das, T. P. (1965), *J. Chem. Phys. 43*, 2199.

Ivanov, V. T., Laine, I. A., Abdulaev, N. C., Sengavina, L. B., Popov, E. M., Ovchinnikov, Y. A., and Shemyakin, M. M. (1969), *Biochem. Biophys. Res. Commun. 34*, 803.

James, T. L., and Noggle, J. H. (1969a), *Proc. Nat. Acad. Sci. U. S. 62*, 644.

James, T. L., and Noggle, J. H. (1969b), *J. Amer. Chem. Soc. 91*, 13.

Jardetzky, O., and Wertz, J. E. (1960), *J. Amer. Chem. Soc. 82*, 318.
Johnson, C. S. (1965), *Advan. Magn. Resonance 1*, 33.
Kilbourn, B. T., Dunitz, J. D., Pioda, L. A. R., and Simon, W. (1967), *J. Mol. Biol. 30*, 559.
Kondo, J., and Yamashita, J. (1959), *J. Phys. Chem. Solids 10*, 245.
Lange, N. A., Ed. (1961), *in* Handbook of Chemistry, 10th ed, New York, N. Y., McGraw-Hill.
Mueller, P., and Rudin, D. O. (1967), *Biochem. Biophys. Res. Commun. 26*, 398.
Ovchinnikov, Y. A., Ivanov, V. T., Evstratov, A. V., Bystrov, V. F., Abdullaev, N. D., Popov, E. M., Lipkind, G. M., Arkhipora, S. F., Efremov, E. S., and Shemyakin, M. M. (1969), *Biochem. Biophys. Res. Commun. 37*, 668.
Pedersen, C. J. (1967), *J. Amer. Chem. Soc. 89*, 7017.
Perrin, F. (1936), *J. Phys. Radium 7*, 1.
Pinkerton, M., and Steinrauf, L. K. (1970), *J. Mol. Biol. 49*, 533.
Pinkerton, M., Steinrauf, L. K., and Dawkins, P. (1969), *Biochem. Biophys. Res. Commun. 35*, 512.
Pioda, L. A. R., Wachter, H. A., Dohner, R. E., and Simon, W. (1967), *Helv. Chim. Acta 50*, 1373.
Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill.
Pressman, B. C., Harris, E. J., Jagger, W. S., and Johnson, J. H. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1949.
Pressman, B. C., and Haynes, D. H. (1969), *in* The Molecular Basis of Membrane Function, Tosteson, D. C., Ed., Englewood Cliffs, N. J., Prentice-Hall, p 221.
Rotunno, C. A., Kowalewski, V., and Cereijido, M. (1967), *Biochim. Biophys. Acta 135*, 170.
Saika, A., and Slichter, D. P. (1954), *J. Chem. Phys. 22*, 26.
Shemyakin, M. M., Ovchinnikov, Y. A., Ivanov, V. T., Antonov, V. K., Shkrob, A. M., Nikhaleva, I. I., Evstratov, A. V., and Malenkov, G. G. (1967), *Biochem. Biophys. Res. Commun. 29*, 834.
Shimizu, H. (1962), *J. Chem. Phys. 37*, 765.
Steinrauf, L. K., Pinkerton, M., and Chamberlin, J. W. (1968), *Biochem. Biophys. Res. Commun. 33*, 29.
Truter, M. R., and Bright, D. (1970), *Nature (London) 225*, 176.
Wipf, H. K., Pioda, L. A. R., Stefanac, A., and Simon, W. (1968), *Helv. Chim. Acta 51*, 377.

On the Identification of Lamellar and Hexagonal Phases in Negatively Stained Phospholipid–Water Systems*

David O. Tinker† and L. Pinteric

ABSTRACT: The principles of projective geometry are used to predict the features of electron micrographs of negatively stained liquid crystals composed of lamellar and two hexagonal phases. A well-characterized phosphatidylethanolamine from pig erythrocytes was hydrated and the resulting liquid crystals negatively stained with phosphotungstic acid and examined at high magnification in the electron microscope. Three types of liquid crystal were identified in these preparations: lamellar (bimolecular lipid leaflets spaced at 46 Å units), H_I (lipid cylinders, diameter 40 Å units, hexagonally packed in an aqueous matrix with a spacing of 61 Å units), and H_{II} (aqueous cylinders, diameter 26 Å units, hexagonally packed in a lipid matrix with a spacing of 56 Å units). All the theoretically predicted features were evident in the micrographs.

In the early stages of hydration, tubules of irregular diameter were seen to grow out of the unstructured lipid particles, later attaining a more uniform diameter and aligning regularly. These tubules evidently give rise to the H_I phase. Tubular structures and H_I phase were evident in the micrographs of Korman *et al.* (Korman, E. F., de Pury, G., Asai, J., Allman, D. W., Kopaczky, K., and Green, D. E. (1970), *Biochemistry 9*, 1318), but the interpretations of their results given by these authors are refuted.

Naturally occurring phospholipids have been shown by X-ray diffraction techniques to form a variety of liquid crystalline phases in aqueous systems (Luzzatti *et al.*, 1960; Luzzatti and Husson, 1962; Luzzatti and Spegt, 1967; Luzzatti *et al.*, 1968a–d; Rand and Luzzatti, 1968; Reiss-Husson, 1967; Small and Bourges, 1966; Small 1967). The most commonly observed phases are the lamellar phase (L) and the two hexagonal phases H_I and H_{II}. Diagrammatic representations of these structures are shown in Figures 1–3.

The structural conclusions drawn as a result of the X-ray studies have been confirmed (at least in part) by electron microscopic investigations. Thus Bangham and his colleagues (Bangham and Horne, 1964; Papahadjopoulos and Miller, 1967) presented convincing evidence for the lamellar structure in negatively stained preparations of hydrated liquid crystals ("liposomes") of a variety of phospholipids. Recently Junger and Reinauer (1969) published high resolution micrographs of negatively stained phosphatidylethanolamine–water preparations in which a hexagonal phase (probably H_{II}) was correctly identified.

* From the Department of Biochemistry, University of Toronto, Toronto 5, Canada. *Received April 20, 1970.* This research was supported by Grants MA-2378 and MA-2355 from the Medical Research Council of Canada.

† To whom correspondence should be addressed.