# Ion Pairing of Ionophore Potassium Complexes. Nuclear Magnetic Resonance Studies with Paramagnetic Anions†

D. J. Boone‡ and Arthur Kowalsky*

ABSTRACT: Ion pairing of positively charged valinomycin–alkali cation complexes with the paramagnetic anions $CoBr_4^{2-}$, $Co(SCN)_4^{2-}$, and $Co(CH_3COCHCOCH_3)_3^-$ has been studied in solvents of varying polarity by observation of the $^1H$ nuclear magnetic resonance spectrum of the valinomycin. Cation complexes of dicyclohexyl-18-crown-6 and dibenzyl-18-crown-6 ethers were also studied with these anions and with $Cr(NH_3)_4(SCN)_2^-$. Pseudocontact shifts were observed in the valinomycin systems while extensive broadening but no shifts were observed in the crown ether systems. Both observations indicate extensive ion pairing. For the latter systems, the ion pairing was dependent on solvent polarity. Assuming models of trigonal or tetragonal symmetry, ion pair distances were calculated. The models with trigonal symmetry yielded distances consistent with distances derived from molecular models. The data suggest that although the cobalt anion may be distorted in the collision to form the ion pair complex, the valinomycin–alkali cation complex is in general resistant to distortion.

The many studies of valinomycin, its cation complexes and their involvement with ion transport (Pressman, 1970; Haynes *et al.*, 1969, 1971; Haynes, 1972) have done much to elucidate the mechanism of valinomycin-facilitated cation transport across membranes. But although crystal structure studies of the solid potassium aurichloride complex have fixed the geometry of the complex in the solid state and have delineated the coordination sites of the cation (Pinkerton *et al.*, 1969), and although nuclear magnetic resonance ($^1H$ and $^{23}Na$) and other spectroscopic techniques have been used to deduce the geometry of the complex in solution (Ivanov *et al.*, 1969) and characterize it structurally and dynamically (Haynes *et al.*, 1971; Haynes, 1972) the actual state of the complex in lipid solvents or in mixtures of lipid and polar solvents is still not clear.

Since the valinomycin–cation complex is positively charged, it may ion pair with a counteranion, either strongly or weakly, to form a dynamic system of charged and neutral species. This is an important consideration since ion pairing offers a means, not only of cation transport by the valinomycin, but also of anion transport.

At the outset there would appear to be two distinct modes for anion translocation: one involving anion for anion exchange either by a chemiosmotic or electrogenic or electrophoretic movement, and the other by ion pair movement. The concept of ion pair movement involving a ionophore complex is a particularly appealing one since an ionophore complex made up of a lipid compatible positive and negative charge can freely exist in the membrane and assist in the transit of the anion across the lipoidal region of the membrane (Pressman and Haynes, 1969). Ion pair formation would serve the purpose of maintaining charge neutrality at the exchange interface avoiding the coulombic interactions which would otherwise be generated in anion for anion exchange. Regions of low dielectric constant such as the hydrophobic surface of a membrane favor ion pair formation.

Magnetic resonance methods have been used to study ion pair formation in both aqueous (Alei, 1964; Gasser and Richards, 1959; Larsen and Wahl, 1965; Larsen, 1966; Walker and Drago, 1968) and nonaqueous solvents (Buchson and Smith, 1964; LaMar, 1964). In these studies paramagnetic anions were used and ion pair formation was detected by either a change in the position of a specific resonance (pseudocontact shifts) (McConnell, 1956; McConnell and Chesnut, 1959), or by a change in the width of specific resonances, both effects arising from the association of the paramagnetic anion with the cation being observed. We have used this approach to determine whether or not ion pairing between potassium complexes of valinomycin and of two crown ethers does occur with paramagnetic anions. Our original hope was that ion pairing could be observed and, since the maintenance of a flux of anionic metabolites is of great importance to functioning mitochondrial systems, that a relative order of anion affinities for valinomycin–cation complexes could be established.

## Experimental Section

*Materials.* Valinomycin was obtained from Calbiochem. Dicyclohexyl-18-crown-6 and dibenzo-18-crown-16 ethers were obtained through the courtesy of Dr. Frensdorff of the E. I. DuPont DeNemours Co. Isotopic solvents, deuteriochloroform, methanol-$d_4$, and acetone-$d_6$ were obtained from Merck, Sharp and Dohme and were used without further purification. Potassium tetrathiocyanatocobalt(II) trihydrate was prepared by mixing stoichiometric amounts of cobaltous thiocyanate and potassium thiocyanate in distilled water, evaporating off the solvent, and recrystallizing from methanol. Potassium reineckate ($KCr(NCS)_4(NH_3)_2(H_2O)$) was prepared by crystallization from an aqueous solution of ammonium reineckate (K&K Chemicals) which had been saturated with potassium bromide at 50°. Potassium tetrabromocobalt(II) trihydrate was prepared by mixing stoichiometric amounts of cobaltous bromide (Alpha Inorganics) and potassium bromide in 50:50 methanol–water. The product was recrystallized from

† From the Department of Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461. *Received June 25, 1973.* This investigation was supported by Grants AM 15251 and P07 RR00636 from the National Institutes of Health, U. S. Public Health Service. A preliminary account of this research has been presented (Boone and Kowalsky, 1972).

‡ Present address: Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52240.

methanol. Tetra-*n*-butylammonium tetrabromocobalt(II) bromide was prepared by refluxing 2 mol of $N(C_4H_9)_4Br$ (Eastman Organics) with 1 mol of cobaltous bromide in acetone. The solution was evaporated to dryness and the solid was recrystallized from acetone. Tetra-*n*-butylammonium tris(acetylacetonato)cobalt(II), $(n\text{-}C_4H_9)_4NCo(acac)_3$,[1] was obtained according to the procedure of Horrocks *et al.* (1966).

Inorganic salts were analyzed for potassium, cobalt, or chromium using a Varian AA-5 atomic absorption spectrometer. Analyses for bromide were performed using the method described by Vogel (1961). *Anal.* Calcd for $K_2Co(NCS)_4 \cdot 3H_2O$: Co, 14.0; K, 18.5; NCS, 55.0. Found: Co, 13.9; K, 17.8; NCS, 50. Calcd for $KCr(NCS)_4(NH_3)_2 \cdot H_2O$: Cr, 13.9; K, 10.4. Found: Cr, 13.4; K, 10.0. Calcd for $K_2CoBr_4 \cdot 3H_2O$: Br, 63.0. Found: Br, 60.5. Calcd for $(N(C_4H_9)_4)_2CoBr_4$: Br, 36.6. Found: Br, 35.5.

Solutions were analyzed following an experiment using the following absorptivities determined in acetone: $Co(NCS)_4^{2-}$, $\epsilon_{662\ nm} = 1830\ M^{-1}\ cm^{-1}$; $CoBr_4^{2-}$, $\epsilon_{622\ nm} = 216\ M^{-1}\ cm^{-1}$; $Co(acac)_3^-$, $\epsilon_{500\ nm} = 49\ M^{-1}\ cm^{-1}$ (Horrocks *et al.*, 1966).

*Methods.* Valinomycin or crown ether $^1H$ nmr spectra were obtained with a Varian HA-100 spectrometer operating at the ambient probe temperature of 30°; a Varian A-60 spectrometer operating at an ambient probe temperature of 35° was used to record some spectra of the crown ethers and in later studies spectra were also obtained on a Jeol PS-100 spectrometer. Chemical shifts were measured relative to either tetramethylsilane or hexamethyldisiloxane added as an internal reference amounting to about 5% of the sample volume in 5-mm sample tubes. Chemical shifts were determined to within ±0.1 Hz with the HA-100 and PS-100 and to ±0.5 Hz with the A-60 spectrometer.

In general the experimental procedure was as follows: (a) a $^1H$ nmr spectrum of the deuterated solvent to be used was obtained checking for chemical shifts and line widths of protonated solvent impurities; (b) valinomycin or crown ether was dissolved in the deuterated solvent and its spectrum was recorded; (c) the valinomycin or crown ether was then titrated with a diamagnetic potassium salt and a spectrum was obtained after each incremental addition; (d) the potassium complex of valinomycin was then titrated with a solution containing the paramagnetic anion and a spectrum was recorded after each addition. For the crown ethers, either a potassium–crown ether complex or the crown ether itself was titrated with the paramagnetic anion. Valinomycin and crown ether concentrations were fixed at between 0.04 and 0.05 M. Potassium bromide, potassium thiocyanate, and potassium benzoate were added in a slight excess or an equivalent amount to the valinomycin or crown ether. Maximum mole ratios of inorganic anion to valinomycin were as follows: (a) $K_2CoBr_4 \cdot 3H_2O$ in $(CD_3)_2CO$, 2.88; (b) $N(C_4H_9)_4Co(acac)_3$ in $CDCl_3$, 2.5; (c) $(N(C_4H_9)_4)_2CoBr_4$ in $CDCl_3$, 0.88; (d) $(N(C_4H_9)_4)_2CoBr_4$ in 50:50 $CDCl_3$–$(CD_3)_2CO$, 1.05; (e) $K_2Co(NCS)_4 \cdot 3H_2O$ in $(CD_3)_2CO$, 2.7; (f) $K_2Co(NCS)_4 \cdot 3H_2O$ in 50:50 $CDCl_3$–$(CD_3)_2CO$, 3.0. The maximum ratios of inorganic anion to dibenzo-18-crown-6 ether were: (a) $K_2Co(NCS)_4 \cdot 3H_2O$ in 50:50 $CDCl_3$–$CD_3OD$, 0.3; (b) $(N(C_4H_9)_4CoBr_4$ in 80:20 $CDCl_3$–$CD_3OD$, 1.0; (c) $KCr(NCS)_4(NH_3)_2 \cdot H_2O$ in 50:50 and 60:40 $CDCl_3$–$CD_3OD$, 0.004 and 0.045, respectively. Maximum mole ratios with the dicyclohexyl-18-crown-6 ether were: (a) $K_2Co(NCS)_4 \cdot 3H_2O$ in 50:50 $(CD_3)_2CO/CDCl_3$, 0.09; (b) $KCr(NCS)_4(NH_3)_2 \cdot H_2O$ in 20:80, 50:50, and 60:40 $CDCl_3$–$CD_3OD$, 0.065, 0.010, and 0.006, respectively.

The paramagnetic anions used were those whose ligands did not readily exchange in the solvents studied. The effect of such solvent substitution would be to reduce the effective negative charge on the anion, which, in the limit, would eliminate its ability to form ion pairs with valinomycin complexes. A change in the coordination number or geometry of the paramagnetic anion resulting from such substitution will also have a drastic effect on the possibility of $^1H$ nmr observation of ion pair formation since the magnitude of the pseudocontact shift is dependent upon the magnetic moment of the anion and its anisotropy. Tetraethylammonium tetrachloronickel(II) when dissolved in 80:20 $CDCl_3$–$CD_3OD$ provides an example of these effects. On addition of the paramagnetic, blue, tetrahedral $NiCl_4^{2-}$ anion to the solvent, the color of the anion changes instantly to a dark green indicative of $Ni(CD_3OD)_6^{2+}$, which, in addition to being a cation, is also diamagnetic. In order to avoid as much as possible both of these effects, substitution inert $Cr(NCS)_4(NH_3)_2^-$ (Adamson, 1958) or tetrahedral cobalt(II) complexes of moderate stability were used. The lability of cobalt(II) complexes toward substitution has been well documented (Hammes and Morrell, 1969; Sutin and Nancollas, 1964). Octahedral cobalt(II) complexes of methanol or water are pink with low molar absorptivities, whereas tetrahedral cobalt(II) complexes with $Br^-$ or $NCS^-$ are blue to violet with relatively high molar absorptivities. Throughout all experiments solutions of all cobalt(II) complexes used in this work maintained the color and intensity of color associated with the established tetrahedral configuration of the crystalline cobalt salt indicating that $CoBr_4^{2-}$ and $Co(NCS)_4^{2-}$ were the predominant species in solution. Selection was also biased toward cobalt(II) complexes for which ion pair nmr studies had been previously carried out and pseudocontact shifts observed (Walker and Drago, 1968; Horrocks *et al.*, 1966). Low solubility of inorganic salts in these nonpolar solvents produced a severe limit to the compounds and solvents which could be used.

## Results

*Valinomycin.* The $^1H$ nmr spectra of valinomycin in the presence of diamagnetic potassium salts, when compared to the spectrum of valinomycin alone in the same solvent, demonstrated upfield resonance shifts for the $C^\alpha$ and $C^\beta$ protons while the lactate methyl resonance was shifted downfield. These changes in resonance positions occurred regardless of the diamagnetic anion present ($Br^-$, $NCS^-$, $C_6H_5CO_2^-$, $CH_3CO_2^-$), or the solvent system used. Such perturbations of these positions are indicative of potassium ion complex formation (Haynes *et al.*, 1969). Complexing of potassium by valinomycin was shown to be essentially complete whenever the mole ratio of potassium ion to valinomycin was 1.0 or greater since no additional chemical shift changes were observed beyond this ratio.

When the thiocyanate salt of $Kval^+$ was titrated with $K_2CoBr_4$ in $(CD_3)_2CO$ solvent, some of the proton resonances of the valinomycin shifted again as shown in Figure 1. Similar results were obtained with $K_2Co(NCS)_4$, $(N(C_4H_9)_4)_2CoBr_4$, and $N(C_4H_9)_4Co(acac)_3$ in this solvent, $CDCl_3$, and mixtures of the two. Both $C^\alpha$- and $C^\beta$-proton resonances shifted further upfield. Shifts for the $\alpha$-proton resonances were two to five times greater than for the $\beta$-proton resonances. In general the largest observed shift was for $C^\alpha$—H resonance of the valine residues. No shift in the resonance positions of the methyl

[1] Abbreviations used are: val, valinomycin; $Kval^+$, valinomycin–potassium complex; DCHE, dicyclohexyl-18-crown-6 ether; DBCE, dibenzo-18-crown-6-ether; acac, acetylacetone.

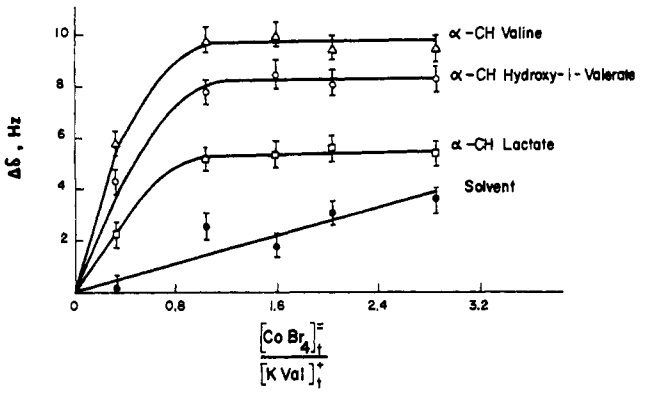


FIGURE 1: Effect of $K_2CoBr_4$ on the resonance positions of KvalBr protons: solvent, $(CD_3)_2CO$. The solvent resonance arises from normal acetone present as an impurity in deuterated acetone. Although not shown here, the solvent resonance position continues to change as the concentration of paramagnetic anion is increased further.

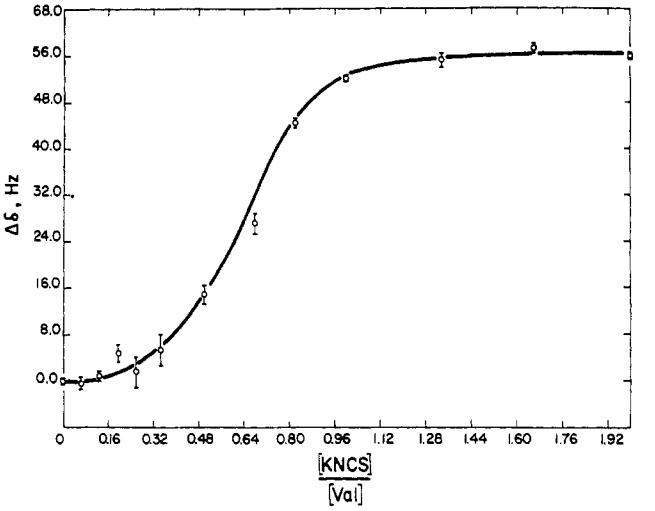


FIGURE 2: Resonance positions of valinomycin peptide protons in the presence of varying amounts of KNCS: solvent, $(CD_3)_2CO$; valinomycin concentration, 0.045 M.

protons or of the N—H protons was observed. Thus, in systems of valinomycin, KSCN, and excess $K_2Co(SCN)_4$ or valinomycin, KBr, and excess $K_2CoBr_4$ in acetone or chloroform–acetone mixtures, the changes in position of the methyl resonances seemed to be random; their averages were less than 0.5 Hz, the experimental error. The change in the position of the amide resonances in these systems was also within the experimental error. Proton resonances for the solvent (as the proton containing impurity in the deuterated compound) shifted upfield linearly as a function of the amount of paramagnetic anion added, but in every case solvent shifts were much less than those for the $\alpha$-proton resonances of the $Kval^+$. These shifts for the $\alpha$-proton resonances followed titration behavior, increasing to a maximum value as the paramagnetic anion was added and then remaining constant beyond a certain mole ratio as is illustrated in Figure 1. The maximum shifts observed with the various cobalt anions for individual $C^\alpha$—H proton resonances are listed in Table I. The variation with anion is expected since the shift depends not only on the spectroscopic properties of the anion and its ligands but also on the geometry of the ion pair complex (see below). The mole ratio, paramagnetic anion to $Kval^+$ at which the maximum shift was obtained also varied from system to system and may be indicative of different ion pair equilibria, of the presence of several species of various states of aggregation or of different conformational forms of the valinomycin complex. Analysis of the data based on a single complex with either a 1:1 or a 2:1 stoichiometry could not account for the experimentally observed titration curves. That more than one conformational form of the complex may exist in solution is suggested by a comparison of the plots of various valinomycin resonance positions as a function of valinomycin complexed by potassium in the absence of any paramagnetic anion. The resonance positions of the lactate methyl protons and the $C^\alpha$ protons are a linear function of the ratio KNCS:val and are constant at mole ratios greater than one (Haynes *et al.*, 1969). However, the resonances of the peptide nitrogen protons exhibit a different behavior; a plot of their position against the ratio KNCS:val shows sigmoidal characteristics (Figure 2).

The pseudocontact shifts observed with the thiocyanate complex anions were much less than those observed with the bromide anions. Because of this, the effect of variation of such parameters as solvent composition on the magnitude of the shift with $K_2Co(NCS)_4$ was difficult to assess (Table I). A decrease in solvent dielectric constant in changing from acetone ($\epsilon$ 20.7) to 50:50 $CDCl_3$–$(CD_3)_2CO$ ($\epsilon$ 13) might have been expected to increase the extent of ion pair formation and therefore the maximum pseudocontact shift. In fact, a slight decrease in the maximum shift, just outside of experimental error was observed (Table I). In another system however, $KvalC_6H_5CO_2 + N(C_4H_9)_4Co(acac)_3$, the maximum shifts did increase when the solvent was changed from 50% acetone–chloroform to pure chloroform. However, these differences (and nondifferences) may just be a reflection of the fact that

TABLE I: Pseudocontact Shifts for Valinomycin Ion Pairs.[a]

| System | Solvent | $C^\alpha$H Valine | $C^\alpha$H Hydroxy-isovalerate | $C^\alpha$H Lactate |
|---|---|---|---|---|
| $KvalNCS + K_2Co(NCS)_4$ | $(CD_3)_2CO$ | 3.6 | 2.5 | 2.5 |
| $KvalNCS + K_2Co(NCS)_4$ | 50:50 $CDCl_3$–$(CD_3)_2CO$ | 2.8 | 1.3 | 1.9 |
| $KvalBr + K_2CoBr_4$ | $(CD_3)_2CO$ | 9.8 | 7.5 | 5.5 |
| $KvalC_6H_5CO_2 + (N(C_4H_9)_4)_2CoBr_4$ | 50:50 $CDCl_3$–$(CD_3)_2CO$ | 4.8 | 4.8 | 4.8 |
| $KvalNCS + (N(C_4H_9)_4)Co(acac)_3$ | $CDCl_3$ | *b* | 6.2 | 3.0 |

[a] All shifts are in Hz upfield from the diamagnetic potassium–valinomycin complex in the same solvent. All values are accurate to ±0.5 HZ or less. [b] A change in coupling constants is evident for the $C^\alpha$H valine resonance, which makes chemical shifts difficult to assign accurately.

TABLE II: Coupling Constants in Potassium–Valinomycin Ion Pairs as a Function of Paramagnetic Anion Concentration.[a]

| System | Solvent | $CoX_4^{2-}$ [c]:Kval$^+$ | $J_{C^\alpha H-NH}$ Valine | $J_{C^\alpha H-NH}$ Hydroxyisovalerate | $J_{CH_3-CH}$ Lactate |
|---|---|---|---|---|---|
| KvalNCS + $K_2Co(NCS)_4$ | $(CD_3)_2CO$ | 0 | 5.0 | 4.0 | 6.6 |
| | | 0.46 | 5.0 | 3.1 | 6.4 |
| | | 1.0 | 5.0 | 3.0 | 6.4 |
| | | 1.5 | 5.0 | *b* | 6.1 |
| | | 2.3 | 5.0 | *b* | 6.0 |
| | 1:1 $CDCl_3$–$(CD_3)_2CO$ | 0 | 4.5 | 3.9 | 7.2 |
| | | 0.40 | 4.8 | 3.5 | 6.8 |
| | | 0.80 | 4.9 | 3.2 | 6.5 |
| | | 1.0 | 5.0 | 3.0 | 6.7 |
| | | 2.0 | 4.9 | 3.0 | 6.1 |
| | | 3.0 | 5.0 | *b* | *b* |
| Kval$C_6H_5CO_2$ + $(N(C_4H_9)_4)_2CoBr_4$ | 1:1 $CDCl_3$–$(CD_3)_2CO$ | 0 | 3.4 | 4.0 | 7.2 |
| | | 0.27 | 4.0 | 4.0 | 7.2 |
| KvalBr + $K_2CoBr_4$ | $(CD_3)_2CO$ | 0 | 5.3 | 3.8 | 7.0 |
| | | 1.1 | 5.0 | 3.6 | 7.0 |
| | | 1.6 | 5.0 | 3.6 | 7.0 |
| | | 2.9 | 5.2 | 3.8 | 7.0 |
| KvalNCS + $N(C_4H_9)_4Co(acac)_3$ | $CDCl_3$ | 0 | 5.0 | 4.0 | 7.1 |
| | | 2.5 | 4.0 | 4.0 | 7.0 |

[a] Uncertainty is ±1 Hz or less. [b] Broadening of resonances prohibited accurate measurement. [c] X indicates NCS, $C_6H_5CO_2$, or Br.

the shift depends not only on the distance between the ions but also on their angular orientation with respect to each other and this may be influenced by the solvent.

Changing the diamagnetic anion (*i.e.*, using potassium thiocyanate or potassium benzoate to form the Kval$^+$ ion) did not seem to markedly affect the maximum pseudocontact shift.

Coupling constants for the $C^\alpha$ protons of the valines and hydroxyisovalerate in general were independent of the concentration of paramagnetic anion when the solvent was acetone (Table II). Addition of chloroform to the solvent in some cases caused changes in the coupling constants although no clear pattern could be discerned. The coupling constant of the lactate methyl protons and the $C_\alpha$ protons were constant irrespective of the solvent when the paramagnetic anion was $CoBr_4^{2-}$ or $Co(acac)_3^-$ but did change when the anion was $Co(NCS)_4^{2-}$.

The line widths of the resonances of the $C^\alpha$ protons were, to some extent, affected by the addition of the paramagnetic anions; however, methyl proton resonances were also broadened slightly. At a ratio of Kval$^+$:$Co(NCS)_4$ = 1, the $C^\alpha$-proton resonance widths increased about 2 Hz. The extent of broadening at a given mole ratio of paramagnetic anion to potassium valinomycin depended upon the anion and the solvent. In general the broadening followed the order $Co(acac)_3^- < Co(NCS)_4^{2-} < CoBr_4^{2-} \ll Cr(NCS)_4(NH_3)_2^-$. In the case of $Cr(NCS)_4(NH_3)_2^-$ which was soluble only in solvents containing large mole fractions of methanol, line broadening of the solvent was so extensive that measurement of resonance positions was precluded at those mole ratios used for the cobalt(II) complexes.

*Crown Ether Complexes.* Titration of potassium complexes of dicyclohexyl-18-crown-6 ether and dibenzo-18-crown-6 ether with the paramagnetic anions, which included the reineckate anion as well as those used previously, and in the same solvents as were used in the valinomycin study produced quite different results than those observed with the potassium–valinomycin complex. There was no observable shift of resonances and by far the largest effect on the resonances of either of the crown ethers was line broadening. Broadening was pronounced at a paramagnetic anion concentration 1:10 of that used in the valinomycin study and ruled our accurate measurement of resonance positions at higher anion:crown ether potassium complex ratios. The line broadening was specific and proportional to the mole ratio of paramagnetic anion to potassium crown ether complex. The line widths of the resonance of the solvent and the reference, hexamethyldisiloxane, were unaffected. The titration curve for DCHE is given in Figure 3. For DBCE it was observed that the line width for the aromatic proton resonance was always less than that of the resonances of the protons comprising the crown portion of the core at any given mole ratio. This greater broadening of the methylene proton resonances as compared to the aromatic proton resonances is reasonable since in an ion pair complex the aromatic protons would be at a greater distance from the paramagnetic anion than the methylene protons.

Ion pair formation in these systems implies competition between diamagnetic and paramagnetic anions for the potassium crown ether complex (eq 1). Addition of a large excess

$$(KDCHE)^+NCS^- + Cr(NCS)_4(NH_3)_2^- \rightleftharpoons (KDCHE)^+Cr(NCS)_4(NH_3)_2^- + NCS^- \quad (1)$$

of diamagnetic potassium salt should favor the diamagnetic ion pair over the paramagnetic ion pair leading to reduced linewidths. Table III shows the results of such an experiment. Addition of excess KNCS clearly shifts the equilibrium in eq 1 in favor of the diamagnetic thiocyanate complex.

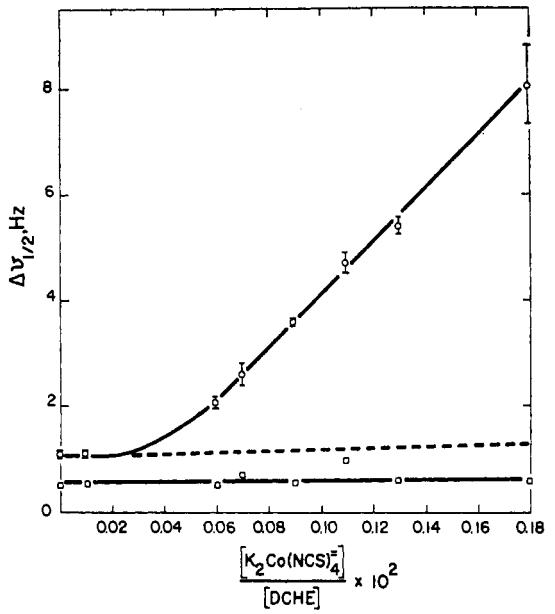


FIGURE 3: Line width of DCHE resonances as a function of paramagnetic anion concentration: solvent, 50:50 $CDCl_3$–$(CD_3)_2CO$ containing 5% hexamethyldisiloxane as reference; crown ether concentration, 0.05 M; crown $CH_2$ resonance, ○; reference resonance, □. The dashed line represents the width of the crown $CH_2$ resonance in the absence of paramagnetic anion but in the presence of a corresponding amount of KNCS.

TABLE III: Effect of Thiocyanate on Broadening of Crown Ether Resonances by Paramagnetic Anions.[a]

| Concn ($\mu$M) | | | Crown $CH_2$ |
|---|---|---|---|
| Crown Ether | KNCS | $KCr(NCS)_4(NH_3)_2$ | Resonance Width (Hz) |
| 20 | 20 | 0.0 | 2.4 ± 0.1 |
| 20 | 0 | 0.12 | 5.8 ± 0.4 |
| 20 | 0.30 | 0.12 | 3.3 ± 0.2 |
| 20 | 20 | 0.12 | 2.7 ± 0.1 |

[a] Solvent, 60:40 $CDCl_3$–$CD_3OD$. Concentration of DCHE, $2.0 \times 10^{-2}$ M.

Solvents with low dielectric constants should decrease charge separation in solution, and favor ion pair formation. In agreement with this, line widths of the DCHE crown methylene proton resonances, when the ether was titrated with $KCr(NCS)_4(NH_3)_2$, increased as the solvent dielectric constant was lowered by increasing the mole fraction of chloroform present. This is shown in Figure 4.

## Discussion

When a paramagnetic ion interacts with, or forms an ion pair with a counterion, the position and width of the resonances depend upon, among other factors, the relative magnitude of the $T_{1e}$, the electron spin–lattice relaxation time of the paramagnetic species. If $T_{1e}$ is long, then the transverse relaxation time of the protons in the counterion being observed becomes very short and the resonance line widths will become broadened. If $T_{1e}$ is short, then the nuclear resonances are not markedly broadened and, if contact or pseudocontact

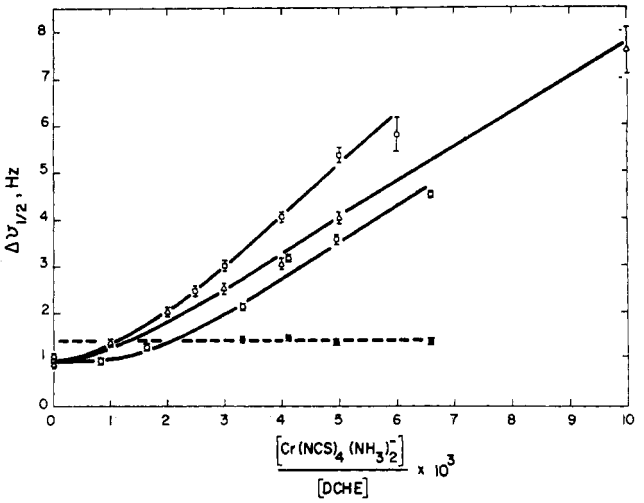


FIGURE 4: Effect of solvent polarity on the line width of crown ether $CH_2$ resonances in the system $KDCHE^+$–$Cr(NCS)_4(NH_3)_2^-$: (○) solvent 80:20 $CD_3OD$–$CDCl_3$; (△) solvent 50:50 $CD_3OD$–$CDCl_3$; (□) solvent 40:60 $CD_3OD$–$CDCl_3$; (●) line width of solvent resonance, 80:20 $CD_3OD$–$CDCl_3$.

interactions are possible, large anomalous shifts arising from these interactions may be observed.

Contact interactions (Fermi, 1930) are a result of delocalization of unpaired electrons (in this case, metal electrons) through overlap of metal orbitals with ligand orbitals. Pseudocontact interactions (Fermi, 1930) arise from a magnetic anisotropy of the paramagnetic metal ion and an incomplete averaging of the dipolar interaction between electron and nuclear spins despite the molecular tumbling. Since no direct covalent bond is possible between the protons in a potassium complex of either valinomycin or a crown ether and an anion, even if ion pair formation does take place, the Fermi contact term is expected to be small. The major term contributing to an isotropic shift in the event of ion pair formation would be the pseudocontact or dipolar interaction. For complexes with axially symmetric $g$ tensors, the change in resonance position would be given by

$$\frac{\Delta\delta}{\nu_0} = \frac{\beta^2 S(S+1)}{45kT}[3g_\parallel + 4g_\perp][g_\parallel - g_\perp]\left[\frac{3\cos^2\theta - 1}{R^3}\right] \quad (2)$$

where $\Delta\delta$ is the change in position of the proton resonance as a result of the interaction with the paramagnetic anion (measured relative to its position when only diamagnetic anions are present), $\nu_0$ is the observing frequency, $g_\parallel$ and $g_\perp$ are the electronic $g$ factors parallel and perpendicular, respectively, to the molecular symmetry axis, $R$ is the distance from the paramagnetic metal atom to the proton whose resonance is being observed and $\theta$ is the angle which $R$ makes with the axis of $g_\parallel$. In addition, eq 2 is derived under the condition that $T_{1e} \ll \tau_r$ where $\tau_r$ is the molecular tumbling time.

If, in the reaction to form an ion pair, there is no specific site of association for either ion, then both cation and anion will essentially tumble independently of each other and the pseudocontact interaction would vanish (McConnell and Robertson, 1958; LaMar, 1965a). The observation that paramagnetic anions induce specific shifts in the resonances of a positively charged potassium–valinomycin complex is proof in itself that ion pairing of these complexes does occur in solution and that the component ions have a preferred orientation with respect to each other. The specific broadening

TABLE IV: Ion Pair Distances in Ion pairs of Potassium–Valinomycin Cations and Tetrahedral Cobalt(II) Anionic complexes.[a,b]

| System | Solvent | Valine | | Hydroxyisovalerate | | Lactate | |
|---|---|---|---|---|---|---|---|
| | | *c* | *d* | *c* | *d* | *c* | *d* |
| KvalBr + $K_2CoBr_4$ | $(CD_3)_2CO$ | 4.9 | 8.2 | 5.3 | 8.5 | 5.5 | 9.1 |
| $KvalC_6H_5CO_2$ + $N(C_4H_9)_{42}CoBr_4$ | 50% $CDCl_3$–$(CD_3)_2CO$ | 5.5 | 9.3 | 5.6 | 9.5 | 5.6 | 9.5 |
| KvalSCN + $K_2Co(SCN)_4$ | $(CD_3)_2CO$ | 5.1 | 8.4 | 4.8 | 7.9 | 4.8 | 7.9 |
| KvalSCN + $K_2Co(SCN)_4$ | 50% $CDCl_3$–$(CD_3)_2CO$ | 5.2 | 8.4 | 5.8 | 9.9 | 5.6 | 9.2 |

[a] Distances in nm, uncertainty estimated as ±0.5 nm. [b] Distance calculations based on the $C^{\alpha}$-H resonance. [c] Tetragonal model. [d] Trigonal model.

of the resonances of the crown ether complexes by paramagnetic anions is indicative that the ion pairing is occurring in these systems also.

No changes in position were observed for the N-H or the methyl proton resonances. The N-H resonances are relatively broad to begin with and the methyl proton resonances of the valine and hydroxyisovalerate form a broad composite peak. Small individual shifts would not be detected. In addition, if the $(3\cos^2\theta - 1)/R^3$ term of eq 2 is small because of $\theta$ (it may even be zero) or $R^{-3}$, a change in resonance position might not be detected.

According to eq 2 a pseudocontact shift will be observed only when the anion possesses an anisotropic *g* tensor. But the tetrahedral anions used in this study have cubic symmetry and on this basis such anisotropy is not expected. It has been suggested that anisotropy in tetrahedral cobalt(II) complexes can result from a superposition of tetragonal or trigonal ligand fields on the original cubic symmetry (Figgis *et al.*, 1964; Walker and Drago, 1968) or by deformation of the anion which would perturb the electronic energy states (Walker and Drago, 1968).

There are numerous orientations of the components of an ion pair complex which can produce an axial field. Walker and Drago (1968) have discussed two possible models in detail. The ionophore systems studied here are comparable to the systems studied by these investigators (a monovalent cation with similar divalent anions), although the ionophore–metal complexes present a more rigid structure than the tetraalkylammonium ions. We have used the models of Walker and Drago and followed their procedure is estimating ion pair distances. The procedure is only outlined here and the reader is referred to the original paper for details of the calculations.

Briefly, estimates of the *g* tensor anisotropy, $\Delta g$, were made for the two models chosen using spectroscopic parameters and various assumed ion pair distances. These calculated $\Delta g$ values were then compared with those required to give the pseudocontact shift observed in the experiments according to eq 2. The experimental values of $\Delta g$ also depend upon the ion pair distance and a reasonable ion pair distance is one which gives agreement between the two values of $\Delta g$.

In both of the models assumed for the $Kval^+$ ion pair, the valinomycin–potassium complexes are colinear with the paramagnetic metal atoms. In the first case, this line passes through one apex of the tetrahedron and the opposite face (trigonal symmetry). In the second case the line passes through an edge of the tetrahedron to the opposite edge (tetragonal symmetry). The values of the parameters used in our calculations for $CoBr_4^{2-}$ are: spin orbit coupling constant, $\lambda$, $-162$ $cm^{-1}$; $10Dq$, 2860 $cm^{-1}$; for $Co(NCS)_4^{2-}$, $\lambda$, $-170$ $cm^{-1}$ and $10Dq$, 4550 $cm^{-1}$. In determining the radial and angular dependent term of eq 2, the distance between the potassium ion and the $C^{\alpha}H$ proton whose pseudocontact shift was observed was determined using known potassium to valine carbonyl oxygen distances (Pinkerton *et al.*, 1969) and normal bond lengths and angles for C—H and C—O bonds. The results of these distance calculations are given in Table IV.

The radii of $K^+$ and $CoBr_4^{2-}$ are 1.3 nm and about 2.5 nm, respectively, while the radius of $Co(NCS)_4^{2-}$ lies between 4.35 and 8.1 nm depending on the $NCS^-$ angle (Ono *et al.*, 1954). The minimum potassium cobalt distance thus is 3.8 nm for the bromine complex and 5.7–9.4 nm for the thiocyanate complex. The potassium is buried in the valinomycin cage and the maintenance of this minimum distance hardly seems likely. The calculated ion pair distances are all above these minima. The uncertainty is estimated at ±0.5 nm, but in view of the crudity of the models it is difficult to select between them. Since the minimum distance for the thiocyante complexes varies from 6 to 9 nm and only the models with trigonal symmetry give values above 6 nm it would seem that these models with trigonal symmetry represent the actual orientation and geometry with greater fidelity. It should be noted that the ion pair distances calculated for any one model using the shifts of any of the three anions agree remarkably well.

The second possibility for the inducing of magnetic anisotropy is the actual distortion of the anion through electrostatic interaction with the cation. From a study of tetrahedrally coordinated Co(II) complexes with a combination of ligands, LaMar (1965b) concluded that compression of the tetrahedrons contributed more to their anisotropy than did the variation in ligand field strength of the different ligands. It should be noted that the observation that the coupling constants of the paramagnetic ion paired valinomycin–potassium complex are in general unchanged from those of a diamagnetic ion paired complex is consistent with, although it does not prove, the distortion of the anion through collision to generate an anisotropic *g* tensor. The unchanged coupling constants would indicate that it is the valinomycin complex which is rigid and nondeformable.

The pseudocontact shifts depend (eq 2) on the geometry not only of the paramagnetic anion but also of its ion pair complex. They also depend, if not so explicitly, on other parameters of the complex, *e.g.*, the position of the ligands of the anion in the spectrochemical series, or the total charge of the anion. The pseudocontact shift can be shown to be inversely proportional to $10Dq^2$. Thiocyanate ion and bromide ion have $10Dq$ values of 4550 and 2860 $cm^{-1}$, respectively

(Cotton and Goodgame, 1961), so that $\Delta\delta$ would be expected to be larger for a $CoBr_4^{2-}$ ion pair than for a $Co(NCS)_4^{2-}$ ion pair. This is actually the case as is seen in Table I (9.8 and 3.6 Hz, respectively).

The effect of charge cannot be easily assessed since it is usually closely linked with the geometry of the system and thus involves not only the factor $(3\cos^2\theta - 1)/R^3$ but also the anisotropy in $g$. We have examined only one complex anion with unit negative charge and octahedral symmetry, $Co(acac)_3^-$, to obtain some idea of the actual magnitude of the differences. The values of $\Delta\delta$ for these $C^\alpha$-proton resonances listed in Table I can be compared with the first two entries in that table. Obviously generalizations cannot be made on the basis of one example, but the shifts in this case are larger.

*Crown Ethers.* The synthetic cyclic polyethers also form complexes with alkali metal ions by an ion dipole interaction with the oxygen atoms of the ether. Recently it has been shown that potassium or sodium complexes of the crown ethers are capable of forming ion pairs with the picrate ion (Frensdorff, 1971) in methylene chloride. The crystal structure of rubidium dibenzo-18-crown-6-thiocyanate has been determined and it was found that the six oxygen atoms are almost coplanar and that each thiocyanate anion is in contact with one cation. The crystal thus consists essentially of ion pairs (Bright and Truter, 1970).

The nmr spectra of these crown ether–potassium complexes show a pronounced broadening in the presence of either cobalt or chromium paramagnetic anions. This broadening is in sharp distinction to the valinomycin system where broadening of resonances was a minor effect and shifting of the resonance positions was a major effect. Table IV shows that there is also a marked difference in the broadening power of the cobalt and chromium anions. The most effective anion by an order of magnitude is the reineckate anion.

These differences cannot be explained quantitatively but their origin does lie in the factors determining the correlation time of the ion pair complex which in turn determines the transverse relaxation time and the width of the resonance. The correlation, $\tau_c$, is given by

$$1/\tau_c = 1/\tau_{ex} + 1/\tau_r + 1/T_{1e} \qquad (3)$$

where $\tau_r$ is the rotational tumbling time of the ion pair (which would be comparable for all systems we have studied here), and $\tau_{ex}$ is the characteristic time of exchange of ionophore–metal complex between free and ion paired forms. The smallest time will obviously dominate and determine $\tau_c$. For octahedral cobalt, $T_{1e}$ is of the order of $10^{-13}$ sec (Chmelnick and Fiat, 1967; Matwiyoff and Darley, 1968) and rotational or exchange processes cannot contribute significantly to $\tau_c$. For tetrahedral cobalt, $T_{1e}$ is not known but it is expected to be very short also. Since pronounced broadening is observed with the crown ether complexes, $T_{1e}$ of the tetrahedral cobalt in ion pairs with the crown ether complexes must be longer than in ion pairs with valinomycin complexes. The reason for this is not known. However, if the cobalt anion is distorted in the formation of valinomycin–potassium ion pairs, the distortion may be responsible for the difference in $T_{1e}$ of the cobalt in the two ion pairs.

The same considerations hold for the chromium anion systems although here the relative magnitude of the various times are different. For chromium, $T_{1e}$ is of the order of $10^{-9}$ sec (Larsen, 1966), comparable to the rotational tumbling time of a small molecule. Consequently both rotational tumbling time and electron spin relaxation time would be expected to contribute to the correlation time. The times are long enough to cause the observed broadening.

The resonances of the methylene protons of the potassium–DBCE complex in the absence of a paramagnetic anion form an $A_2B_2$ pattern. It was observed that as this complex was titrated with the paramagnetic anion, the resonances, although becoming broader, retained the $A_2B_2$ characteristics and their center of gravity. The pattern of course depends upon the relative magnitudes of the coupling constants and the chemical shift between the A and B resonances. Thus it would seem that the crown ethers also are not distorted in the formation of the ion pair complex. The X-ray studies of the crystalline rubidium–DBCE thiocyanate compound show that the rubidium is easily accessible to the anion (Bright and Truter, 1970). It may be that the large paramagnetic anions can also approach the positively charged metal closely and if, as may occur in the valinomycin case, $T_{1e}$ is modified by distortion of the anion, such distortion is not necessary with these crown ether ion pairs.

It was originally envisaged that, if ion pairing could be detected by nmr techniques, competition studies with simple anionic metabolites would allow a relative order of anion affinities for the valinomycin–potassium complex to be established. Because of the small shifts involved and problems of solubility and stability of the paramagnetic anions this has not been possible. Since $^{13}C$ magnetic resonance shifts are very large, the use of $^{13}C$-labeled monovalent anions in ion pairing studies may allow such a series to be established. Such studies are currently in progress (Kowalsky, 1973).

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# Effects of Detergents and High Pressures upon the Metarhodopsin I ⇄ Metarhodopsin II Equilibrium†

A. A. Lamola,* T. Yamane, and A. Zipp‡

ABSTRACT: The effects of high pressure upon the equilibrium (3°) between metarhodopsin I ⇌ metarhodopsin II has been examined in several different preparations of bovine visual pigment. The equilibrium is shifted to the left for sonicated rod outer segments (ROS) ($\Delta V$(1 atm) $\geq$ 60 cm$^3$/mol) and digitonin extracts of ROS ($\Delta V \simeq$ 10 cm$^3$/mol). Pressure has no effect on the equilibrium ($\Delta V \simeq 0$) for extracts of ROS made with the detergents emulphogene or lauryldimethylamine oxide. These observations support the proposal that in outer segment disk membranes rhodopsin–phospholipid interactions are coupled with the transformation metarhodopsin I → metarhodopsin II.

Functional interactions between the rhodopsin molecules and lipids in the outer segment membranes of vertebrate photoreceptor cells are implied in most models for signal transduction in photoreception (see Hagins, 1972; Blasie, 1972; Abrahamson and Wiesenfeld, 1972; Cone, 1972; Robinson and Weir, 1974). What is suggested in some models is that key structural changes in the disk membrane accompany one or more of the steps in the rhodopsin bleaching sequence. We have been seeking evidence for such rhodopsin–lipid coupling by measuring changes in the thermodynamics of bleaching which occur as a consequence of disrupting the lipid field surrounding the rhodopsin (*cf.* Applebury *et al.*, 1974).

It occurred to us that the molar free volume change ($\Delta V$) might be a useful parameter to consider in this regard because large $\Delta V$ values are expected for processes in which the exposure of hydrophobic groupings to water is altered and because of the large compressibility of assemblies of hydrocarbon chains. Thus, we examined the pressure dependence of the equilibrium (3°) between the intermediates, metarhodopsin I (478 nm), and metarhodopsin II (380 nm) in the bleaching of bovine rhodopsin to obtain the $\Delta V$ for the transformation metarhodopsin I → metarhodopsin II. We found modest values of $\Delta V$ in sonicated rod outer segments (ROS)[1] and digitonin extracts of ROS but $\Delta V \simeq 0$ for extracts using detergents which displace nearly all the lipid surrounding the pigment protein. We describe these pressure experiments in this report.

## Materials and Methods

*Detergents, Buffers, and Reagents.* Digitonin (Fisher Certified Reagent) and commercial lauryldimethylamine oxide (a 30% aqueous solution trade named Ammonyx LO was provided by the Onyx Chemical Co.) were used without further purification. Emulphogene BC720 (General Aniline and Film Corporation), a polyether detergent, was filtered through Celite. Imidazole (Calibiochem, A grade), recrystallized from ethanol, was neutralized with hydrochloric acid. Cacodylic acid (Fischer Reagent) was used as the sodium salt.

† From the Bell Laboratories, Murray Hill, New Jersey 07974 (A. A. L. and T. Y.), and the Department of Chemistry, Princeton University, Princeton, New Jersey 08540. *Received August 8, 1973.* Part of this work carried out at Princeton University was supported by a grant from the National Science Foundation.

‡ Present address: Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, Calif. 94122.

[1] Abbreviation used is: ROS, rod outer segments.