hyde are indicative of the significant role of $^{1,3}(n,\pi^*)$ states in this molecule. A large positive phosphorescence polarization beyond the 0–0 emission band has also been found in this molecule, suggesting vibronic involvement of (n,π^*) states in the spin–orbit interactions. However, whether vibronic coupling in the triplet manifold is more important than vibronic coupling in the singlet manifold has not been determined. It is felt that, for complicated molecules such as indole-3-aldehyde and other analogs, both vibronic coupling schemes are important in the over-all vibronic spin–orbit coupling. For example, Lamola has shown that both mechanisms are equally likely in acetophenone (^3L_a).[71] It would be instructive to make a rough estimate of the one-center spin–orbit contribution of the (n,π^*) localized on oxygen in indole-3-aldehyde. Using the procedure given by Plotnikov,[72] and adopting 47.7 cm^{-1} for the spin–orbit coupling constant of atomic oxygen, the absolute square of the spin–orbit coupling matrix element was found to be 303 cm^{-2}. The P–P–P wave function was used for this calculation. The calculated value is somewhat smaller in magnitude than was found for benzaldehyde (454 cm^{-2}).[72] Like the other indoles studied in this work, the singlet–triplet split is large (7100 cm^{-1}) for indole-3-aldehyde and is assigned as ^3L_a. Lowest triplet states of the $^3(n,\pi^*)$ type have been thoroughly investigated by Goodman and his coworker.[73]

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Chlorine-35 Nuclear Magnetic Resonance Study of Zinc Nucleotide Diphosphate Complexes[1]

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Abstract: In 0.5 *M* NaCl solutions, Zn(II)–nucleotide diphosphate complexes increase the ^{35}Cl nuclear relaxation rate. The added relaxation is due to the formation of Zn(II)–Cl^- bonds and has been studied by measuring the ^{35}Cl nmr line width. The relaxation produced by unprotonated nucleotide diphosphate complexes is dependent on the nucleotide base structure. Maximum line broadening was found for the adenosine diphosphate complex, Zn(ADP)$^-$, while the inosine diphosphate complex, Zn(IDP)$^-$, has the least effect. The relaxation produced by a given complex is influenced by protonation of the NDP ligand or by hydrolysis of the metal ion. The formation of $Zn(ADP)_2^{4-}$ is evident. The results strongly suggest that Zn^{2+} is chelated between the nucleotide base and phosphate chain in Zn(ADP)$^-$ but is chelated only to the phosphate chain in Zn(IDP)$^-$.

Both metal ions and enzymes are essential cofactors in the biochemical reactions of nucleotides.[2] It is also well known that there exists a high degree of enzyme specificity toward both the metal ion and nucleotide base structures. With this in mind, the structures of metal ion–ATP complexes have been studied extensively,[3] these being biochemically the most important nucleotides. A number of recent studies[4-10] have sought to determine whether or not internal chelates were formed in which the metal ions were chelated both to the phosphate chain and to the nucleotide base moieties of ATP. All experimental evidence indicates that Mg^{2+} and Ca^{2+} bind only to the phosphate chain, whereas for other ions, such as Zn^{2+}, Cu^{2+}, and Mn^{2+}, ring binding has often been indicated. There is no general agreement, however, on whether internal chelate formation takes place to a minor extent ($\backsimeq$15%) or whether it becomes essentially complete.[10] Little is known regarding the structures of the metal ion–nucleotide diphosphate complexes. The proton nmr results of Cohn and Hughes,[4] however, indicate that the Cu^{2+} and Mn^{2+} complexes of ADP may exist in the internally chelated form.

We wish to report a study of the Zn^{2+} complexes of several nucleotide diphosphates by means of ^{35}Cl nmr. Preliminary results on the ADP complexes have been reported earlier.[11] In these studies, Cl^- ions have been used to monitor the Zn^{2+} environment in these complexes as the nucleotide base structure is changed. Processes such as hydrolysis of the metal ion, the formation of complexes other than the 1:1 species, and protonation of the Zn(NDP)$^-$ complexes have been ex-

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.
(2) Nucleotide abbreviations used are: ATP for adenosine 5′-triphosphate; ADP, CDP, GDP, and IDP for adenosine, cytidine, guanosine, and inosine 5′-diphosphate; and NDP for a general nucleotide 5′-diphosphate. Where necessary, the charges and number of ionizable protons are indicated for a given molecule or ion, *e.g.*, Zn(ADPH$_2$)$^+$, Zn(ADPH), Zn(ADP)$^-$, ADPH$_2^-$, etc. The formulas do not indicate proton positions.
(3) For a review of the literature prior to 1967, see R. Phillips, *Chem. Rev.*, **66**, 501 (1966).
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plored and found clearly to influence the metal ion environment as sensed by Cl^- ions. The results strongly suggest that internal chelation is extensive for $Zn(ADP)^-$ and minimal for $Zn(IDP)^-$. Recent studies[12,13] of Hg^{2+} and Zn^{2+} complexes by this technique have demonstrated the potential of the method. The way in which Cl^- ions are used to probe the metal ion environment in a complex has been described by Stengle and Baldeschwieler.[14] It is summarized in the text so that a discussion of the results might be clearer. The experimentally measured parameter is the ^{35}Cl nmr line width in 0.5 *M* NaCl solutions. Under suitable conditions, the interaction of Cl^- ions with free or complexed metal ions can lead to changes in the observed line width. An analysis of these line width changes can be related to structurally dependent environmental features of the complexed metal ion.

Experimental Section

The ^{35}Cl spectra were obtained at 5.88 Mc using a Varian V 4311 fixed-frequency radiofrequency unit. A PAR Model HR8 lock-in amplifier was used for field modulation and phase sensitive detection. The spectrometer was equipped with a field-frequency lock system. The radiofrequency field used was low enough so that saturation effects were absent. The ^{35}Cl line widths were measured from recorded spectra as the full width at half-maximum. Reported values are the average of six measurements. The scatter about the mean value was about 5%. Line widths are related to the ^{35}Cl relaxation time by $\pi\Delta\nu = 1/T_2$.

Sample temperature in all of the experiments was 32.9°. The ionic strength of the samples was 0.5. The nucleotide diphosphates used were obtained from P-L Biochemicals, Inc., in the form of the sodium salts. A Corning Model 12 pH meter was used to measure sample pH. Ultraviolet difference spectra were measured with a Cary 14 spectrophotometer using cells with a 0.1-mm light path.

Results

The dominant relaxation mechansim for ^{35}Cl nuclei in the liquid state is quadrupolar in nature. In the extreme narrowing limit, nmr line widths for spin $^3/_2$ nuclei, like ^{35}Cl, are therefore given by[15]

$$\Delta\nu = \frac{2\pi}{5}(e^2qQ)^2\tau \qquad (1)$$

where $\Delta\nu$ is the full line width measured in cycles per second (cps) at half-maximum. In eq 1, q is the electric field gradient at the nucleus of quadrupole moment, Q, and is a tensor quantity assumed to be axially symmetric with a reorientation time, τ.

In 0.5 *M* NaCl solutions, the solvation of chloride ions is nearly symmetric and the resultant field gradient at ^{35}Cl nuclei approaches zero. For this reason, a narrow nmr line is observed ($\Delta\nu \approx$ 11–12 cps). We shall consider systems, however, in which chloride can also form chemical bonds with Zn^{2+}. The bound ^{35}Cl nuclei experience a large electric field gradient and therefore have broad nmr lines.

In the systems to be considered, a number of different Zn^{2+} ion environments may be present, each providing a potential site at which ^{35}Cl relaxation might be produced. Thus, at any given time, several types of bound Cl^- environments may exist, each with its characteristic nmr line width, $\Delta\nu$. The chemical exchange process, which for $ZnCl^+$ occurs with a rate constant[16] near 10^8 sec^{-1}, can provide a mechanism for effectively averaging the various $\Delta\nu_i$ with the line width for aqueous chloride ions, $\Delta\nu_{Cl^-}$. In the rapid exchange limit, a composite line width results, given by

$$\Delta\nu_{obsd} = \frac{[Cl^-]}{[Cl^-]_T}\Delta\nu_{Cl^-} + \frac{[ZnCl]_i}{[Cl^-]_T}\Delta\nu_i + \frac{[ZnCl]_i}{[Cl^-]_T}\Delta\nu_j + \ldots \qquad (2)$$

where $[ZnCl]_i$ denotes the concentration of bound chlorides associated with zinc environment (i), and $\Delta\nu_i$ is the ^{35}Cl line width for this type of chloride in the absence of exchange. The coefficient of $\Delta\nu_i$ in eq 2 represents the probability (P) of finding Cl^- ions bound at site i. For experiments at a given NaCl concentration, in which the chloride ion concentration is high enough so that it remains essentially equal to Cl^-_T, these probabilities are proportional to the formal concentrations of the various Zn(II) sites present. Hence, we may write eq 2 in the form

$$(\Delta\nu_{obsd} - \Delta\nu_{Cl^-}) = \bar{\nu}_i[Zn(II)]_i + \bar{\nu}_j[Zn(II)]_j + \ldots \qquad (3)$$

Here, $\bar{\nu}_i = k_i\Delta\nu_i$, the k_i being a proportionality constant involving $[Cl^-]_T$ and relevant $Zn(II)_i + Cl^-$ association constants. The symbol $[Zn(II)]_i$ denotes the total concentration of zinc environments, at which halide ions may or may not be bound. The units of the $\bar{\nu}_i$ are (cycles/sec) $\times$ M^{-1} and will be referred to as "molar relaxivity parameters" for the various zinc environments. The $\bar{\nu}_i$ thus contain information regarding the structurally dependent quantities, q, τ, and the probability of chloride binding.

We have reported earlier[11] that the addition of Zn^{2+} to 0.5 *M* NaCl solutions increases the ^{35}Cl line width by an amount $(\Delta\nu_{obsd} - \Delta\nu_{Cl^-}) = 2.0 \times 10^3[Zn^{2+}]$ and, that when the Zn^{2+} ions are complexed by ADP, far greater broadening of the ^{35}Cl resonance occurs. In the absence of Zn^{2+}, the ^{35}Cl line width is not affected by pH variations or by the addition of the NDP ligands. Neither does the addition of Mg^{2+} or Ca^{2+}, either in the presence or absence of the NDP ligands, cause a measurable change in the ^{35}Cl line width. Thus, in the experiments reported here, line widths in excess of $\Delta\nu_{Cl^-}$ can only be attributed to the formation of Zn^{2+}–Cl^- bonds, which persist for a time long as compared to τ. It is this added relaxation of ^{35}Cl nuclei which will concern us; therefore, the line width data are presented in the form $(\Delta\nu_{obsd} - \Delta\nu_{Cl^-})$, where it is understod that the natural line width actually measured for Cl^- ions (usually between 11 and 12 cps) has been substracted.

The results presented below report the pH dependence of the ^{35}Cl line width for solutions containing Zn^{2+} and NDP in the ratios of 1:1, 2:1, and 1:5. The data for equimolar solutions and those containing excess Zn^{2+} will be used to evaluate $\bar{\nu}$ parameters for the various Zn^{2+} complexes.

A value of $\bar{\nu}$ estimated for the corresponding complex of Zn^{2+} with γ-phenyl isopropyl diphosphate is also reported. This complex was investigated in order to provide a model complex in which the metal ion en-

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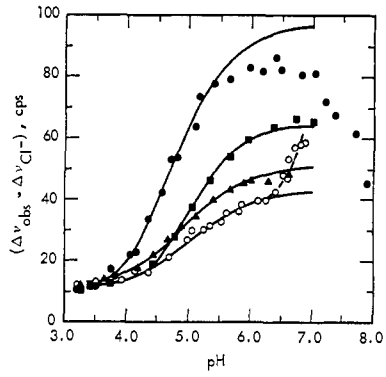

Figure 1. Broadening of ^{35}Cl nmr line in solutions with [Zn] = [NDP] = 4.76×10^{-3} *M*: ●, ADP; ■, CDP; ▲, GDP; ○, IDP.

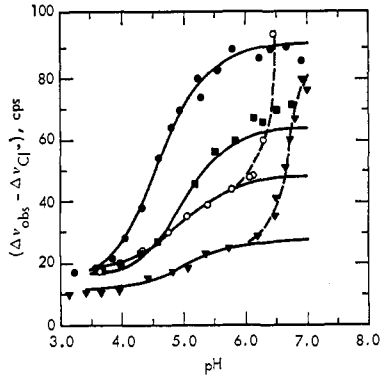

Figure 2. Broadening of ^{35}Cl nmr line in solutions with [Zn] = 2.0[NDP] = 7.14×10^{-3} *M*: ●, ADP; ■, CDP; ○, IDP; ▼, IDP [$(Zn^{2+}] = 4.76 \times 10^{-3}$).

vironment could not be influenced by the presence of a nucleotide base.

Equimolar and Excess Zn^{2+} Solutions. ^{35}Cl line width *vs.* pH profiles are shown in Figure 1 for equimolar Zn^{2+}–NDP solutions and in Figure 2 for solutions containing excess Zn^{2+}. At pH 6.0 the dominant zinc complex in each case is the unprotonated $Zn(NDP)^-$ species. The line widths observed at this pH are measures of the relaxation of ^{35}Cl by Zn^{2+} in the different $Zn(NDP)^-$ complexes. The line width data show that the different nucleotide diphosphate complexes are not equivalent. It is evident that the $Zn(ADP)^-$ and $Zn(CDP)^-$ complexes are more effective in ^{35}Cl relaxation than are the $Zn(GDP)^-$ or $Zn(IDP)^-$ complexes.

For each system the ^{35}Cl line width decreases as the solution pH is lowered. Part of this behavior must be attributed to a decrease in the concentration of bound zinc. The observed line width *vs.* pH profiles need not be described entirely by changes in the concentration of bound zinc, since at lower pH's protonation of the $Zn(NDP)^-$ complexes occurs. This leads to species which might differ from $Zn(NDP)^-$ in their effectiveness at producing ^{35}Cl relaxation. For example, protonation of a $Zn(NDP)^-$ complex might induce a structural change which could significantly change τ. This would, of course, provide an additional contribution to the observed change in ^{35}Cl line width with pH. To investigate this, we have analyzed the data in such a way as to derive estimates of the $\bar{\nu}$ parameters for the various protonated forms of the ZnNDP complexes. This is outlined below.

Derivation of $\bar{\nu}$ Parameters. To derive the $\bar{\nu}$ parameters from the ^{35}Cl line widths, it was necessary to first calculate the concentrations of the various Zn^{2+} species as a function of pH. The line width data can then be related to these concentrations by expanding eq 3. The following general equilibria were considered relevant to these solutions.

$$NDPH_2^- \overset{k_1}{\rightleftharpoons} NDPH^{2-} + H^+$$

$$NDPH^{2-} \overset{k_2}{\rightleftharpoons} NDP^{3-} + H^+$$

$$Zn^{2+} + NDPH_2^- \overset{K_1'}{\rightleftharpoons} Zn(NDPH) + H^+$$

$$Zn(NDPH) \overset{K_2'}{\rightleftharpoons} Zn(NDP)^- + H^+$$

$$Zn(NDPH_2)^+ \overset{K_3'}{\rightleftharpoons} Zn(NDPH) + H^+$$

$$Na^+ + NDP^{3-} \overset{K_{Na}}{\rightleftharpoons} Na(NDP)^{2-}$$

Related equilibria are

$$Zn^{2+} + NDP^{3-} \overset{K_1}{\rightleftharpoons} Zn(NDP)^-$$

$$Zn^{2+} + NDPH^{2-} \overset{K_2}{\rightleftharpoons} Zn(NDPH)$$

$$Zn^{2+} + NDPH_2^- \overset{K_3}{\rightleftharpoons} Zn(NDPH_2)^+$$

The equilibrium expressions, together with the conservation equations for total Zn^{2+}, T_{Zn}, and total NDP, T_{NDP}, can be combined to give[17]

$$[Zn^{2+}]^2 + (T_{NDP} - T_{Zn} + C)[Zn^{2+}] - CT_{Zn} = 0 \quad (4)$$

where

$$C = \frac{[H^+]^2 + k_1[H^+] + (K_{Na}[Na^+] + 1)k_1k_2}{(K_1'/K_3')[H^+]^2 + K_1'[H^+] + K_1'K_2'}$$

$$[Zn(NDP)^-] = \frac{T_{Zn} - [Zn^{2+}]}{1 + \dfrac{[H^+]}{K_2'} + \dfrac{[H^+]^2}{K_2'K_3'}} \quad (5)$$

$$[Zn(NDPH)] = \frac{T_{Zn} - [Zn^{2+}] - [Zn(NDP)^-]}{1 + ([H^+]/K_3')} \quad (6)$$

$$[Zn(NDPH_2)^+] = T_{Zn} - [Zn^{2+}] - [Zn(NDP)^-] - [Zn(NDPH)] \quad (7)$$

If eq 3 is written in a more explicit form and both sides divided by $[Zn^{2+}]_{bound}$, a molar relaxivity for bound Zn^{2+}, $\bar{\nu}_{Zn_b}$, may be defined as follows

$$\bar{\nu}_{Zn_b} = \frac{(\Delta\nu_{obsd} - \Delta\nu_{Cl^-}) - \bar{\nu}_1[Zn^{2+}]}{[Zn^{2+}]_b} = \bar{\nu}_2\frac{[Zn(NDPH_2)^+]}{[Zn^{2+}]_b} + \bar{\nu}_3\frac{[Zn(NDPH)]}{[Zn^{2+}]_b} + \bar{\nu}_4\frac{[Zn(NDP)^-]}{[Zn^{2+}]_b} \quad (8)$$

(17) For IDP, ionization of the nucleotide base ($pk \approx 9.0$) was neglected. The use of k_2, K_2', K_{Na}, and $Zn^{2+} + IDPH^{2-} \rightleftarrows (K_1')$ $Zn(IDP)^- + H^+$ lead to eq 4, with $C = ([H^+] + (1 + K_{Na}[Na^+])k_2)/K_1'(1 + [H^+]/K_2')$. The expressions $[Zn(IDP)^-] = (T_{Zn} - [Zn^{2+}])/(1 + [H^+]/K_2')$ and $[Zn(IDPH)] = T_{Zn} - [Zn(IDP)^-] - [Zn^{2+}]$ also result.

or

$$\bar{\nu}_{Zn_b} = \bar{\nu}_2 F_{ZnNDPH_2^+} + \bar{\nu}_3 F_{ZnNDPH} + \bar{\nu}_4 F_{ZnNDP^-} \quad (9)$$

where $F_{ZnNDP^-} = [Zn(NDP)^-]/[Zn^{2+}]_b$ and the other F_{Zn_i} are defined similarly so that $\Sigma_i F_{Zn_i} = 1.0$.

The IDP and GDP systems were easiest to treat. Over the pH region used for the analysis, the species $Zn(IDPH_2)^+$ is not relevant since protonation of the uncharged inosine ring does not occur. The species $Zn(GDPH_2)^+$ was also neglected since little error resulted from neglecting protonation of the guanosine ring ($pk = 2.9$) over the region pH 3.6 to 6.0. For these two systems then eq 9 reduces to $\bar{\nu}_{Zn_b} = \bar{\nu}_3 + (\bar{\nu}_4 - \bar{\nu}_3)F_{ZnNDP^-}$ and a plot of ν_{Zn_b} *vs.* F_{ZnNDP^-} should be linear. Extrapolation of F_{ZnNDP^-} to the limits 1.0 and 0.0 gives $\bar{\nu}_4$ and $\bar{\nu}_3$, respectively.

To make the indicated plots for these two systems, trial values for the constants K_1' and K_2' were chosen, and the concentrations of the various Zn^{2+} species were calculated at ten pH values between 6.0 and 3.6. At each pH, F_{ZnNDP^-} was evaluated and plotted against ν_{Zn_b} which was evaluated using $\nu_1 = 2.0 \times 10^3$, the observed ^{35}Cl line width, and calculated concentrations of bound and free Zn^{2+}. Initial values of K_2' were estimated by assuming $(pk_2 - pK_2') = 2.0$ as reported by Handschin and Brintzinger[18] for the Zn^{2+}–ATP system. Trial values for this difference were chosen in the range between 2.0 and 2.6. The constants pk_2 are listed in Table I. For a given value of K_2', several trial values of K_1' were tested. These were chosen so that the related value of $K_1 (= K_1'/k_2$ for IDP and $K_1'K_2'/k_1k_2$ for GDP) would vary between the limits 1.0×10^4 and 7×10^4. A number of investigators have measured this constant for transition metal binding to ADP and have generally reported it to be in this range.[3] Linear plots, as shown in Figure 3, were obtained using the equilibrium constants listed in Table I. The resulting values of $\bar{\nu}_3$ and $\bar{\nu}_4$ are included in Table II.

Table I. Equilibrium Constants Used to Calculate the Concentrations of Zn(II) Species in Zinc-Nucleotide Diphosphate Solutions[a]

			pK_a			
	pk_1[b]	pk_2[c]	Nucleotide base-Zn complex	Terminal phosphate-Zn complex	pK_1'	Log K_1
ADP	3.9	6.78	3.9	4.18	1.70	4.83
IDP		6.79		4.39	2.16	4.63
GDP	2.9	6.84	2.9	4.44	0.67	4.63
CDP	4.6	6.82	4.6	4.42	2.19	4.63

[a] A value of 5.5 was used for K_{Na}: N. C. Melchior, *J. Biol. Chem.* **208**, 615 (1954); R. M. Smith and R. A. Alberty, *J. Phys. Chem.*, **60**, 180 (1956). [b] R. M. Bock, *et al.*, *Arch. Biochem. Biophys.*, **62**, 253 (1956). [c] Evaluated at $\mu = 0.1$ according to R. C. Phillips, *et al.*, *J. Biol. Chem.*, **240**, 4393 (1965).

The analysis of ADP and CDP data was similar. Values for the association constants were chosen so as to be consistent with those for GDP and IDP. Thus K_1 was taken to be 4.27×10^4 and Zn^{2+} binding was assumed to lower pk_a for the terminal phosphate proton by 2.40 units. In view of the lack of published data it was assumed that metal ion binding to CDP and ADP does not influence the pk for ring ionization. For the purpose of estimating the $\bar{\nu}$ parameters, this assumption was found to be adequate and is reasonable in view of the minor effect observed in metal ion–ATP complexes.

(18) U. Handschin and H. Brintzinger, *Helv. Chim. Acta*, **45**, 1037 (1962).

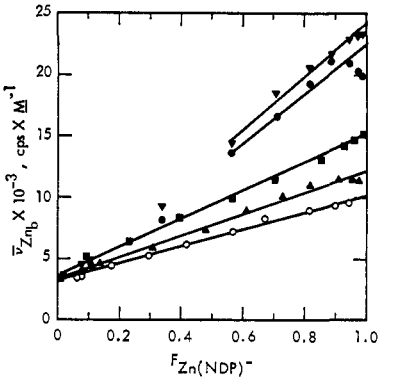

Figure 3. Plot of molar relaxivity of bound zinc, $\bar{\nu}_{Zn_b}$, *vs.* fraction of bound zinc present as $Zn(NDP)^-$, F_{ZnNDP^-}. The intercept at $F_{ZnNDP^-} = 1.0$ gives $\bar{\nu}_{ZnNDP^-}$: ●, (1:1) ADP; ■, (1:1) CDP; ▲, (1:1) GDP; ○, (1:1) IDP; ▼, (2:1) ADP.

Table II. Molar Relaxivity Parameters for Zinc Complexes with Nucleotide Diphosphates and with γ-Phenyl Isopropyl Diphosphate

Complex	$\bar{\nu} \times 10^{-3}$, cps × M^{-1}		Complex	$\bar{\nu} \times 10^{-3}$, cps × M^{-1}	
$ZnADP^-$	22.5[a]	24.1[b]	ZnADPH	2.4[a]	3.3[b]
$ZnCDP^-$	15.3	16.0	$ZnADPH_2^+$		
$ZnGDP^-$	12.1		ZnCDPH	3.5	3.6
$ZnIDP^-$	10.1	11.1	$ZnCDPH_2^+$		
$Zn(PhIPDP)^-$	8.4		ZnGDPH	3.5	
Zn^{2+}(aq)	2.0		ZnIDPH	3.3	3.5

[a] Results from 1:1 titrations. [b] Results from 2:1 titrations.

As before, the concentrations of the various Zn^{2+} species were calculated at a number of pH values and the quantities $\bar{\nu}_{Zn_b}$ and F_{ZnNDP^-} evaluated. The curves obtained by plotting $\bar{\nu}_{Zn_b}$ *vs.* F_{ZnNDP^-} at each pH could be extrapolated to $F_{ZnNDP^-} = 1.0$ to obtain $\bar{\nu}_4$ (Figure 3). By substracting the ^{35}Cl line broadening (or relaxation) due to $Zn(NDP)^-$ from the total line broadening due to bound Zn^{2+}, similar plots could be made to obtain $\bar{\nu}_2$ and $\bar{\nu}_3$. Thus, an expression analogous to eq 8 is obtained by dividing eq 3 by $[Zn(NDPH_2)^+] + [Zn(NDPH)]$ giving

$$\frac{\Delta\nu_{obsd} - \Delta\nu_{Cl^-} - \bar{\nu}_1[Zn^{2+}] - \bar{\nu}_4[Zn(NDP)^-]}{[Zn(NDPH_2)^+] + [Zn(NDPH)]} = \bar{\nu}_2 \frac{[Zn(NDPH_2)^+]}{[Zn(NDPH_2)^+] + [Zn(NDPH)]} + \bar{\nu}_3 \frac{[Zn(NDPH)]}{[Zn(NDPH_2)^+] + [Zn(NDPH)]} \quad (10)$$

Inspection of eq 10 indicates that a plot of the left side of the equation *vs.* the quantity $[Zn(NDPH)]/([Zn(NDPH_2)^+] + [Zn(NDPH)])$ should be linear with intercepts $\bar{\nu}_2$ and $\bar{\nu}_3$.

For the CDP system, the results indicated approximately equal values for $\bar{\nu}_2$ and $\bar{\nu}_3$, namely 3500. For the ADP system, a slightly higher value of K_2 was re-

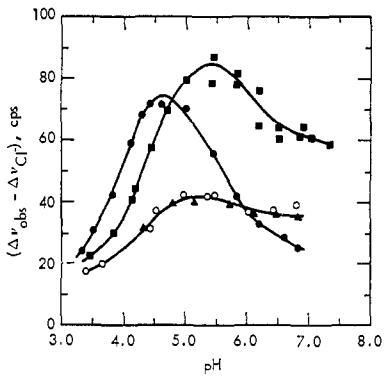

Figure 4. Broadening of ^{35}Cl nmr line in solutions with $[Zn^{2+}] = 0.20[NDP] = 5 \times 10^{-3}$ *M*: ●, ADP; ■, CDP; ○, IDP; ▲, GDP.

quired to eliminate curvature in the plot. The use of $(pk_2 = pK_2') = 2.6$ resulted in essentially equal values for the two molar relaxivity parameters. The final value of K_2' for the ADP system is the same as that reported by Kahn and Martell for the Zn(ADPH) complex.[19]

The various $\bar{\nu}_i$ estimated in this way for the Zn^{2+}–CDP, –ADP, –IDP, and –GDP systems are summarized in Table II together with a $\bar{\nu}$ for the unprotonated complex of Zn^{2+} with γ-phenyl isopropyl diphosphate (PhIPDP). Results from both the equimolar and excess Zn^{2+} experiments are included. It appears that excess Zn^{2+} does not influence the $\bar{\nu}$ parameters obtained and therefore it is likely that only the 1:1 complexes of Zn^{2+} and nucleotide diphosphates are significant in these solutions. The $\bar{\nu}$ parameters listed in Table I, together with the calculated concentrations of the various Zn^{2+} species, were used to calculate ^{35}Cl line width *vs.* pH profiles by means of eq 9. The calculated profiles shown as the solid curves in Figures 1 and 2 give an adequate representation of the data in the pH region below pH 6.0.

For the ADP system, it appears that the observed ^{35}Cl line widths begin to deviate from the calculated curve in the pH region near 6.0. Such a deviation might arise from either hydrolysis of bound Zn^{2+} or from the formation of $Zn(ADP)_2^{4-}$. The rapid decrease in ^{35}Cl line width above pH 7.0 can be attributed to the formation of $ADPZn(OH)^{2-}$. The formation of this species has been reported by Khan and Martell[19] and leads to the formation of dimers. The hydrolysis reaction, which was reported to have a p*k* near 8.5, apparently leaves the Zn^{2+} ion essentially inaccessible to Cl^- ions.

In both the equimolar and excess Zn^{2+} experiments with IDP, a surprising increase in ^{35}Cl line width takes place above pH 6.0. It appears that in the region 6.0 to 6.8, a pH-dependent process gives rise to a change in the Zn^{2+} environment in $Zn(IDP)^-$. No similar rise in ^{35}Cl line width has been detected in solutions containing Zn^{2+} alone or for solutions containing the model complex, $Zn(PhIPDP)^-$, up to the point at which a precipitate forms near pH 7.2. Thus the increased relaxation of ^{35}Cl cannot conveniently be attributed to normal hydrolysis of bound Zn^{2+} with a resultant increase in the *q* experienced by bound Cl^-. It therefore appears that the inosine ring is involved in the environmental change.

(19) M. M. T. Khan and A. E. Martell, *J. Amer. Chem. Soc.*, 84, 3037 (1962).

Protonated ZnNDP complexes show only a minor enhancement in $\bar{\nu}$ over that for Zn^{2+} ions alone. No major dependence of $\bar{\nu}$ on nucleotide base structure was evident for these species. An enhancement in ^{35}Cl relaxation of about 1.8 seems to be appropriate for the protonated complexes relative to that for aqueous Zn^{2+} ions.

Excess NDP Solutions. The pH dependence of the ^{35}Cl line width is shown in Figure 4 for solutions containing a fivefold excess of NDP. Again it is evident that, as sensed by halide ions, the different Zn^{2+}–NDP systems are not equivalent.

In the low pH region, the line width *vs.* pH profiles are analogous to those for the equimolar solutions which contained about the same concentration of Zn^{2+}. For pH values <5.0, the presence of excess NDP leads to a greater ^{35}Cl line broadening than observed in equimolar solutions. This can reasonably be attributed to an increased binding of Zn^{2+} which is far from complete in the equimolar solutions. In the pH region near 5.0, the excess NDP should be in the $NDPH^{2-}$ form in which the terminal phosphate group is protonated. The dominant zinc complex at this pH is $Zn(NDP)^-$. Since the observed line widths are at a maximum here, it seems clear that these two species do not interact to form a new complex characterized by a significantly lower $\bar{\nu}$.

The analogy with the equimolar pH profiles does not hold beyond pH 5.0–5.5, however. In the solutions containing excess NDP, the ^{35}Cl line width passes through a maximum near pH 5.0 and then decreases. The decrease from the maximum observed line width is dramatic for the Zn–ADP system, being about 65% as compared to 20% for the GDP, CDP, and IDP systems. We attribute the decrease in ^{35}Cl line width to the formation of $Zn(NDP)_2^{4-}$ concomitant with the titration of excess $NDPH^{2-}$ to NDP^{3-}. The $Zn(ADP)^-$ complex appears to be particularly susceptible to the addition of a second ADP^{3-} ligand. This may be contrasted with the $Zn(CDP)^-$ complex which, like $Zn(ADP)^-$, produces relatively high ^{35}Cl relaxation. Although the ^{35}Cl line width decreases somewhat at higher pH's for CDP solutions, it still remains broad at pH 7.0.

It appears that ADP^{3-} forms a 2:1 complex with $Zn(ADP)^-$, but $ADPH^{2-}$ does not. Since $ADPH^{2-}$ and ADP^{3-} have identical nucleotide bases, it can be concluded that the unprotonated phosphate chain of ADP^{3-} is a major requirement for the formation of a stable $Zn(ADP)_2^{4-}$ complex. The most direct interpretation of the results is that the Zn^{2+} ion of $Zn(ADP)_2^{4-}$ is chelated between the two pyrophosphate chains. The relative affinities of $Zn(ADP)^-$ toward ADP^{3-} and $ADPH^{2-}$ is certainly in agreement with the weak complexing properties of the protonated diphosphate chain. On the other hand, this model for $Zn(ADP)_2^{4-}$ is not entirely satisfactory. Coordination of Zn^{2+} between four negatively charged oxygen atoms should lead to an inaccessible Zn^{2+} ion. We do not, however, observe complete removal of ^{35}Cl relaxation by bound Zn^{2+} even at greater concentrations of excess ADP. Thus, other models[9] for $Zn(ADP)_2^{4-}$ are possible, but probably must be evaluated by other methods.

To aid in an understanding of the enhanced ^{35}Cl relaxation by $Zn(NDP)^-$ complexes, uv difference spectra were measured for the Zn–ADP system, for which

^{35}Cl relaxation was maximum. The spectra obtained for 0.5 M NaCl solutions containing 2.87×10^{-3} M ADP and 5.74×10^{-3} M Zn^{2+}, Mn^{2+}, or Mg^{2+} are shown in Figure 5. Similar spectra have been reported by Schneider, Brintzinger, and Erlenmeyer,[8] for ATP solutions of these metal ions. The spectra are obtained by measuring the absorption of the metal complexes relative to that of the free ligand. For the ATP systems, the spectra are due to a red shift in the 260-mμ band of the ligand. This band is due to the heterocyclic ring and is shifted by metal ion chelation. The intensity of the uv difference spectra for ATP systems was taken to be a measure of the extent to which metal ions bind to the nucleotide base. If the same interpretation is given to the spectra in Figure 5, the results are certainly indicative of extensive chelation of Zn^{2+} by the nucleotide base. The intensity of the 280-mμ peak for ZnADP is about five times that reported for the corresponding ZnATP peak, which has a maximum at 270 mμ.

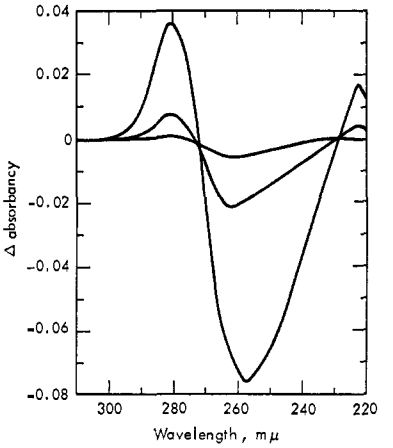

Figure 5. Differential uv absorption spectra of 2.87×10^{-3} M ADP in the presence of 5.74×10^{-3} M metal ions using 0.10-mm cells. From top to bottom at 280 mμ, Zn^{2+}, Mn^{2+}, Mg^{2+}.

Discussion

The results obtained in these studies show that a detailed description of the metal ion environment in solutions containing Zn^{2+} and nucleotide diphosphates is complicated due to the formation of species other than the $Zn(NDP)^-$ complexes. Even in dilute equimolar solutions, where the 1:1 complexes are dominant, the metal ion environment, as sensed by chloride ion probes, is dependent on the nucleotide base structure and on the degree of protonation of the complexes.

The results show that the zinc environment, as represented by $\bar{\nu}$, is not always the same in various $Zn(NDP)^-$ complexes. As the following analysis shows, it is therefore unlikely that all of the complexes have open configurations. First of all, the $\bar{\nu}$ values for the different complexes depended on both the probability of Cl^- binding and on q for bound ^{35}Cl. If Zn^{2+} were bound only to the phosphate chain in the various $Zn(NDP)^-$, then in each case it would have been equally accessible to Cl^- and the electric field gradient experienced by bound ^{35}Cl nuclei would have been nearly the same. We expect, therefore, that these two parameters would not have been variables. If we next consider that $\bar{\nu}$ also depends on τ, there is the possibility that this parameter could change even if all the complexes had open structures. This is because of size differences which might lead to a division of τ values according to whether a given complex had a purine or pyrimidine base. Further, differences in intermolecular hydrogen bonding could lead to different τ values. A consideration of dimerization, however, based on reported association constants[9,20] near 5 M^{-1}, reveals that NDP molecules in 4.7×10^{-3} M solutions should be 95% monomeric. Therefore, intermolecular interactions can probably be ignored. Further, our observed $\bar{\nu}$ values do not correlate with the expected size of open complexes. For example, $Zn(IDP)^-$ and $Zn(ADP)^-$ have very different $\bar{\nu}$ but have nearly the same open size; also the $\bar{\nu}$ for $Zn(CDP)^-$ with its pyrimidine ring is larger than the $\bar{\nu}$ for $Zn(IDP)^-$ with its purine ring. We conclude, therefore, that in certain of the complexes the nucleotide base is involved in binding Zn^{2+} and this is revealed in the different $\bar{\nu}$'s.

(20) A. D. Broom, M. P. Schweizer, and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, 89, 3612 (1967).

Although the ^{35}Cl line width may be used to examine the Zn^{2+} ion environment in the various ZnNDP complexes, an interpretation of measured specific relaxation by a given complex is obviously difficult. The measured parameter is proportional to the product of q^2, τ, and P for a given complex and it is not possible as yet to clearly correlate over-all differences in this product with changes in the individual components. For example, the observation of different $\bar{\nu}$ parameters for $Zn(ADP)^-$ and $Zn(IDP)^-$, while pointing out a dependence of the metal ion environment on the nucleotide base structure, does not provide an answer to the question of which nucleotide base interacts with the metal ion. This observation would not rule out the possibility that both nucleotide bases bind the metal ion.

The results of two experiments, in particular, indicate that internal chelation of Zn^{2+} is extensive in $Zn(ADP)^-$ but not in $Zn(IDP)^-$. First, the uv difference spectrum obtained for $Zn(ADP)^-$ strongly suggests a metal ion–nucleotide base interaction. Second, the $\bar{\nu}$ obtained for the model complex $Zn(PhIPDP)^-$ should approximate that for a $Zn(NDP)^-$ complex in which the metal ion is bound only to the phosphate chain. The complexes $Zn(IDP)^-$ and $Zn(PhIPDP)^-$ have similar $\bar{\nu}$ parameters. Therefore, we suggest that enhanced ^{35}Cl relaxation by $Zn(ADP)^-$ relative to that for $Zn(IDP)^-$ can be attributed primarily to an increased τ_{rot} for Cl^- ions bound to an internally chelated Zn^{2+} ion in the adenosine complex. It will be shown below that the observed effects of higher pH and excess NDP^{3-} on ^{35}Cl relaxation by the $Zn(NDP)^-$ complexes can also be explained on this basis.

In the presence of excess ADP^{3-}, the Zn^{2+} environment in $Zn(ADP)^-$ that gives rise to enhanced ^{35}Cl relaxation is altered by the interaction of the complex with a second ADP^{3-} molecule. Because the process evidently involves a rather large decrease in either q, τ, or probability of Cl^- binding, it is of interest to consider two possible interpretations. It is possible that excess ADP^{3-} competes with Cl^- for the available Zn^{2+} coordination sites of $Zn(ADP)^-$. This would decrease Cl^- binding and sharpen the ^{35}Cl nmr line. Competition of this sort does not seem to be as important, however, in the other NDP systems. Furthermore, broadening by $Zn(ADP)^-$ did not seem to approach zero when

the excess of ADP^{3-} was made large; rather, it decreased to a level near that of aqueous Zn^{2+}. For these reasons, it seems necessary to offer an alternate interpretation of line narrowing by excess ADP^{3-}. A reasonable possibility is that excess ADP^{3-} forms a new complex by reacting with the nucleotide base of $Zn(ADP)^-$ rather than with the bound Zn^{2+}. This type interaction could, by blocking a nucleotide base atom normally used for internal chelation of Zn^{2+}, lead to a less rigid configuration for the Zn^{2+} site and a corresponding decrease in τ. Intermolecular hydrogen bonding between nucleotide bases as well as stacking of the bases are reactions of this type, which are well substantiated for NDP concentrations $\geq$ those used in excess NDP experiments.

It has been noted that the Zn^{2+} environment in $Zn(CDP)^-$ (which, like that in $Zn(ADP)^-$, is characterized by a relatively high $\bar{\nu}$) appears to be more stable toward an interaction with a second CDP^{3-} molecule. It is tempting to attribute this to a stronger metal ion chelation to the nucleotide ring in the $Zn(CDP)^-$ complex. Certainly this would correlate well with the basicities of the adenosine and cytidine rings. An internal chelate involving the cytidine ring ($pk = 4.6$) would on this basis be more stable than one involving the adenine ring ($pk = 3.9$).

The changes which occur in the ^{35}Cl relaxation produced by $Zn(ADP)^-$ and $Zn(IDP)^-$ as the solution pH is raised beyond 6.0 are also indicative of the structure of the complexes. For $Zn(ADP)^-$, hydrolysis of the metal ion leads to very extensive removal of ^{35}Cl relaxation by bound Zn^{2+}. $Zn(ADP)^-$ produces a maximum ^{35}Cl line broadening near pH 6.5 but only one-third of this remains at pH 8.7. If the Zn^{2+} ion of $Zn(ADP)^-$ were chelated between two phosphate groups and the nucleotide base, then hydrolysis of the Zn^{2+} would most likely produce a coordinately saturated metal ion inaccessible to Cl^- ions. This would account for the observed behavior.

For $Zn(IDP)^-$ it has been noted that a pH-dependent change in the complex takes place over the pH region 6.0 to 7.0. The resulting complex produces ^{35}Cl relaxation to a considerably greater extent than does $Zn(IDP)^-$. This was particularly evident in solutions containing excess Zn^{2+} where ^{35}Cl relaxation in the ADP and IDP systems became nearly the same at pH 7.0. We tentatively suggest that hydrolysis of the Zn^{2+} ion in $Zn(IDP)^-$ leads to the formation of an internal chelate structure characterized by a longer τ_{rot}, essentially equal to that for $Zn(ADP)^-$. The apparent stability of the structure may be due to hydrogen bonding between the inosine OH and $>Zn(OH)^-$ moiety. Removal of a proton from the inosine ring ($pk \approx 9.0$) would seem unlikely in this pH region.

As a result of these studies, we conclude that internal chelation of Zn^{2+} occurs in $Zn(ADP)^-$ and $Zn(CDP)^-$, while the Zn^{2+} is bound only to the phosphate chain of $Zn(IDP)^-$. It appears that the latter complex may also attain a folded configuration as a result of metal ion hydrolysis. It also seems clear that conditions of pH and molar ratio of Zn^{2+}:NDP can readily influence the Zn^{2+} ion environment in these solutions. Results quite similar in nature have been obtained for Zn^{2+}–nucleotide triphosphate complexes and will be the subject of a later report.

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The Carbonic Anhydrase Catalyzed Hydrolysis of 2-Hydroxy-5-nitro-α-toluenesulfonic Acid Sultone

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Abstract: An ionizable group in bovine carbonic anhydrase (BCA) with a pK of 7.3 appears to be involved in the enzyme-catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I). Similar observations have been reported previously for the pH–rate behavior of the BCA-catalyzed hydration of CO_2, hydration of carbonyl compounds, and hydrolysis of nitrophenyl esters of carboxylic acids. The BCA-catalyzed hydrolysis of I is subject to sulfonamide inhibition as are the other reactions mentioned above. Also, human carbonic anhydrases B and C have been demonstrated to be effective catalysts for the hydrolysis of I. On the basis of our observations taken in conjunction with those of other investigations we have proposed that a zinc bound hydroxide ion is the active catalytic species in carbonic anhydrase action and we have suggested a cyclic mechanism for the carbonic anhydrase catalyzed solvolysis of I, involving no net proton transfer to the solvent.

A previous communication from this laboratory presented preliminary information on the carbonic anhydrase catalyzed hydrolysis of a new sulfonate ester substrate, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I).[2] In the present article we report a full account of our findings with this compound which appears to be the most rapidly hydrolyzed ester substrate of carbonic anhydrase known. We have explored the esterase properties of bovine erythrocyte carbonic anhydrase (BCA) and human carbonic anhydrases B and C (HCAB and HCAC).

(1) Fellow of the Alfred P. Sloan Foundation.

(2) K.-W. Lo and E. T. Kaiser, *Chem. Commun.*, 834 (1966).