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## *HALIDE IONS AS CHEMICAL PROBES FOR NMR STUDIES OF PROTEINS**

BY THOMAS R. STENGLE† AND JOHN D. BALDESCHWIELER

DEPARTMENT OF CHEMISTRY, STANFORD UNIVERSITY

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There has been considerable recent interest in the application of nuclear magnetic resonance techniques to the study of biological macromolecules.[1] NMR is in general not sufficiently sensitive for the direct study of biomolecules in solution at realistic concentrations. Some of the most promising developments have therefore involved the use of nuclear relaxation effects to probe gross molecular structural features.[2] These techniques frequently rely on the effects of small concentrations of paramagnetic atoms on the relaxation times of solvent nuclei,[3–5] or on the binding and exchange of small molecules with proteins.[6] Although paramagnetic relaxation effects have been widely exploited to yield structural information on relatively simple inorganic complexes in solution,[7–9] these techniques usually suffer from the inherent complexity of the relaxation process.[2] The observed effects on nuclear relaxation are usually the result of several competing mechanisms of comparable significance, which makes interpretation of line shapes in terms of molecular events hazardous.

For nuclei with spin greater than $^1/_2$, the interaction of the nuclear electric quadrupole moment with fluctuating field gradients at the nucleus can provide a simple and dominant relaxation mechanism.[10] It is thus of interest to explore the possible application of this mechanism. In this paper it is shown that the relaxation and exchange of quadrupolar nuclei at suitable sites can provide a general technique for the study of biomolecules in solution. Suitable sites for the binding and exchange of halide ions can be readily inserted at interesting places in proteins, and information analogous to that accessible from spin-labeled biomolecules by ESR[11] can be inferred from the line width of the halide nuclear resonance.

*Line-Width Theory.*—Fluctuations in the orientation and magnitude of the electric field gradient $q$ at the site of a nucleus with electric quadrupole moment $Q$ provide an efficient nuclear relaxation mechanism. In the extreme narrowing approximation, the contribution to the nuclear resonance line width from quadrupole relaxation is[10]

$$\Delta\nu = K(e^2qQ)^2\tau_c, \qquad (1)$$

where $\Delta\nu$ is the full line width at half height in cps, $(e^2qQ)$ is the quadrupole coupling constant in cps, $\tau_c$ is the correlation time for molecular rotation in sec, $I$ is the spin quantum number, and

$$K = \frac{3\pi}{10}\frac{2I+3}{I^2(2I-1)}(1+\eta^2/3),$$

where $\eta$ is the asymmetry parameter. For a nucleus of spin $^3/_2$ such as $Cl^{35}$ or $Br^{81}$, $K$ reduces to simply $2\pi/5$ if the asymmetry parameter is neglected.

It is apparent that equation (1) can be applied to obtain $(e^2qQ)$ from the line width if $\tau_c$ is known, or $\tau_c$ if $(e^2qQ)$ is known. A large range of line widths is possible depending on the values of these quantities. For example, for $Cl^-$ in dilute aqueous solution, the solvation of the ion is essentially symmetric, and the electric field gradient at the nucleus is nearly zero. Chloride line widths observed for dilute solutions of NaCl in water are of the order of 10 cps.[12] On the other hand, for carbon-chlorine bonds, the values of $(e^2qQ)$ range from 40 to 90 Mc/sec.[13] Thus in $CCl_4$ where $e^2qQ = 81.3$ Mc/sec, and $\tau_c \cong 1.7 \times 10^{-12}$ at 25°C, the line width is of the order of 14.5 kc/sec.[14] Even greater line widths are of course expected for molecules larger than $CCl_4$ with longer $\tau_c$.

If a quadrupolar nucleus can be located at different kinds of sites in solution, then the resulting line shape depends on the relative concentrations of the various sites, the values of $(e^2qQ)$ and $\tau_c$ at each site, and the rate of exchange of $Cl^-$ among the various sites. For example, in an aqueous solution of NaCl and $HgCl_2$, the chloride ion might be expected to be either symmetrically solvated by water, or complexed to the mercuric ion as $HgCl_4^=$. The symmetrically solvated chloride is expected to yield a sharp line, whereas the chloride associated with $HgCl_4^=$ has nonzero $(e^2qQ)$ and should yield a broad resonance. In the limit of slow chloride exchange between these two environments, the resulting spectrum is expected to be a simple superposition of broad and sharp lines with areas proportional to the concentrations of the two sites.[7, 15] For low $HgCl_4^=$ concentration, the broad $Cl^{35}$ signal would be difficult to detect. If the exchange between sites is more rapid than the reciprocal of the width of the broad line expressed in $sec^{-1}$, then a composite signal is observed. The line width of the composite signal is given by

$$\Delta\nu = (\Delta\nu_a)\,P_a + (\Delta\nu_b)\,P_b, \qquad (2)$$

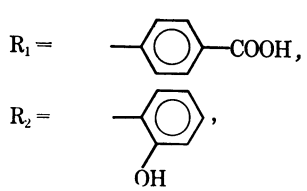
where $\Delta\nu_a$ and $\Delta\nu_b$ are the line widths at sites $a$ and $b$, and $P_a$ and $P_b$ are the probabilities that the nucleus is at sites $a$ and $b$. For intermediate exchange rates, the composite line shape is complex and depends in detail on the exchange rate. It is apparent from equation (2) that if $\Delta\nu_b$ is very large, a very small value of $P_b$ may produce an observable effect on $\Delta\nu$. If, as in the above example, site $a$ corresponds to 1 $M$ $Cl^-$ in aqueous solution where $\Delta\nu_a = 16$ cps, and site $b$ corresponds to the $HgCl_4^=$ with $\Delta\nu_b = 8{,}000$ cps, then $[HgCl_4^=] = 3 \times 10^{-5}$ $M$ will give a 1-cps contribution to $\Delta\nu$. A 1-cps change in a line width of 16 cps is easily measured by standard NMR techniques. From a study of $\Delta\nu$ it is thus possible to detect the presence and accessibility of suitable $b$ sites and to obtain information on the concentration of $b$ sites, $q(b)$, and $\tau_c\,(b)$. In experiments of this type, the chloride ion is used as a probe for interesting sites in low concentrations, and the exchange process functions as a chemical amplifier. The sensitivity of this scheme improves

as the square of $Q$ so that $Br^-$ and $I^-$ are superior probing ions to $Cl^-$. A general list of useful probing ions is easy to compile, depending on the detailed application for the technique.

It is clear that the choice of the exchange site is reasonably restricted. The probing ion must be able to enter the first coordination sphere of the site, and form a sufficiently strong bond to give a large value of $q(b)$. The ion must remain bound for a time long compared with $\tau_c$, but exchange with ions in the bulk solvent must occur in a time short compared with $1/\pi\Delta\nu_b$. Although general criteria are apparently not available for the choice of exchange sites with these properties, at least $Hg^{++}$ and $Pb^{++}$ are suitable.[16] The following discussion will be focussed primarily on results obtained with $Cl^-$ and mercuric compounds, but the general applicability of the technique is by no means limited to these ions.

Since mercuric ion is reasonably selective for the halide binding and exchange process, it is of interest to label specific sites in the biomolecules with mercury, and thus use the halide ion as a probe to examine these sites. Fortunately, mercury has a rich organometallic chemistry. Condensations of $HgCl_2$ and RHgCl with R'SH groups occur readily, providing a variety of methods of binding mercury to functionally important sites in macromolecules. Once the mercury is bound as a label, the properties of the site can be inferred from the halide ion NMR line widths even though the concentration of sites may be very low. A wide range of information on the properties of the macromolecules is available, analogous to the information obtained using spin labels in ESR.[11] It is, of course, not so obvious that the delicate conditions for halide binding and exchange will be maintained for the organomercury derivatives. Preliminary results demonstrating the feasibility and applications of this general scheme are given below.

*Results.*—The line widths of $Cl^{35}$ NMR signals observed for various aqueous solutions are given in Table 1. $Hg^{++}$ ion in low concentration is very effective in broadening the $Cl^{35}$ resonance observed in 2 $M$ NaCl. This result indicates that the orders of magnitude discussed above are appropriate. Two compounds of the type RHgCl, where




also are effective in broadening the $Cl^{35}$ resonance. For both of these examples, the

TABLE 1

$Cl^{35}$ LINE WIDTHS IN AQUEOUS SOLUTIONS

| Molar conc. | Component | Molar conc. | Component | $\Delta\nu - \Delta\nu_{NaCl}$ (cps) | Remarks |
|---|---|---|---|---|---|
| 2.0 | NaCl | 0.001 | $HgCl_2$ | 17 | |
| 2.0 | NaCl | 0.001 | $R_1HgCl$ | 11 | pH < 10 |
| 2.0 | NaCl | 0.001 | $R_2HgCl$ | 30 | pH < 10 |
| 2.0 | NaCl | 0.001 | $R_1$-Hg-$SR_3$ | <1 | |
| 2.0 | NaCl | 0.002 | $R_3S$-Hg-$SR_3$ | 6 | * |

* This value approaches 0 in excess $R_3SH$ as shown in Fig. 2.

pH must be less than about 10 for the line broadening to occur. It is possible that at higher pH the $Cl^-$ ions on the $Hg^{++}$ are displaced by $OH^-$.

The reaction of $HgCl_2$ and RHgCl with sulfhydryl groups is well known, and in fact $R_1HgCl$ has been used to substitute Hg at cysteine sites in protein molecules for X-ray studies.[17] The molecule $R_1$-Hg-$SR_3$ where $R_3$ is $-CH_2$-COOH is readily obtained by condensation of $R_1HgCl$ with $R_3SH$. However, neither this compound nor $R_3S$-Hg-$SR_3$ have any apparent effect on the $Cl^{35}$ line width. More information on these reactions can be obtained by performing an effective titration of $R_1HgCl$ or $HgCl_2$ versus $R_3SH$ using the $Cl^{35}$ line width as an indicator. As shown in Figure 1, the reaction of $R_1HgCl$ with $R_3SH$ seems to go simply to completion. However, the reaction of $HgCl_2$ with $R_3SH$ shown in Figure 2 may involve species of the type $R_3SHgCl$ since $Cl^-$ binding sites are still available in equilibrium with $(R_3S)_2Hg$ at the equivalence point. These results do not appear to be consistent with previous polarographic studies.[18] The lack of a line-broadening effect for these examples can arise if the chloride is blocked from binding with the mercury, if the value of $q$ is very small in the complex, or if the $Cl^-$ is so tightly bound that the exchange is slow. Various routes to the study of this effect include the effects of temperature, variation of $Q$ by using different isotopes of halide ions, and variation of substitutents on the R groups.

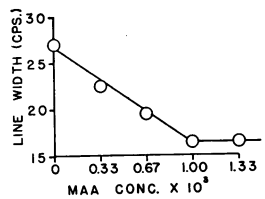



FIG. 1.—Titration of paramercurichlorobenzoic acid (PMCB) with mercaptoacetic acid (MAA); [NaCl] = 2 $M$, [PMCB] = 0.001 $M$.

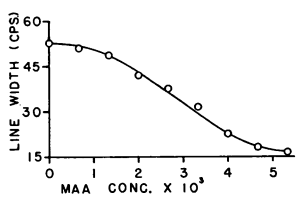



FIG. 2.—Titration of mercuric chloride with mercaptoacetic acid (MAA); [NaCl] = 2 $M$, [$HgCl_2$] = 0.002 $M$.

Reaction of $HgCl_2$ directly with $-SH$ groups on protein molecules is expected to produce sites of the type P-S-HgCl since it is generally sterically awkward to form P-SHgSP′ links. The results of the reaction of $HgCl_2$ with equine hemoglobin (Pentex, Inc., twice crystallized, ferric form, mol wt $\approx$ 68,000) in saline solution are shown in Figure 3. The $Cl^{35}$ resonance of 0.5 $M$ aqueous NaCl is shown in Figure 3$A$. The line width is 16 cps. The $Cl^{35}$ resonance of $7 \times 10^{-5}$ $M$ hemoglobin in the 0.5 $M$ NaCl solution is shown in Figure 3$B$. The line width for this mixture is 22 cps. The addition of $14 \times 10^{-5}$ $HgCl_2$ to this mixture yields a $Cl^-$ line width of 210 cps as shown in Figure 3$C$. The effect of the mercury on the $Cl^{35}$ line width in the presence of hemoglobin is very large. If it is assumed that the mercuric ions react to completion with the two accessible $-SH$ groups on hemoglobin and that $q$ is about the same for the hemoglobin-mercuric chloride complexes and $HgCl_4^=$, then $\tau_c$ for Hg-hemoglobin site must be about 100 times as long as that for the small $HgCl_4^=$ complex. This result is consistent with what might be expected from other estimates of protein $\tau_c$'s.[4, 11]

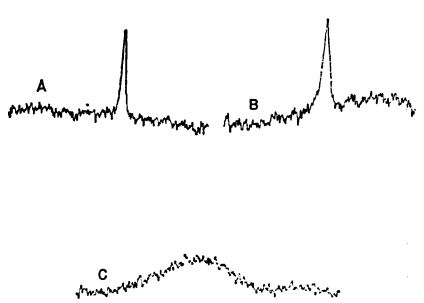



FIG. 3.—Chloride ion resonance in ($A$) 0.5 $M$ NaCl, ($B$) 0.5 $M$ NaCl plus $7 \times 10^{-5}$ $M$ hemoglobin, ($C$) 0.5 $M$ NaCl plus $7 \times 10^{-5}$ $M$ hemoglobin plus $14 \times 10^{-5}$ $M$ $HgCl_2$.

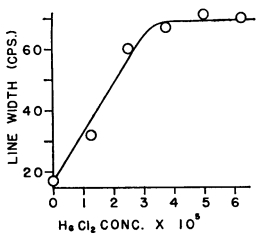



FIG. 4.—Titration of hemoglobin (Hb) with mercuric chloride; [NaCl] = 0.5 *M*, [Hb] = $1.4 \times 10^{-5}$ *M*; pH adjusted to 7.0 with 0.05 *M* phosphate buffer.

The titration of a NaCl-hemoglobin mixture with $HgCl_2$ is illustrated in Figure 4. The end point at a mole ratio $[HgCl_2]/[Hb] = 2$ ($[HgCl_2] = 2.8 \times 10^{-5}$) is reasonably well defined. However, many processes can contribute to this curve; for example, in NaCl solutions hemoglobin will dissociate to some extent[19] and expose normally inaccessible —SH groups. This effect was minimized, but not entirely eliminated, by keeping the concentration of $Cl^-$ relatively low. Furthermore, some of the mercury sites may be trapped in hydrophobic regions of the protein where $Cl^-$ cannot follow, or the mercury sites may be more or less rigidly bound to the protein giving a variation in $\tau_c$.

*Discussion.*—The limiting sensitivity of halide ions as chemical probes for NMR studies of proteins can be estimated from these preliminary results. If it is assumed that $(e^2qQ)$ for the mercuric chloride complex is 40 Mc/sec and $\tau_c$ for the mercury site on a protein is $10^{-10}$ sec, then $\Delta\nu_b$ is approximately $2 \times 10^5$ cps. Thus if $\Delta\nu_a$ for 1 *M* NaCl is 16 cps, a concentration of $5 \times 10^{-6}$ *M* Hg-protein sites gives a 1-cps change in $\Delta\nu$. Since $Q(Br^{79})/Q(Cl^{35}) = 4.14$, a minimum detectable concentration of Hg-protein sites of $3 \times 10^{-7}$ *M* is predicted with the same assumptions if the $Br^{79}$ nucleus is used as a probe. For small values of $[b]$,

$$\Delta\nu - \Delta\nu_a = \frac{[b]}{[a]}(\Delta\nu_b). \tag{3}$$

Thus reducing $[a]$ also improves the sensitivity of the technique. The minimum value of $[a]$ is determined by the sensitivity of the NMR spectrometer for the observed nucleus. This sensitivity is essentially proportional to the square of the nuclear magnetogyric ratio. Since $\gamma(Br^{79})/\gamma(Cl^{35}) = 2.5$, an additional reduction of a factor of 6 in the minimum detectable concentration is available using $Br^-$ as the probing ion. Reduction in $[a]$ of course has additional advantages for the study of solutions of realistic biochemical composition.

The concentrations of Hg-protein sites that can be easily studied with this technique are in a range appropriate to a wide variety of biochemical applications. Changes in gross protein structure that change $\tau_c$ should be easy to follow. Helix-coil transitions as a function of pH or temperature should give rapid changes in $Cl^{35}$ line width. The trapping of the mercury label in hydrophobic regions, or in regions where the motion of an organomercury group is seriously restricted, should also be apparent from the $Cl^{35}$ line width. The line-shape effect of Hg bound to a substrate molecule should be additionally changed when the substrate is incorporated into the active site of an enzyme. Considerable data on biomolecule reaction chemistry should be available through refined versions of the $Cl^{35}$ line-width titrations. These preliminary results and the evident variety in the chemistry of the sites and probes indicate that the use of quadrupolar nuclei as chemical probes has considerable promise as a general tool for the NMR study of biomolecules in solution.

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† On leave from the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts.

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# *THERMODYNAMIC FACTORS IN THE SYNTHESIS OF TWO-STRANDED NUCLEIC ACIDS*

BY LEONARD PELLER*

CARDIOVASCULAR RESEARCH INSTITUTE, UNIVERSITY OF CALIFORNIA (SAN FRANCISCO)

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A thermodynamic analysis of the extents of certain enzyme-catalyzed polymerizations and copolymerizations has been presented earlier.[1, 2] It was one of the principal conclusions of these calculations that only very small extents of polymerization (number average degrees of polymerization less than ten) were realizable at equilibrium for the readily reversible reactions catalyzed by the polysaccharide and polyribonucleotide phosphorylases. Long chains detectable with *in vitro* studies of these systems arise only transiently from kinetic effects in the catalytic process.

More recently, this analysis has been extended and applied to the *de novo* synthesis from nucleoside triphosphates of double-stranded nucleic acids. It has been found that enormous extents of polymerization (equilibrium number average degrees of polymerization of the order of $10^{13}$) are predicted as a consequence of the driving force due to chain association coupled with the opportunity to "push" the