reduced mononucleotide, the C_6H undergoes a larger perturbation (4.4 Hz) compared with C_2H (1.4 Hz). Following the arguments of Schweizer, *et al.*,[59] one may conclude that the preferred conformation around the glycosidic linkage may be anti (II) for β-NMNH. Such conclusion is compatible with the X-ray data on lactate dehydrogenase reduced coenzyme complex.[63]

The conclusion that in β-NMN and β-NMNH the preferred conformations may be syn and anti, respectively, does not imply that the same arrangement may exist in the parent oxidized and reduced dinucleotides, although there is no reason to believe that this may not be the case. Our continuing studies indicate that in β-NMN, β-DPN, and β-TPN, the preferred conformation of the nicotinamide moiety is likely to be syn and that in β-NMNH, β-DPNH, and β-TPNH to be anti. A preliminary communication on this subject has been published from this laboratory[64] and this will be discussed in detail in paper II of this series.

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(63) M. J. Adams, A. McPherson, Jr., M. G. Rossman, R. W. Schevitz, I. E. Smiley, and A. J. Wonacott in ref 9, pp 157–174.

(64) R. H. Sarma and R. J. Mynott, *J. Chem. Soc., Chem. Commun.*, 977 (1972).

Bromide Ion Probe Nuclear Magnetic Resonance Studies of Protein Conformation. Application to Methemoglobin

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Abstract: The application of the bromide ion as an nmr probe of protein conformation has been investigated both theoretically and experimentally. It is shown that the bromide ion has some advantages over the more commonly used chloride ion, the most important being its ability to yield values for the free-to-bound exchange rate constant of the probe itself, in addition to the correlation time for reorientation of the bound probe. It is shown experimentally that the exchange rates are fast, near the diffusion limit, and are sensitive to protein conformational effects near the binding site. Also, each of two equally abundant Br isotopes can be studied for an independent check of the conformational parameters. The bromide probe technique has been tested by measurements of signal-averaged ^{81}Br and ^{79}Br free induction decay constants. The reorientational correlation time for the bromide probe bound *via* mercury to the F9(93)β-sulfhydryl groups of horse methemoglobin decreased from $(1.48 \pm 0.04) \times 10^{-10}$ sec at pH 7.0 to $(0.8 \pm 0.2) \times 10^{-10}$ sec at pH 10, while the halide exchange rate decreased from $(3.27 \pm 0.06) \times 10^7$ to $(1.2 \pm 0.2) \times 10^7$ l. mol^{-1} sec^{-1}. These effects are interpreted in terms of conformational differences between acid and alkaline methemoglobin.

There has been considerable interest in the application of nuclear magnetic resonance (nmr) and other probes to the study of the conformation of proteins.[1] The nmr halide probe method[2] detects changes in the halide nuclear transverse relaxation time, T_2, as a result of halide exchange between symmetrically solvated free halide ion (T_2 long) and a probe site where the quadrupolar halide nucleus is bound tightly to a specific region within a protein (T_2 short). The resulting relaxation time is dependent on the relative concentrations of free and bound sites, and the physical environment of the bound halide. In its most common application, a solution of protein is prepared in aqueous sodium halide, and specific binding sites on the protein may be titrated with a metal such as mercury which binds only to these sites and which subsequently complexes halide from the solution. Stengle and Baldeschwieler have shown[2] that the method leads in principle to the determination of the number of metal binding sites on the protein and the correlation time for reorientation of the halogen–metal bond attached to the protein. A feature of the technique is that small concentrations of labeled protein (10^{-4}–10^{-6} *M*) can cause observable changes in the halogen nucleus T_2, due to a "chemical amplifier" effect originating in the very large difference in the field gradient present at the halogen nucleus in its free and bound sites.

In most halide-probe studies on proteins the number and availability of metal ion binding sites were determined by measurement of the ^{35}Cl steady-state nmr line width. Stengle and Baldeschwieler[3] titrated the sulfhydryl groups of equine and human hemoglobin with mercuric chloride, finding two sites in the native protein. A similar study by Ellis, *et al.*,[4] revealed one and

(1) G. M. Edelman and W. O. McClure, *Accounts Chem. Res.*, 1, 65 (1968).

(2) T. R. Stengle and J. D. Baldeschwieler, *Proc. Nat. Acad. Sci. U. S.*, 55, 1020 (1966).

(3) T. R. Stengle and J. D. Baldeschwieler, *J. Amer. Chem. Soc.*, 89, 3045 (1967).

(4) W. D. Ellis, H. B. Dunford, and J. S. Martin, *Can. J. Biochem.*, 47, 157 (1969).

perhaps two sulfhydryl groups in bovine hemoglobin and one group in horseradish peroxidase. Byrant[5] has reported detecting a 0.5 sulfhydryl group in bovine mercaptalbumin and has evaluated the relative strengths of protein–metal bonding for the group IIb metals. In all three of the above studies, the line width was found to be dependent on pH and the presence of denaturants, presumably reflecting some structural change within the protein. Some nonspecific binding of halide ions to the protein has also been observed; this phenomenon has been studied in carboxypeptidase A and in several other proteins.[6] The binding of zinc as a coenzyme has been demonstrated for carbonic anhydrase[7] and for pyruvate kinase.[8] The ^{35}Cl nmr line-width method has also been used in the titration of the sulfhydryl groups on the erythrocyte membrane.[9]

The present study is an evaluation of the feasibility of using pulsed nmr to observe protein structural changes by following the T_2 of ^{81}Br and ^{79}Br rather than ^{35}Cl nuclei. Pulsed nmr has in principle some advantages over broad-line steady-state nmr techniques. (1) Linewidth measurements by broad-line techniques are subject to possible errors from saturation effects and modulation distortion which are not present in the pulse method. (2) Efficient signal averaging is possible with pulsed nmr. For measurements taken over the same time interval, the pulsed nmr method with signal averaging can thereby yield greater sensitivity than continuous-wave techniques.

The present work shows that, in addition to the correlation time for local motion at the bound site, it is possible in certain cases to obtain the rate of halogen exchange from the bound site. The measured exchange rates are found to be fast, near the diffusion limit, and thus can be dependent on the steric and electrostatic environments of the bound site. The mercuric bromide probe is intrinsically more sensitive to changes in the rate of exchange between the free and bound sites than is the case for the chloride probe. Consequently, the bromide ion can be a more powerful tool for examining protein conformational changes than is the chloride ion.

The sensitivity of the bromide probe to conformational changes in proteins was examined using horse methemoglobin and some other proteins. The hemoglobin molecule has the advantage of having a known tertiary structure based on the X-ray studies of Perutz,[10,11] and a well-studied natural conformational change induced by changing the nature of the ligand on the heme iron. Further, our results can be compared with those obtained by other probes[12–15] attached to the same sulfhydryl group, that of cysteine F9(93)β. In the preliminary study reported here, the bromide probe is shown to be sensitive to the small conformational difference between acid and alkaline methemoglobin. The differences in the correlation times we measured are interpreted in terms of a shift in the equilibrium, postulated by Perutz[10] and Moffat,[13] between the free and immobilized conformations of penultimate tyrosine HC2(145)β near the bound site.

Theory

The dominant spin–lattice relaxation mechanism for a quadrupolar ($I > 0.5$) nucleus is normally the interaction between the nuclear quadrupole moment (eQ) and the fluctuating electrostatic field gradient (eq) at the nucleus, the fluctuation being due to Brownian motion reorientation of the covalent bond to the quadrupolar nucleus, or to changes in the ion pair geometry around the nucleus in the case of a solvated ion. The fluctuation is characterized by a correlation time τ_c usually much shorter than the nuclear Larmor period ($\omega_0\tau_c \ll 1$ —the extreme narrowing limit) and in this limit the spin–lattice decay rate, R_1, is[16]

$$R_1 = \frac{3\pi^2}{10}\frac{2I+3}{I^2(2I-1)}\left(\frac{e^2qQ}{h}\right)^2\tau_c = \kappa\tau_c \qquad (1)$$

for an axially symmetric field gradient, where e^2qQ/h is the quadrupole coupling constant in hertz.

For symmetrically solvated halide ions, the electric field gradient, eq, is relatively small at the nucleus, leading to $R_1(^{35}Cl, I = {}^3/_2)$ values near 50 sec^{-1} in aqueous chloride solutions, and to $R_1(^{81}Br, I = {}^3/_2)$ values near 1200 sec^{-1} in aqueous bromide. The field gradient is much larger in a covalent bond, and, for the mercury complexes $HgCl_4^{2-}$ and $HgBr_4^{2-}$, the R_1 values are estimated at[17] 5×10^4 sec^{-1} for ^{35}Cl and 10^6 sec^{-1} for ^{81}Br.

For moderately fast chemical exchange of a halogen nucleus between a free solvated environment and a covalently bound environment, the time-average nuclear spin–lattice decay rate is the weighted mean of the individual rates R_{1F} and R_{1B} in the two environments

$$R_1 = P_F R_{1F} + P_B R_{1B} \qquad (2)$$

where P_F and P_B are the fractional populations in free and bound environments. Equation 2 is the basis of previous interpretations[2–9] of the chloride probe studies; Stengle and Baldeschwieler[2] have pointed out that the wide spectrum of values in R_{1F} and R_{1B} leads to a "chemical amplifier" effect, in which small concentrations (10^{-4}–10^{-5} M, $P_B \approx 10^{-5}$) of bound environments yield observable effects on the measured decays. Thus the halide probe can be used to study interesting biological sites at low concentrations. However, for the simple equation (2) to be valid, the nuclear lifetime in the bound site against chemical exchange, τ_{BF}, has to be short compared to $T_{1B} = 1/R_{1B}$ and long compared with the correlation time, τ_c, for reorientation of the field gradient in the bound site. Under these conditions the average spin–lattice decay rate R_1 is equivalent to the average transverse decay rate R_2, which is the actual quantity

(5) R. G. Bryant, *J. Amer. Chem. Soc.*, **91**, 976 (1969).
(6) M. Zeppezauer, B. Lindman, S. Forsen, and I. Lindquist, *Biochem. Biophys. Res. Commun.*, **37**, 137 (1969).
(7) R. L. Ward, *Biochemistry*, **8**, 1879 (1969).
(8) G. L. Cottam and R. L. Ward, *Arch. Biochim. Biophys.*, **132**, 308 (1969).
(9) H. E. Sandberg, R. G. Bryant, and L. H. Piette, *ibid.*, **133**, 144 (1969).
(10) M. F. Perutz, *Nature (London)*, **228**, 726 (1970).
(11) M. F. Perutz, *Proc. Roy. Soc. London, Ser. B*, **173**, 113 (1969).
(12) H. M. McConnell, W. Deal, and R. T. Ogata, *Biochemistry*, **8**, 2580 (1969).
(13) J. K. Moffat, *J. Mol. Biol.*, **55**, 135 (1971).
(14) C. Ho, J. J. Baldassare, and S. Charache, *Proc. Nat. Acad. Sci. U. S.*, **66**, 722 (1970).
(15) (a) S. Ogawa, Ph.D. Thesis, Stanford University, 1967; (b) M. A. Raftery, W. H. Huestis, and F. Millett, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 541 (1972); *Biochemistry*, **11**, 1648 (1972).
(16) A. Abragam, "The Principles of Nuclear Magnetism," Oxford University Press, London, 1961, p 314.
(17) D. E. O'Reilly, G. E. Schacher, and K. Schug, *J. Chem. Phys.*, **39**, 1756 (1963).

monitored by line widths in steady-state experiments and by free induction decay rates in pulse experiments.

When the exchange rate is slower than that demanded by eq 2, and $\tau_{BF} \gtrsim T_{1B}$, the average transverse decay rate becomes at least partially limited by the free to bound exchange rate. This situation has been treated by O'Reilly, *et al.*,[17] and their results can be modified for the case of homogeneous exchange of free aqueous halide ion with mercury halide bound to specific sulfhydryl (-SH) sites on a protein molecule

$$X^{*-} + RSHg\text{-}X \underset{k_2}{\overset{k_2}{\rightleftharpoons}} X^- + RSHg\text{-}X^* \qquad (3)$$

Here k_2 is the bimolecular chemical rate constant for the equivalent forward and reverse reactions. If the free halide concentration, [X], is much greater than the concentration [B] of halide bound as RSHg-X, then O'Reilly's result reduces to

$$R_2 - R_{1F} = \left[\frac{1}{k_2[B]} + \frac{1}{R_{1B}}\frac{[X]}{[B]}\right]^{-1} \qquad (4a)$$

$$= [\tau_{BF} + T_{1B}]^{-1}\frac{[B]}{[X]} = \alpha'[B] \qquad (4b)$$

with R_{1B} given by eq 1 applied to the species RSHg-X, and

$$\tau_{BF}^{-1} = k_2[X]$$
$$\tau_{FB}^{-1} = k_2[B] \qquad (5)$$

In the alternate case where the exchange rate is faster than that demanded by eq 2, so that the exchange lifetime for bound halogen, τ_{BF}, becomes comparable to or less than the rotational correlation time of the bound site, τ_c, one must account for the decrease in the spin-lattice relaxation rate due to the additional fluctuation in the electrostatic field gradient accompanying the exchange reaction. This situation has been treated by Marshall,[18a] who finds that eq 1 still applies with replacement of τ_c by an apparent correlation time τ_{app}

$$1/\tau_{app} = 1/\tau_c + 1/\tau_{BF} \qquad (6)$$

The result is to modify the α' of eq 4 to α given by

$$R_2 - R_{1F} = \alpha[B] \qquad (7a)$$

$$\alpha^{-1} = 1/k_2 + 1/\kappa\{[X]/\tau_c + k_2[X]^2\} \qquad (7b)$$

$$\alpha^{-1}/[X] = \tau_{BF} + 1/\kappa\{1/\tau_c + 1/\tau_{BF}\} \qquad (7c)$$

where κ is defined by eq 1. Equations 7a–7c now describe the concentration dependences of the experimental decays over the entire range of exchange rates.

The parameter α defined by eq 7 may be determined experimentally from the measured [B] dependence of the observed halogen nuclear R_2 values. If successive aliquots of HgX_2 solution are added to a solution containing initially known halide and protein concentrations, then α is the slope of the R_2 *vs.* [B] = [added Hg^{2+}] plot, *i.e.*, the slope of the relaxation titration curve up to the end point. This end point is marked by a pronounced decrease in slope on such a plot due to the discontinuous jump from the relatively long correlation times ($\tau_c \approx 10^{-9}$–10^{-10} sec) associated with the bound protein complex RSHg-X being formed below the end point, to the much shorter correlation times ($\tau_c \approx 10^{-11}$–10^{-12} sec) of the simple complexes HgX_4^{2-} being formed beyond.

We note that the α parameter of eq 7 is isotope dependent *via* the isotopic values of eQ carried by the κ. Thus, a useful experimental redundancy can be afforded in the separation of τ_c and τ_{BF} effects by comparison of results from the isotope pairs ^{35}Cl:^{37}Cl or ^{79}Br:^{81}Br.

Consideration of eq 7c leads to three limiting cases for the bound-site lifetime τ_{BF}.

(i) $T_{1F} \gg \tau_{BF} \gg T_{1B}$ (**Slow Exchange Limit**). All halogen nuclei relax during the interval when they are bound to the protein, *i.e.*, the bound site acts as a magnetization sink for spins transferred from the free site where they are observed. The measured α is governed only by the rate of exchange out of the free site.

(ii) $T_{1B} \gg \tau_{BF} \gg \tau_c$ (**Fast Exchange Limit**). If this condition holds, relaxation in the protein site occurs primarily through changes in the direction of the electric field gradient as the protein or a portion thereof tumbles in solution; the molecule tumbles quickly enough so that rotation occurs during the interval that the halide nucleus is bound. In this limit, α is dominated by T_{1B} and thus τ_c and eq 7 reduces to eq 2, in the limit $P_B = [B]/[X] \rightarrow 0$. This case is the one assumed by Stengle and Baldeschwieler.[2,3]

(iii) $\tau_{BF} \ll \tau_c$ (**Extremely Fast Exchange Limit**). The exchange lifetime is so short that appreciable reorientation of the protein cannot occur in this interval; instead relaxation occurs because the direction of the electric field gradient tensor is altered each time there is a change in environment. α is again dependent only on k_2. This limit has not been reached in halide-exchange studies to date.[17]

The three limiting regions resulting from eq 7a–7c become apparent if α is plotted as a function of the exchange rate for ^{35}Cl, ^{81}Br, and ^{127}I (Figures 1–3). For slow exchange ($k_2 \sim 10^5$, 10^6) and long correlation times ($\tau_c \sim 10^{-9}$, 10^{-10}), α is dependent only on k_2 (linear portion of Figures 1–3) and measurements of α under these conditions can only yield values for the exchange rate. The extremely fast exchange limit (τ_c long, k_2 large) presents a similar case where only k_2 can be determined. In the fast exchange limit, α is dependent exclusively on τ_c (flat regions of Figures 1–3) and only this quantity can be determined.

Intermediate regions prove to be the most informative, with measures of α under these conditions yielding values for both k_2 and τ_c from the [X] dependence of α^{-1}. Bromide-mercury complexes (Figure 2) fall between the slow and fast exchange limits, where eq 7 becomes eq 4. O'Reilly, *et al.*,[17] have obtained values of $(3.7 \pm 0.4) \times 10^7$ l. mol^{-1} sec^{-1} for k_2, and $(1.46 \pm 0.07) \times 10^6$ sec^{-1} for R_{1B} in the $[HgBr_4]^{2-}$ ion at 27°. The latter quantity places τ_c for this complex at 3.6×10^{-12} sec, assuming[17] a quadrupolar coupling constant of 320 MHz. Under the experimental conditions encountered in this work, hemoglobin-bound bromide was found to have exchange rates at 23° which placed it in this intermediate region also. This result is of some importance, as it is then possible with a bromide probe to measure two parameters characteristic of a protein binding site: the local correlation time and the exchange rate, the former quantity providing a measure of the rigidity of the sulfhydryl side chain and the latter indicating the

(18) (a) A. G. Marshall, *J. Chem. Phys.*, 52, 2527 (1970); (b) J. C. Mensch, private communication, 1969.

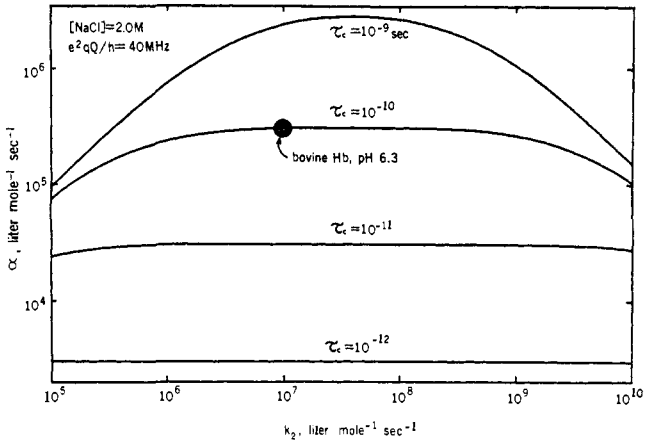

Figure 1. Calculated dependence of α on the rate of halide exchange for 2.0 M chloride. Literature values of k_2[17] and τ_c[4] for bovine hemoglobin place these quantities within the cross-hatched area.

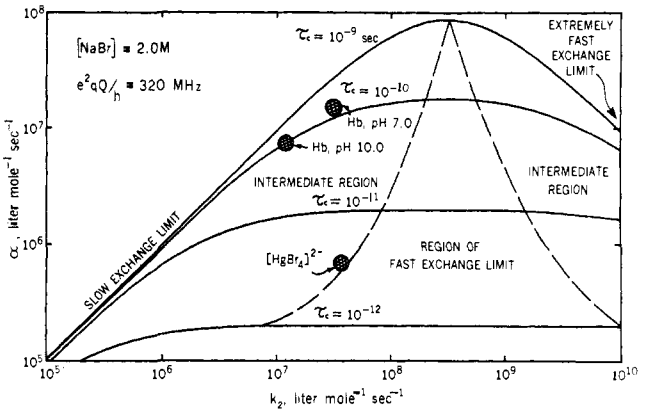

Figure 2. Calculated dependence of α on the rate of halide exchange for 2.0 M bromide. Literature values[17] of k_2 and τ_c for the mercuric tetrabromide ion place these quantities within the cross-hatched area (α corrected for the four sites available for exchange). Present values of k_2 and τ_c for equine methemoglobin at pH 7.0 and 10.0 have been included for comparison.

accessibility of that site to the surrounding environment. Iodide–mercury exchange processes may be expected to show behavior analogous to that of the bromide complex. For the $[HgI_4]^{2-}$ complex, k_2 has been estimated[17] to be $(8.8 \pm 1.8) \times 10^7$ l. mol^{-1} sec^{-1} at 27°, with $\tau_c = 1.4 \times 10^{-11}$ sec. These values place the iodide exchange process also inside the intermediate region between the slow and fast exchange limits (Figure 3).

However, it is likely that only with the bromide probes can both k_2 and τ_c be easily measured for sulfhydryl groups by this method, since the larger quadrupole moment for the ^{127}I nucleus combined with the greater polarizability of the solvated I^- ion reduces the T_2 value for ^{127}I in aqueous iodide solution to the inconveniently short time of around 170 μsec.[17] Accurate measurements of the reduction of this value by a bound iodide probe are difficult to attain, and this fact attests the virtuosity of earlier workers.[17]

The correlation times estimated by Mensch[18b] for papain and Ellis[4] for bovine hemoglobin are of the same order as those measured here, 10^{-10} sec, and it seems likely that this is a typical value for sulfhydryl-bound halide probes. If the halide exchange rate is 9×10^7, 4×10^7, and $\sim 10^7$ l. mol^{-1} sec^{-1} for I^-, Br^-, and Cl^-, respectively,[17] this correlation time would situate bromide and iodide exchange well into the intermediate region between the slow and fast exchange limits (Figures 2 and 3) while placing chloride exchange nearly at the fast exchange limit (Figure 1).

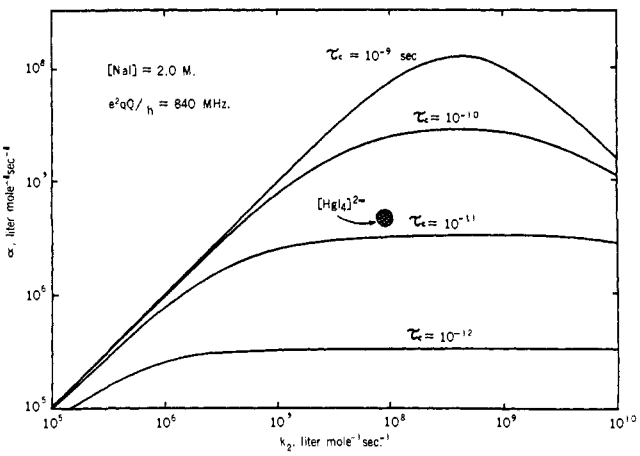

Figure 3. Calculated dependence of α on the rate of halide exchange for 2.0 M iodide. Literature values[17] of k_2 and τ_c for the mercuric tetraiodide ion place these quantities within the cross-hatched area (α is corrected for the four sites available for exchange).

The behavior of chloride complexes should deviate significantly from the fast exchange limit only at correlation times of the order of 10^{-9} sec or longer. The information available from bromide exchange is therefore potentially greater than that obtainable from chloride exchange.

Experimental Section

Apparatus and Methods. The 15-MHz ^{81}Br and ^{79}Br nmr signals were obtained as signal-averaged phase-detected free induction decays by standard pulse techniques using a Varian 12-in. magnet, NMR Specialties PS-60A pulse unit, and a home-built probe.[19] The spectrometer was not field-frequency locked. For the ^{81}Br resonance from 2 M aqueous NaBr solution and 1-μsec output time constant, the final signal available at the beginning of a single free induction decay was typically 2 V with a signal-to-noise ratio of 10:1. With signal averaging, it was possible to obtain usable R_2 values from NaBr solutions with concentrations down to 0.5 M. Figure 4 shows typical raw and averaged signals from NaBr in the concentration range 0.5–2.0 M. The samples were contained either in Wilmad polished high resolution nmr tubes of 15 mm o.d. and 1 mm wall thickness or in 15 mm test tubes. The sample was not spun.

The value of the bromide relaxation time could be obtained to within 15% from photographs of direct display of decay signals on a Tektronix 549 storage oscilloscope. The precision in all relaxation time measurements was increased by signal averaging. Usually 512–2048 scans were performed using a Fabritek 1064 signal averager with SW-2 sweep plug-in and SD-2 digitizer operating at 10 bit resolution. These plug-ins were limited to a minimum dwell time per channel of 50 μsec, rather longer than optimum for dealing with the moderately small values of ^{81}Br and ^{79}Br T_2 values experienced here (250–750 μsec).

Errors in the measured decay rates due to H_0 inhomogeneity were minimized by shimming the magnet homogeneity on the sharp nearby ^{23}Na resonance. The resonance condition was tuned by maximizing the decay amplitude over the time interval between one and two T_2 values.

Within experimental error, the measured decays were exponential over at least two T_2 intervals. R_2 was calculated from the time interval between two points with known amplitude ratio on the signal averaged decay curve. The measurement was repeated several times,

(19) A. Brooke, M. Kaplansky, and E. J. Wells, unpublished results.

retuning to resonance each time. The first points of the decay were excluded from the interval to avoid effects from pulse feed-through.

Methemoglobin was titrated in 0.5–3.0 *M* NaBr at pH 7.0 (0.05 *M* sodium phosphate buffer) and 0.5–2.0 *M* NaBr at pH 10.0 (0.05 *M* sodium bicarbonate buffer). Oxford micropipets were used to add aliquots of $HgCl_2$, which was used instead of $HgBr_2$ because of its greater solubility. The sample volume was 10 ml and the temperature was 23 ± 1°. At pH 7.0, two titrations were performed for each halide concentration, with the value of R_2 for each point on the titration curve being the average of decay-rate measurements on two separate hemoglobin samples. Titrations at pH 10.0 were repeated four times for each halide concentration. In order to maintain the decay rate within the range where accurate measurements could be performed, methemoglobin and $HgCl_2$ concentrations were made proportional to the halide concentration. Values of α used in the determination of T_{1B} and k_2 were calculated by a linear least-squares fit of the titration data.

Some difficulty was experienced with the solubility of the methemoglobin at pH 7.0. The solutions became turbid after approximately 5 min in 3 *M* NaBr and 20–30 min in 0.5 *M* NaBr. Separate fresh solutions were therefore used for each measurement. At pH 10.0, methemoglobin solutions in 0.5–2.0 *M* NaBr showed no sign of turbidity for over 2 hr. During this period, measurements of R_2 performed on a sample of 4.1×10^{-5} *M* methemoglobin made 5×10^{-5} *M* in $HgCl_2$ did not detect any changes in R_2 exceeding the error in the decay rate measurement. At this pH, therefore, titrations could be performed by adding successive portions of $HgCl_2$ to a hemoglobin solution. Protein instability precluded titrations in 3.0 *M* NaBr at pH 10.0; under these conditions, methemoglobin solutions became turbid shortly after mixing, and precipitation of the majority of the protein occurred within minutes.

Materials. Twice-recrystallized equine methemoglobin, lot no. U3770, prepared by the method of Drabkin[20] was purchased from Mann Research Laboratories, and stored at 4° in a desiccator over anhydrous magnesium perchlorate. A similar product purchased from Pentex Inc. was used initially but was abandoned because of variations in the sulfhydryl content between different shipments. All of the hemoglobin samples from Mann were mixed together thoroughly to ensure homogeneity of the sample. All other chemicals were reagent grade. The hemoglobin content was determined from the absorbance at 540 nm of the cyanomethemoglobin prepared according to the method of Wootton and Blevin,[21] assuming an absorptivity of 11.0×10^3 cm^2 mol^{-1}.[22] The assay of three portions of Mann hemoglobin yielded a hemoglobin content of 81 ± 2%. All hemoglobin concentrations reported here have been corrected to this hemoglobin content. Gel filtration of the hemoglobin on Sephadex G-75 revealed identical elution profiles for the heme (405 nm) and protein (275 nm) absorbances, suggesting that no significant amount of proteinaceous impurities was present.

Absorption spectra recorded using a Cary-14 spectrophotometer showed the heme to be in the ferric form. No significant change in the visible spectrum occurred on the addition of a 100-fold excess of ferricyanide, indicating that the amount of oxyhemoglobin present was negligible.

The pK for interconversion of acid and alkaline methemoglobin was determined spectrophotometrically at λ 575 nm to be approximately 8.5 in 2 *M* NaBr at 23°. This is in agreement with the value obtained by extrapolation of pK values previously observed[23] at ionic strengths below 1.0 with various other salts. Thus, the high NaBr concentrations used in the bromide nmr experiments do not cause any marked changes in the binding of OH^- by the heme.

Amperometric titrations with mercuric ion[24] and spectrophotometric titrations using *p*-mercuribenzoate[25] were done, varying the solvent conditions, to assure that the sulfhydryl reactivity was not affected by high bromide concentrations, and to check that the bromide nmr probe was indicating accurately the sulfhydryl titer. With the Mann methemoglobin, both methods revealed a titer in the range of 1.1–1.4 per tetramer (mol wt 64,500) which did not depend significantly on the solvent ionic strength or type of anion. In 0.05 *M* phosphate buffer, pH 7.0, and 2 *M* in NaBr the amperometric method indicated 1.2 SH groups (*p*-mercuribenzoate precipitated at this ionic strength). Since there are normally two reactive sulfhydryls per tetramer of horse hemoglobin, it appears that some of the cysteines have become oxidized in this commercial preparation, as has previously been noted for papain.[26]

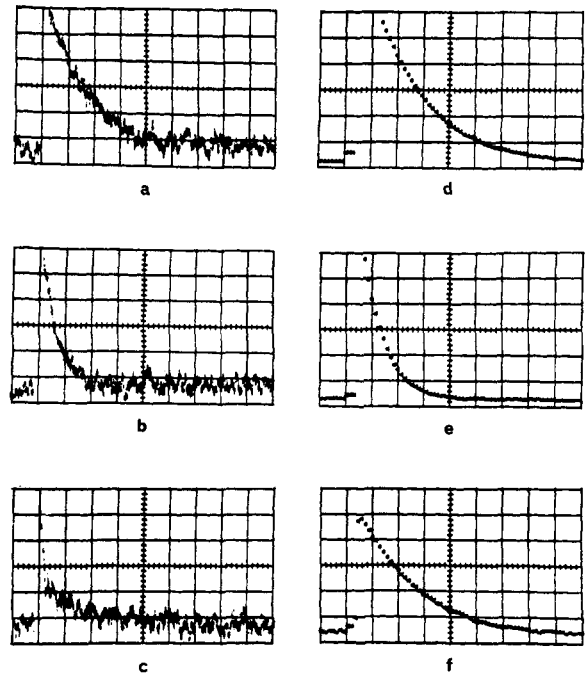

Figure 4. Typical raw and averaged ^{81}Br free-induction decays from aqueous NaBr solutions: a–c, raw signal; vertical scale, 1.4 V/division; horizontal scale, 500 μsec/division; d–f, averaged signal; vertical scale, 1.25 V/division; horizontal scale, 50 μsec/channel (~350 μsec/division). (a) 2 *M* NaBr; (b) 2 *M* NaBr + 7×10^{-6} *M* $HgCl_2$; (c) 0.5 *M* NaBr; (d) 2 *M* NaBr, average of 512 sweeps; (e) 2 *M* NaBr + 7×10^{-5} *M* $HgCl_2$, average of 512 sweeps; (f) 0.5 *M* NaBr, average of 1024 sweeps.

Results

Nmr titrations of methemoglobin with mercuric ion in the presence of 2.0 *M* NaBr at pH 7.0 and 10.0 are shown in Figure 5. The end points indicate approximately 1.3 reactive sulfhydryls per tetramer at pH 7 and approximately 1.1 per tetramer at pH 10; these are in the range of values we found by the amperometric and spectrophotometric methods (see Materials).

Measurements of the slope α should not be affected by the low sulfhydryl titer, and hence the τ_c and k_2 derived from it are also not affected. In 2 *M* NaBr (Figure 5), α is 1.7 times greater for acid methemoglobin than for alkaline methemoglobin; however, α for $HgBr_4^{2-}$ (above the end point) is the same at both pH values. In other experiments[27] we have observed α for $HgBr_4^{2-}$ to be independent of pH over the range pH 2–11.

The contributions of k_2 and R_{1B} to the values of α for the sulfhydryl groups were calculated from the dependence of α^{-1} on [Br^-], in Figure 6, according to eq 4. A least-squares fit of the data in Figure 6 produced values of k_2 and T_{1B} given in Table I. Stated errors represent one standard deviation. Values for the correlation time of the bound halide nucleus were calculated assuming[17] an ^{81}Br quadrupolar coupling constant of 320 MHz.

An independent check on the calculated values of k_2

(20) P. B. C. Drabkin, *Amer. J. Med. Sci.*, **209**, 268 (1945).
(21) I. D. P. Wootton and W. R. Blevin, *Lancet*, No. 7357, 434 (1964).
(22) D. A. Morningstar, G. Z. Williams, and P. Suutarinen, *Amer. J. Clin. Pathol.*, **46**, 603 (1966).
(23) P. George and G. Hanania, *Biochem. J.*, **55**, 236 (1953).
(24) I. M. Kolthoff, W. Stricks, and L. Morren, *Anal. Chem.*, **26**, 366 (1954).
(25) P. D. Boyer, *J. Amer. Chem. Soc.*, **76**, 4331 (1954).
(26) L. A. Sluyterman, *Biochim. Biophys. Acta*, **139**, 430 (1967); I. B. Klein and J. F. Kirsch, *Biochem. Biophys. Res. Commun.*, **34**, 575 (1969).
(27) T. R. Collins, Z. Starčuk, E. J. Wells, and A. H. Burr, unpublished results.

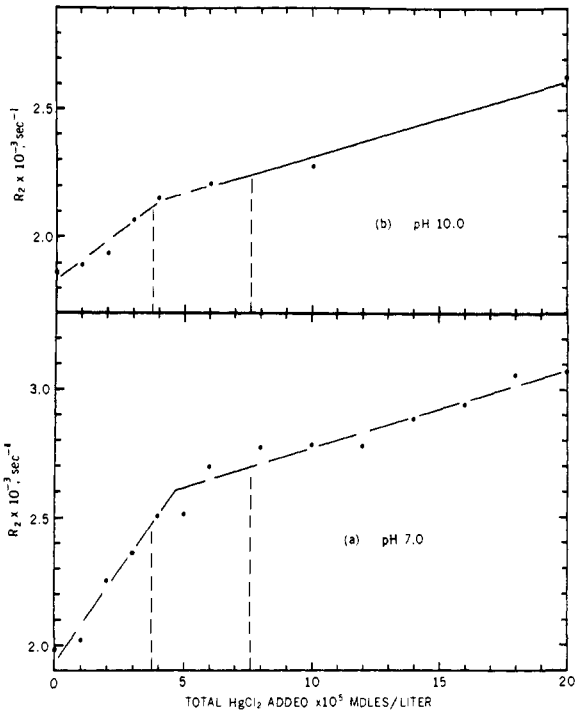

Figure 5. Titration of 3.8×10^{-5} M equine methemoglobin in 2.0 M NaBr: (a) 0.05 M phosphate buffer, pH 7.0, $\alpha = (1.38 \pm 0.2) \times 10^7$ l. mol^{-1} sec^{-1}; (b) 0.05 M bicarbonate buffer, pH 10.0, $\alpha = (0.8 \pm 0.1) \times 10^7$ l. mol^{-1} sec^{-1}. Vertical dashed lines represent the $HgCl_2$ concentration at which the first and second sulfhydryl groups would be titrated. Each point is the average of two titrations. Estimated error in R_2 is 100 sec^{-1} at pH 7.0 and 40 sec^{-1} at pH 10.0.

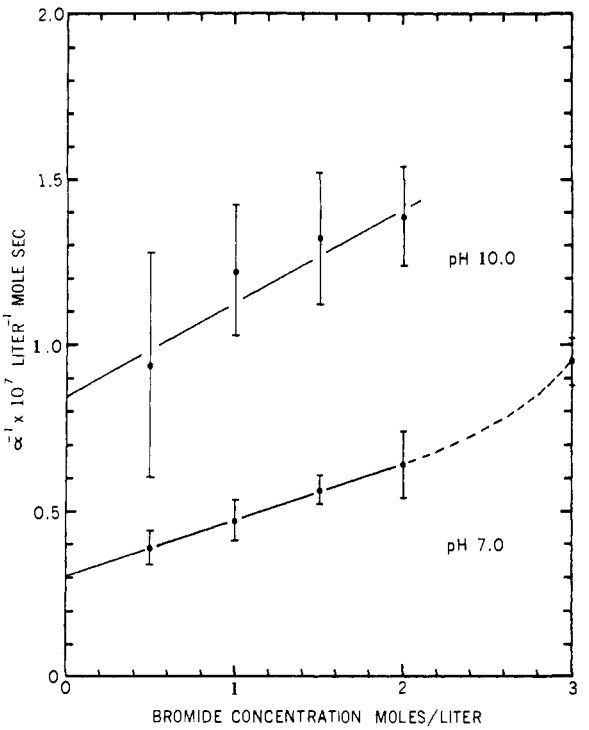

Figure 6. Bromide concentration dependence of α^{-1} for equine methemoglobin at pH 7.0 and 10.0. Error bars represent the greater of either the average of the standard deviation of the individual α values, or the range of values of α. The solid line is the least-squares fit to the data, excluding the 3 M pH 7 data point.

and τ_c was obtained by measurement at pH 7.0 with ^{79}Br. Although instrumental difficulties precluded extensive measurements on ^{79}Br, two titrations in 2 M NaBr yielded an average α value of $(1.65 \pm 0.2) \times 10^7$ l. mol^{-1} sec^{-1} for ^{79}Br. Assuming that the exchange rate for the bound probe at pH 7.0 is the same for both ^{81}Br and ^{79}Br, it is possible to estimate the magnitude of T_{1B} and τ_c for ^{79}Br from this value of α (Table I). The estimated values of τ_c for ^{79}Br are in reasonable agreement with correlation times calculated for ^{81}Br. The small values of τ_c deduced ($\sim 10^{-10}$ sec) and the apparent consistency of the separation of the exchange and reorientation effects as determined from the two Br isotopes validate the extreme narrowing approximation made for the nuclear relaxation in the bound site.

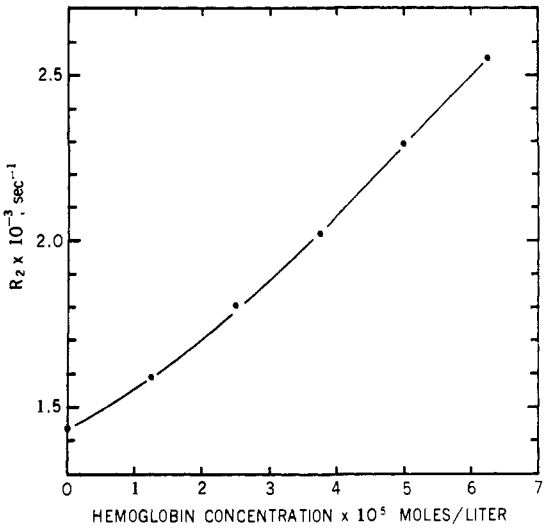

Figure 7. Nonspecific binding of bromide in the absence of Hg. Methemoglobin samples dissolved in 0.5 M NaBr in 0.05 M phosphate buffer, pH 7.0. Estimated error in R_2 is 80 sec^{-1}.

Table I. Experimental Values of k_2 and T_{1B}

Isotope	pH	$k_2 \times 10^{-7}$, l. mol^{-1} sec^{-1}	$T_{1B} \times 10^8$, sec	$\tau_c \times 10^{10}$, sec
^{81}Br	7.0	3.27 ± 0.06	1.68 ± 0.04	1.48 ± 0.04
^{81}Br	10.0	1.2 ± 0.2	3 ± 1	0.8 ± 0.2
^{79}Br	7.0	3.27[a]	1.5 ± 0.1	1.1 ± 0.3

[a] Assumed value.

Besides halide exchange at the bound mercury atoms, hemoglobin was found to exhibit nonspecific binding of bromide ions, as evidenced by the increase in the decay rate with increasing protein concentration shown in Figure 7. Zeppezauer, *et al.*,[6] concluded that this phenomenon is the result of quadrupolar relaxation resulting from interactions between bromide ions and undetermined groups on the surface of the protein. Binding of Br^- to the Fe of methemoglobin is slight[28] even in the presence of 3 M NaBr. Viscosity measurements performed by us showed that the increase in viscosity with increasing protein concentration contributes only slightly to this change in R_2. Rather, the nonlinearity in Figure 7 is probably due to dissociation into subunits at the lower protein concentration.

Papain (EC 3.4.4.10) was titrated by this method but no end point was seen. It is likely that the single free sulfydryl group[29] had been oxidized to a mixed disulfide

(28) H. Uchida, L. J. Berliner, and M. H. Klapper, *J. Biol. Chem.*, **245**, 4606 (1970).

(29) J. Drenth, J. N. Jansonius, R. Kockock, H. M. Swem, and B. G. Wolthen, *Nature (London)*, **218**, 929 (1968).

with traces of cysteine present in the preparation.[26] Mensch[18b] titrated a partially active preparation of this enzyme using ^{35}Cl nmr, calculating a correlation time of 10^{-10} sec for the bound chloride probe. We found ovalbumin to bind 2 equiv of mercury in sites where exchange cannot occur, resulting in titration curves similar to those that we have observed[27] in some low molecular weight thiols.

Discussion

The correlation time of 1.48×10^{-10} sec calculated at pH 7.0 is in agreement with the value of 10^{-10} sec estimated by Ellis[4] (using the Cl^- probe) for the sulfhydryl groups of bovine hemoglobin at pH 6.3. These correlation times obtained by the nmr halide-probe method are shorter by two orders of magnitude than those found by other methods; Haugland and Stryer,[30] for example, used depolarization of fluorescence to calculate a rotational correlation time of 5×10^{-8} sec for anthraniloyl chymotrypsin. It is apparent that the nmr probe is indicating motion at the binding site rather than rotation of the macromolecule as a whole. The decrease of τ_c from 1.48×10^{-10} sec at pH 7.0 to 0.8×10^{-10} sec at pH 10.0 must result from enhanced motion of the 93β-cysteine at the higher pH.

Of some consequence is the fact that the bromide exchange rate constants calculated here for protein-bound mercury are close in magnitude to those found for the mercury tetrabromide ion, since the latter rates are close to the theoretical diffusion limit.[17] In this limit, the rate constant does not depend on the chemistry of the mercury–bromide bond (the mercury–sulfur bond is not dissociated above pH 5[27,31]) but is sensitive only to steric or electrostatic effects limiting the rate of diffusion to and from the binding site; thus it could reflect the configuration and charge in the neighborhood of the binding site in a protein. Thus, the k_2 measured in the present experiments, as well as τ_c, are undoubtedly changed by conformational differences between acid and alkaline methemoglobin. By comparison, we found the α value of the tetrabromide ion to be independent of pH.

The structural models of Perutz[10] show that there are major differences in tertiary structure between deoxyhemoglobin in which (high spin) Fe^{2+} lies about 0.8 Å out of the plane of the heme, and acid methemoglobin in which the (high spin) Fe^{3+} lies about 0.3 Å out of the plane. The conformation of oxyhemoglobin, with (low spin) Fe^{2+} lying in the plane of the heme, Perutz found[32] to be essentially identical with that of methemoglobin. Perutz concluded that the small movement of the iron upon conversion of deoxyhemoglobin to oxyhemoglobin is propagated to the surface of the subunits in the neighborhood of the C terminal residues. There is a pocket between the H and F chains of each subunit in which the side chain of the penultimate tyrosine is firmly bound in the deoxy conformation; this becomes narrower in oxyhemoglobin, and the tyrosine chain is in equilibrium between the bound position and a free configuration. The resulting destabilization of the subunit conformation leads to the breaking of salt bridges between the subunits, and provides an explanation for cooperativity and the Bohr effect.[10]

The cysteine F9(93)β which reacts with the Hg in the nmr titration of hemoglobin is ideally located for probe studies. This amino acid is next to proximal histidine F8(92)β, whose imidazole side chain is coordinated with the iron of the heme. Movement of Fe relative to the heme can therefore be expected to alter the position of the probe relative to its surroundings. Further, the tyrosine pocket between helices F and H is only a few ångströms away from F9(93)β cysteine.[11] A probe attached to the cysteine may therefore sense the relative occupancy of the bound and free positions of tyrosine.

The latter was shown elegantly by McConnell and others with spin labeled probes linked to the cysteine by iodoacetamide. The esr spectra in the case of methemoglobin and liganded hemoglobin consists of two components, A and B, corresponding, respectively, to strongly immobilized and weakly immobilized isomeric states of the probe.[12] It was shown by X-ray difference Fourier techniques that the spin label in the A state occupies the tyrosine pocket and in the B state is displaced by tyrosine and lies free in solution on the surface of the β subunits.[13] The proportion in the two states appears to be dependent on the amount of displacement of iron from the plane of the heme; the fraction of label in state B is 1.0 in deoxyhemoglobin, 0.45 in acid methemoglobin, and 0.3 in liganded hemoglobins.[12,13] With one of the probes used, isosbestic points are not seen in esr spectra when cooperative binding of ligands occurs, but are observed on addition of cyanide and azide ligands to acid methemoglobin,[33,34] and also OH^- ligand to acid methemoglobins, the fraction of B decreasing with pH.[14] The spectra of spin-labeled CO-hemoglobin and azide methemoglobin, which have the same heme iron displacement, are identical. Likewise, spin-labeled acid methemoglobins and fluoride methemoglobins have the same spectrum.[12] Thus, it appears that the less the displacement of heme iron from the plane of the ring, the smaller is the proportion of penultimate tyrosine in the bound state.

Interpretation of our results on acid and alkaline methemoglobins leads to the same conclusion. The observed α value would be expected to be the average of the α of each state at equilibrium, and the conversion from acid to alkaline methemoglobin would correspond to a decrease in the proportion of the tyrosine-bound conformation. The linear S–Hg–Br complex, however, is shorter and smaller than the spin labels, and would not be expected to exchange with tyrosine for the pocket between the F and H helices. Rather, the probe should more likely indicate the state of the cysteine to which it is bound. The probe should be *less* mobile in the tyrosine-bound conformation of the protein, as it is reasonable to expect that motion of the F helix and cysteine F9(93)β is constrained in that conformation by van der Waals contacts with bound tyrosine.[10] Since the observed (average) τ_c is shorter for alkaline methemoglobin, it follows that the proportion of the tyrosine-bound conformation must be lower for alkaline than for acid methemoglobin.

(30) R. P. Haugland and L. Stryer, "International Symposium on the Conformation of Biopolymers," Vol. I, Academic Press, New York, N. Y., 1967, p 321.
(31) W. Stricks and I. M. Kolthoff, *J. Amer. Chem. Soc.*, **75**, 5673 (1953).
(32) M. F. Perutz and L. F. Ten Eyck, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 295 (1972).
(33) S. Ogawa, A. Horwitz and H. M. McConnell, 1968, unpublished data cited in ref 12.
(34) V. J. Di Pasquale and C. Ho, unpublished results cited in ref 14.

The above interpretation, however, cannot be distinguished from one in terms of a pH-dependent conformational change not linked to the change in the ligand and spin state of the heme Fe^{3+}. Such a possibility is indicated by other probe studies on oxyhemoglobin[15a,33] and must be checked using other ligands. Further experiments should also lead to an adequate explanation of the lower exchange rate constant observed at higher pH. Since the exchange reaction is diffusion limited, and hence sensitive to steric or electrostatic effects, one could expect that either the positions of the C-terminal residues or the ionization of residues in the neighborhood of cysteine F9(93)β should affect the exchange rates.

It is evident that measurement of the exchange rate and correlation time with the bromide probe may yield considerable new information concerning the nature of the conformation at specific sites in macromolecules. Application to the study of the conformational changes near cysteine F9(93)β of hemoglobin has supplied information which apparently is complementary to that previously obtained at that site with esr probes. The smaller mercury bromide nmr probe may not compete with penultimate tyrosine for the tyrosine pocket, as do the esr probes, and thus may give more realistic information on the parent cysteine site. Continued work with this probe, at the least, should provide independent confirmation of the esr probe conclusions, and in addition may indicate the influence of bulky or charged groups near the site on the accessibility of bromide to the mercury.

Potential problems in the application of this probe, as indicated by the present study, may include protein instability at the higher ionic strengths, and dependence of the conformation near the probe on ionic strength or bromide concentration. A more sensitive apparatus, now being tested, should enable future studies to be done at lower concentrations of bromide.

Acknowledgments. We thank Dr. M. Kaplansky and Mr. A. Brooke for help in the construction of the nmr probe. The pulse spectrometer was purchased through grants (to E. J. W.) from the National Research Council of Canada and the Canadian Donner Foundation. This work has been supported by the National Research Council of Canada through operating grants to A. H. B. and E. J. W., and by an N.R.C. postgraduate scholarship to T. R. C.

Communications to the Editor

General Syntheses for New Pentadentate Ligands. The Crystal Structure of α,α'-{2-(2'-Pyridyl)ethyl}-ethylenebis(salicylideniminato)cobalt(II)-Ethanol

Sir:

Some biological systems impose upon a metal a pentacoordinate environment consisting of an unsaturated planar tetradentate group and a heterocyclic base. Notable examples are hemoglobin[1] and myoglobin[2] in which the globin protein provides a pocket for the porphyrin complex in such a way that an imidazole group on the protein is positioned as an axial ligand affording square-pyramidal coordination. Another example of this situation is vitamin B_{12}[3] in which the fifth ligand, 5,6-dimethylbenzimidazole, is chemically attached to the corrin affording similar coordination geometry. Synthetic pentadentate ligands capable of assuming this geometry are rare.[4] We wish to report a general scheme for the preparation of such ligands and an X-ray diffraction study of a typical complex.

Our synthetic strategy is based on the observation that vicinal diamines such as 1,2-ethylenediamine (en) and 1,3-propylenediamine (pn) when condensed with carbonyl derivatives (salicyladehyde, 1,3-diketones, oximino ketones, etc.) afford unsaturated tetradentate ligands capable of planar coordination. We, therefore, prepared a series of 1,2-en and 1,3-pn derivatives containing pyridine groups appended by alkyl chains of lengths likely to permit five-coordination (**1** and **2** in Scheme I). These schemes were based on the premise that aldehyde and malonic amide derivatives could serve as antecedents of en and pn groups, respectively. The procedures were cumbersome but uneventful, the only difficulty arising from finding suitable neutral reducing conditions to transform malonic amide derivatives into the pn group without interference from the acidic C–H group. Diborane sufficed.

Condensation of salicylaldehyde with **1a** afforded the expected pentadentate ligand which was transformed (using the divalent metal acetates under N_2) into its Co(II) and Fe(II) complexes.[5]

X-Ray structure analysis was used to establish pentacoordination and investigate its stereochemical consequences. The compound α,α'-{2-(2'-pyridyl)ethyl}-ethylenebis(salicylideniminato)cobalt(II)-ethanol, $CoC_{25}N_3O_2H_{21}\cdot C_2H_5OH$, was selected for this work. It crystallizes in space group $P\bar{1}$ of the triclinic system with four molecules in a unit cell of dimensions a = 15.049(2), b = 15.127(2), c = 10.258(1) Å; α = 90.53(1), β = 93.53(1), and γ = 105.81(1)°. Least-squares refinement of positional and isotropic thermal parameters for the two independent formula units has led to a conventional R factor of 0.064 for 1237 reflections having $F^2 > 3\sigma(F^2)$.

The two independent, separate, neutral, complex molecules display the same distorted trigonal-bipyr-

(1) M. F. Perutz, *Nature (London)*, **228**, 726 (1970).
(2) J. C. Kendrew, *Science*, **139**, 1259 (1963).
(3) C. Brink-Shoemaker, D. W. J. Cruickshank, D. C. Hodgkin, M. J. Kamper, and D. Pilling, *Proc. Roy. Soc., Ser. A*, **278**, 1 (1964).
(4) W. M. Coleman and L. T. Taylor, *J. Amer. Chem. Soc.*, **93**, 5446 (1971). For this example experimental evidence concerning coordination of the fifth ligand (a secondary amine) is equivocal.
(5) All of these compounds gave acceptable elemental analyses.