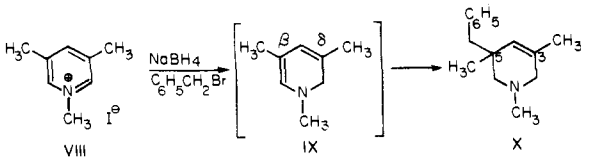
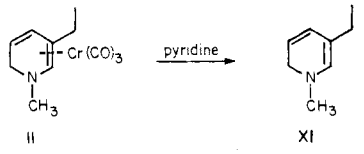
Thus in the above studies, the pyridinium iodide, initially present in the aqueous phase, is reduced and the resulting dihydropyridine III is transferred to the organic phase whereupon it reacts immediately with benzyl bromide to form a salt. The latter intermediate then transfers to the aqueous layer and is reduced with borohydride and the end product finally transfers to the organic layer from where it is extracted. These rapid transfers of the intermediates undoubtedly minimize side reactions in these studies. All of our earlier investigations, prior to the utilization of this technique, led essentially to intractable tars.

The above studies provided the first clear indication that the 1,2-dihydropyridine III was being alkylated at the β-position and in that sense was similar in reaction to that of dienamines studied previously.[4]

To extend the alkylation studies to dihydropyridines bearing an alkyl substituent at the β-position, the above procedure was applied to *N*-methyl-3,5-dimethylpyridinium iodide (VIII) and the C-alkylated product X was isolated in overall 48% yield. In this instance it was unnecessary to expose the reaction mixture to the debenzylation procedure mentioned above. The nmr data, δ 0.86 (C_5-CH_3, s), 1.16 (C_3-CH_3, s), 2.11 (C_6-CH_2, q), 2.28 (N-CH_3, s), 2.63 ($CH_2C_6H_5$, s), 2.71 (C_2-CH_2, q), 5.11 (olefinic, s), and 7.15 (aromatic), and the mass spectrum with fragments at *m/e* 215 (M^+), 172, 158 and 157, (base peak) 124, 123, 122, and 91 substantiated the structure X for the alkylation product.



It was now desirable to prepare the novel 1,6-dihydropyridine system XI for further studies. From various investigations conducted in our laboratory it was not possible to obtain XI directly *via* reduction of *N*-methyl-3-ethylpyridinium iodide. The chromium complex II obtained previously[1] was of particular value here and indeed represents presently the only synthetic route available to such systems. Thus reaction of II with pyridine at room temperature affords a product for which spectral data allow assignment of the expected structure XI. The nmr spectrum (100 MHz, C_6D_6)



with signals at δ 1.02 (CH_3CH_2, t), 1.98 (CH_3CH_2, q), 2.24 (N-CH_3, s), 3.50 (C_6-H, d), 5.18 (C_5-H, m), 5.52 (C_2-H, s), and 5.58 (C_4-H, d) and the uv spectrum (λ_{max} 332 nm) were fully in accord with expectation. Studies on the alkylation and other reactions on XI are now in progress.

In conclusion the above investigations have provided some information about the chemistry of several novel dihydropyridine systems. It is already clear from these preliminary results that such systems will indeed provide some interesting avenues for synthetic and biosynthetic studies in natural products[1] as well as in heterocyclic chemistry. With respect to the latter, for example, extension of the present studies will allow a versatile synthetic pathway to 3- and 3,5-disubstituted pyridines from readily available pyridine systems. There are presently only a limited number of approaches to the direct synthesis of such compounds.[5,6]

Acknowledgment. Financial aid from the National Research Council of Canada is gratefully acknowledged. One of us (V.E.R.) is grateful for a fellowship from Consejo National de Ciencia y Tecnologia, Mexico.

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Nuclear Magnetic Resonance Studies of Hemoproteins.[1] Restricted Rotation of a Heme Side Chain Methyl Group in Some Ferric Myoglobin Complexes and Its Implication in van der Waals Contact in the Heme Side Chain Environments

Sir:

The heme in hemoproteins is embedded in a hydrophobic cleft made up by the polypeptide chain. It has been revealed[2] that the porphyrin side chains, directed toward the interior of myoglobin and hemoglobin, are in van der Waals contact with nonpolar groups of the polypeptide chain. Some of the important contact in these hemoproteins has been visualized by the X-ray crystallographic study.[2] Although the nuclear magnetic resonance(nmr) method has been proved[3] to be potentially useful for the studies of electronic structure and conformation of hemoproteins, there has been no nmr work on the interaction between heme side chain and apoproteins. In the present communication we wish to report the nmr studies of restricted rotation of the heme side chain methyl group in some ferric myoglobin complexes and to show that the methyl rotational barrier could serve as a sensitive probe for the studies of this heme-apoprotein interaction.

Wüthrich, *et al.*, have observed[4] at an appropriate temperature doubling of one heme ring methyl signal in sperm whale and gray whale cyanoferrimyoglobins, which was tentatively ascribed to methyl hindered rotation without further verification.[4] Here we have obtained more confirmative evidence in the nmr spectra[5] for methyl hindered rotation in horse ferricyanomyoglobin, which is amenable to numerical estimation of this rotational barrier. We have also studied the effect of some ionic additives or different axial ligands on this methyl restricted rotation in order to shed light on van der Waals contacts in the heme side chain environments.

Figure 1 shows the temperature dependence of hyperfine-shifted signals of a heme side chain group at the lower field side in horse ferricyanomyoglobin($MbCN^-$). The most striking feature of this figure is collapse of one methyl signal (1) when the temperature is lowered. At the temperature above 35°, four methyl signals have the same line width (see Figure 1a). However, the highest field methyl signal (1) at −13.5 ppm behaves itself quite differently from the other three methyl signals below 30° at which it broadens and the signal line becomes unsymmetrical. At

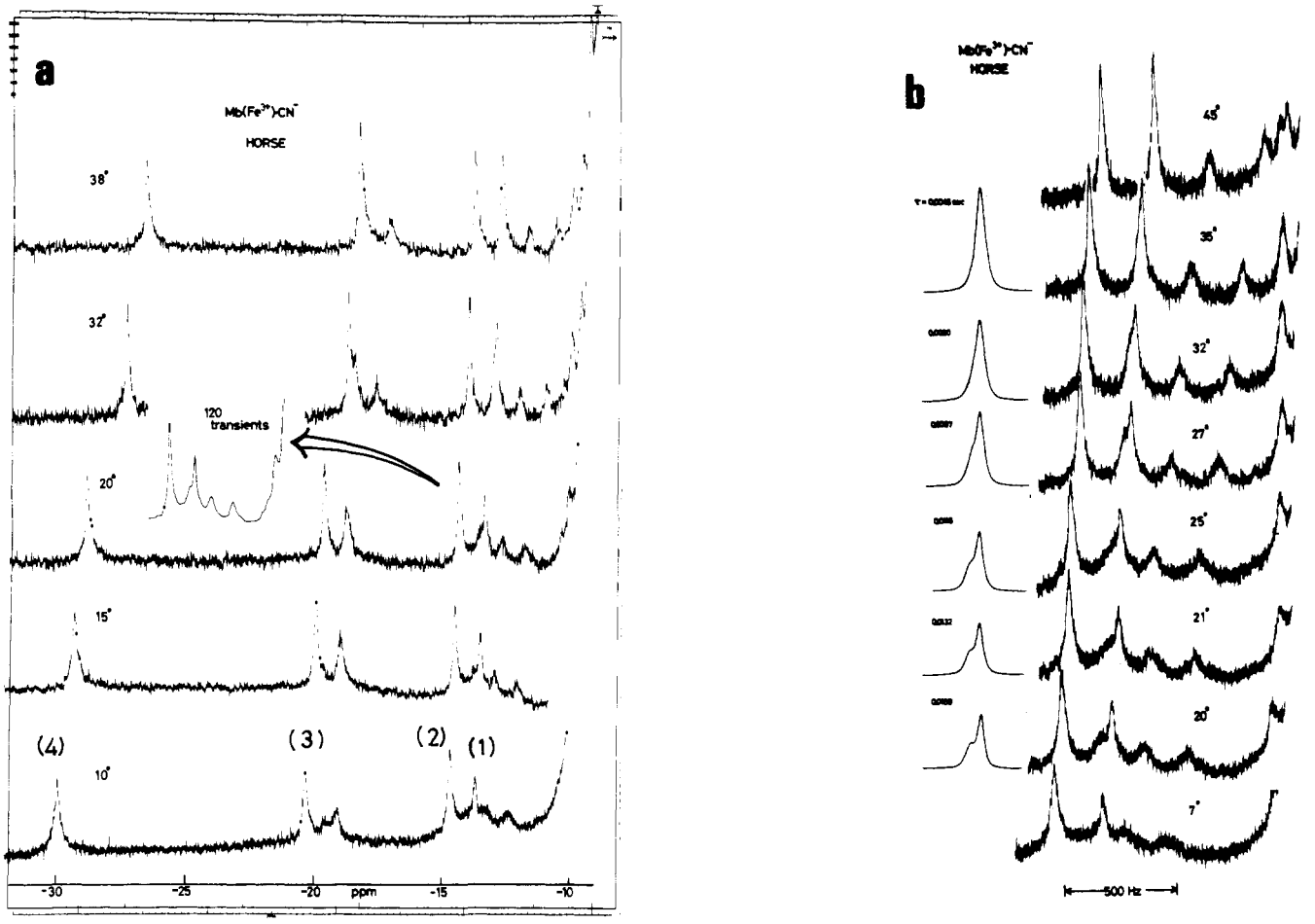

Figure 1. (a) Proton nmr spectra at 220 MHz of ferric horse cyanomyoglobin at various temperatures. The hyperfine-shifted signals of heme side chain at lower field side. (b) Observed and computer-simulated spectra of the methyl signal (1) at various temperatures. Simulated spectra are given with varying values of τ, the life time (τ = 0.0046, 0.0060, 0.0087, 0.0115, 0.0132, and 0.0158 sec, as depicted in the figure).

20° the signal is split into two broad lines, indicating nonequivalence of the methyl protons. This spectral perturbation was reversible with raising or lowering of the temperature. This temperature dependence of the methyl signal (1) is attributable to its hindered rotation, as analyzed in the following. When three hydrogens of the methyl group are located in different environments, the methyl protons should give an ABC pattern when the rotation is frozen. In the real case seen in Figure 1, however, this frozen spectrum could not be obtained due to paramagnetic broadening and/or intermediate collapsing of the frozen signal. Therefore, we have to assume the parameters of the chemical shifts and coupling constants to simulate the spectral pattern by using the LAOCOON program. The best fit values of these parameters were $\nu_1 = \nu_1$, $\nu_2 = \nu_1 + 10$, and $\nu_3 = \nu_1 + 65$ Hz for the chemical shifts and $J_{HH} = -12.58$ Hz for the geminal coupling constant.[6] Using these parameters, computer simulation[7] was also performed to obtain the rate constant of the exchange at various temperatures (see Figure 1b). Correspondence between observed spectral variation at various temperatures and simulated spectra obtained with the line width of 20 Hz is quite satisfactory (see Figure 1b). The Arrhenius plot with least-square treatment yielded the activation energy of E_a = 14.8 ± 1.8 kcal/mol, ΔH^* = 14.2 + 1.8 kcal/mol (at 25°), and ΔS^* = −2.1 ± 0.6 eu(25°). To our best knowledge, this is the first case of quantitative estimation of the restricted rotational barrier of methyl group buried in the protein. This value appears to be quite large for the methyl hindered rotation. However, it is to be noted that Nakamura, *et al.*, have reported[8] 14.1 kcal/mol of methyl hindered rotation for 9-methyltriptycene derivatives in which the interaction between the methyl protons and chlorine atom attached to benzene may cause this hindered rotation.

This rotational barrier should be compared with the case of sperm whale ferricyanomyoglobin in which the hyperfine-shifted methyl signal (1) at −12.5 ppm experienced doubling with the relative intensities of 1:2 and spacing of 100 Hz even at 60°, implying that the methyl rotational barrier is much greater than 14.8 kcal/mol. According to Wüthrich, *et al.*,[4] this methyl signal (1) in sperm whale $MbCN^-$ has been assigned tentatively to that attached to the pyrrole ring PL,[9] where Arg-45 would form the ionic bond with the propionate COO^- group in the ring PL. This salt bridge may cause the restricted rotation of the adjacent methyl group (1).[4] Hindered rotation of the methylene group in octaethylporphyrin has been reported[10] to occur due to adjacent ethyl-ethyl interaction. Therefore, the difference in this methyl rotational barrier in going from horse to sperm whale could be resulting from small perturbations of steric interaction between the methyl group and the adjacent propionate group induced by the change in the 45th amino acid residue from arginine (sperm whale) to lysine (horse).[4] The direct van der Waals contact between this methyl group and a part of apoprotein could also contribute to this hindered rotation of the heme side chain methyl group. Since such methyl hindered rotation has not been reported for isolated protoheme, the above heme-apoprotein interaction could be responsible for steric inhibition of heme side chain methyl rotation.

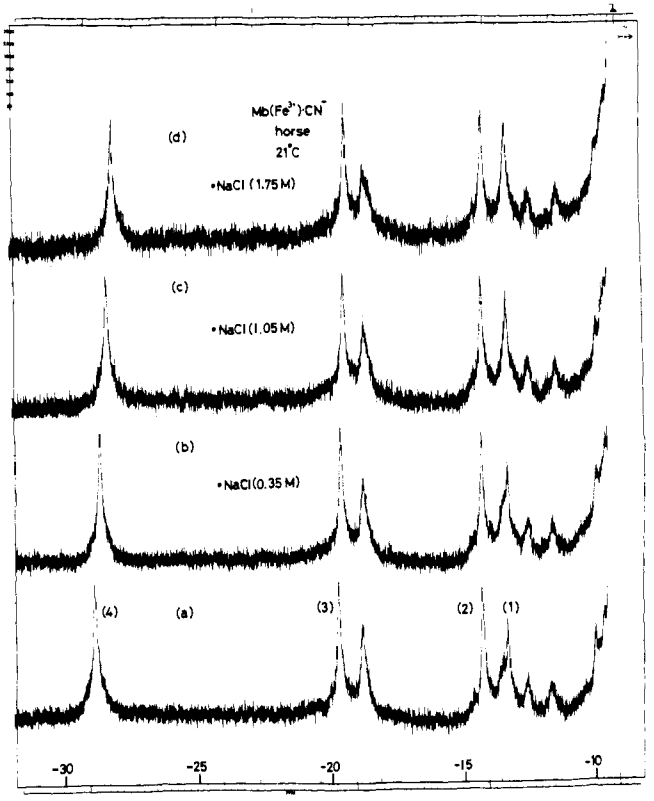

Figure 2. The effect of ionic additives to the nmr spectra (at 220 MHz) of horse Mb · CN^-. The spectra are taken at 21° in the absence (a) and presence (b-d) of NaCl. The heme concentration is 6 m*M* in D_2O.

In order to gain further insight into this restricted methyl rotation, we have examined the effect of some ionic additives on it. Addition of NaCl or $(NH_4)_2SO_4$ caused sharpening of the methyl signal(1) at 21° (see Figure 2), indicating that restricted rotation of this methyl group is released by the enhanced ionic strength of the horse myoglobin solution.[11a] Doubling of the methyl signal(1) in sperm whale $MbCN^-$, however, was not affected by the addition of NaCl or $(NH_4)_2SO_4$. Nonequivalence of these methyl proton signals in horse and sperm whale $MbCN^-$ also was not perturbed by the change of the pD value. These findings may allow us to expect that van der Waals contact at the methyl group(1) in horse $MbCN^-$ may be broken, presumably by perturbing the salt bridge, when NaCl is added and the ionic strength of the solution is increased.[11b]

In azido complexes of ferric horse and sperm whale myoglobin, there was no nmr spectral evidence for restricted rotation of heme side chain methyl group. For imidazole complex of sperm whale myoglobin, however, two of the four hyperfine-shifted methyl signals exhibited specific broadening, which could be interpreted in terms of restricted rotation.[12] The studies on the effect of some axial ligands on the hindered rotation of heme side chain methyl group are now under way.[13,14]

It is, therefore, tempting to expect that monitoring of rotational barrier of heme side chain methyl group could serve as a sensitive probe to detect some van der Waals contacts or steric interaction between heme side chain and apoprotein.[13]

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(12) The proton nmr studies of ferric azido and imidazole, with attention to thermal equilibrium between high spin and low spin states, have been published (ref 1). Spectral features of these complexes are seen in these references.
(13) Details of the nmr study on the van der Waals interaction between heme side chain and apoproteins will be published in a separate paper (I. Morishima and T. Iizuka, submitted to *Biochim. Biophys. Acta.*)
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Enantiomer Differentiation in Transport through Bulk Liquid Membranes[1]

Sir:

Transport in biological systems of amino acids through lipophilic cell walls up concentration gradients (active transport) is linked to H^+, Na^+, or K^+ transport down concentration gradients (passive transport).[2] Metal cation transport with natural or synthetic multiheteromacrocycles through thin synthetic membranes and organic bulk liquid membranes has been studied extensively.[3] Results have appeared of a study of amino acid and dipeptide transport by lipophilic anions or cations through bulk toluene from one aqueous solution to a second.[4]

We report that *enantiomer differentiation* occurs when designed, neutral, lipophilic, and chiral host compounds[5] carry amino ester salts (guest compounds) from one aqueous solution through bulk chloroform to a second aqueous solution. Entropy of dilution and inorganic salt "salting out" of the organic salt provided the thermodynamic driving force for transport. Optically pure compounds **1, 2,** and **3** of established configurations were hosts,[5a] and **4, 5,** and **6** were guests.[5] Rate constants for transport were measured for the faster moving A enantiomer (k_A) and for the slower moving B enantiomer (k_B). Table I records the conditions and results. Transport of guest in the absence of host was at