in the methyl group is perturbed by the 1,3 diaxial interaction, thus increasing the vibrational frequency and hence the zero point energy of methyl groups in the axial position. The CD_3 group has a lower zero point energy and thus is raised *less* in energy than the CH_3 group. If methyl groups in the equatorial position are unperturbed, as is likely, the energy difference between the conformations is simply due to this factor.[17-18]

It is important to be aware of the possible presence of conformational equilibrium isotope effects when using intrinsic chemical shift isotope effects for assignment of ^{13}C resonances. While rigid molecules pose no problem, systems in which deuterium substitution either breaks the conformational degeneracy, or perturbs a nondegenerate equilibrium, can potentially show a conformational equilibrium isotope effect, as well as an intrinsic chemical shift isotope effect.[19]

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(11) The CD_3 carbons should have a very long T_1 relaxation time. Since the interval between pulses (tip angle of ~40°) was 2 s, these carbons should show the effects of saturation. In addition, the nuclear Overhauser effect may be small and coupling to the deuterons will result in the resonance being a multiplet. All of these factors greatly reduce the signal-to-noise ratio of the signal.
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Interaction of an Iron Macrocyclic Complex with Apohemoglobin and Apomyoglobin

Sir:

For many years, attempts have been made to prepare iron complexes which could react reversibly with dioxygen. One of the main objectives of these efforts was to establish the mode of binding of the oxygen molecule to the iron atom. The realization that Fe(II) complexes undergo irreversible oxidation via a dioxygen bridged dimer, Fe^{II}-O-O-Fe^{II}, have led Baldwin[1] and Collman[2] independently to a successful solution to this problem. They reasoned that the dimerization can be impeded by a sterically hindered ligand and, thus, in Baldwin's case, that the steric hindrance was built into the periphery of a macrocyclic ligand, while Collman prepared the now famous "picket fence" porphyrin. It was later realized that the steric hindrance is not obligatory for the reversible uptake of dioxygen and that simple iron porphyrins, such as Fe^{II}-TPP, can also bind dioxygen reversibly at low temperature.[3]

We thought that it would be of interest to use globin as the sterically hindered environment and investigate its interaction with relatively simple macrocyclic iron complexes. We reasoned that, if such complexes could enter the heme cavity, then, with the right choice of iron macrocycle, reversible oxygenation would take place. In this communication, we report the interaction of human apohemoglobin and horse heart apomyoglobin with the iron complex of the macrocyclic ligand, 5.14-dihydrodibenzo[*b,i*][5.9.14.18]tetraaza[14]annulen (L)[4,5] (Figure 1).

Refluxing stoichiometric amounts of L and $Fe(CH_3COO)_2$ in DMF led to the isolation of the red brown complex Fe(L)-(CH_3COO).[6] Apohemoglobin and apomyoglobin were prepared from human hemoglobin and horses heart myoglobin respectively, by the acid-butanone method.[7] The protein preparations were checked by reconstitution and reaction with dioxygen.

The electronic spectra of Fe(L)(CH_3COO) and its imidazole adduct are shown in Figure 2. Addition of the complex dissolved in a minimum amount of DMF to an aqueous solution of globin gave rise to the electronic spectra depicated in

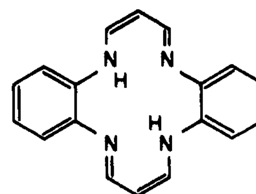

Figure 1. The macrocyclic ligand used in the study.

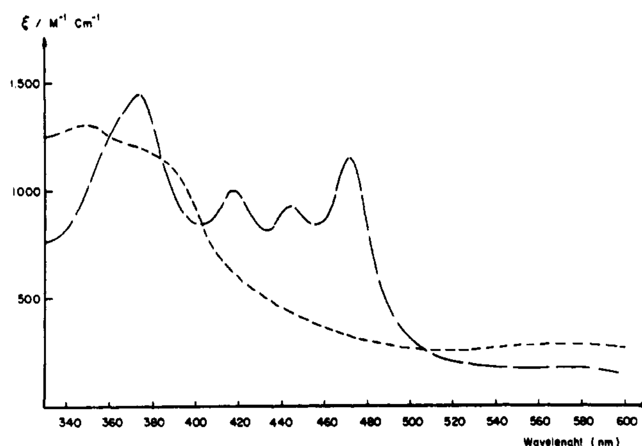

Figure 2. Visible spectra of FeL^+ (---) of its imidazole adduct (— —) in DMF.

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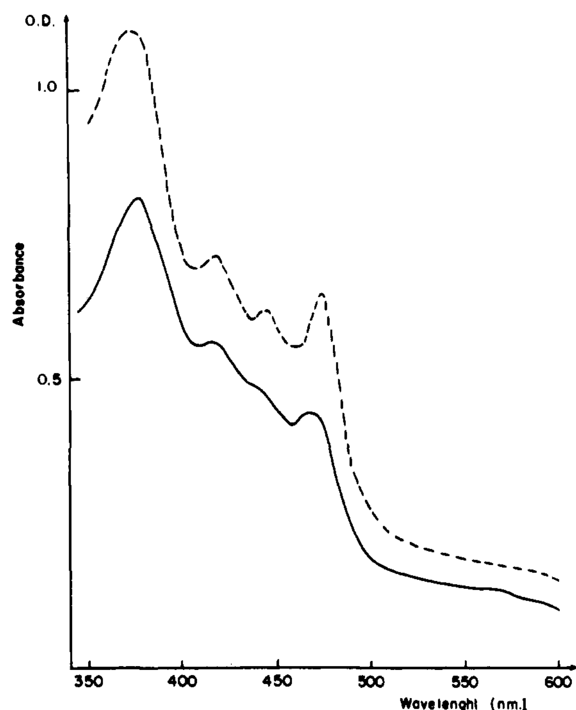

Figure 3. The visible spectra of the FeL^+ complex with globin (—) and with albumin (---).

Figure 3. The similarity between the two spectra is apparent, and, since addition of histidine to the complex results in an electronic spectrum which is virtually identical with that of the imidazole adduct, it is suggested that the complex binds to the protein via the imidazole ring of a histidine residue. The attachment of the complex to the histidine residue is not specific to globin but occurs also with albumin, as can be seen from Figure 3.

To establish the stoichiometry of the interaction between this iron complex and globin, a solution of the globin was treated with excess complex. The unbound complex was removed by chromatography on a Sephadex G-10 column. The amount of bound iron was determined by the Folin–Ciocalteau method.[9] The results of several determinations clearly indicate that one complex molecule is bound to one subunit of globin. It should be pointed out that, in these determinations, the total protein and iron content is determined; thus the results are independent of the state of aggregation of the globin in solution, which is known only for the Rossi-Fanelli preparation.

Spectrophotometric titration of a globin solution with the complex is shown in Figure 4, where the concentration of bound globin is plotted as a function of added complex.[10] The sigmoid nature of the titration curve is quite apparent. This interesting result indicates a cooperative process in the binding of the complex to the protein, suggesting that the binding of the first complex molecule facilitates the binding of additional ones. This behavior is reminiscent of the interaction of heme with globin subunits. Addition of one heme to the α,β dimer of globin leads exclusively to the incorporation of two hemes, and there is no evidence for the existence of an α,β dimer with only one heme, suggesting that the binding of the second heme molecule is more facile than the binding of the first.[11] However, because of the high affinity of apohemoglobin for the heme molecule, the sigmoid nature of this interaction has never been observed experimentally. Also it was shown by Beychok that the attainment of the native structure of hemoglobin from its subunits is effected by the heme molecule.[12]

By the same procedures described above it has also been shown that the Fe(III) complex binds to the heme site of myoglobin, and that the stoichiometry of binding is 1:1. As expected, the curve which results from spectrophotometric

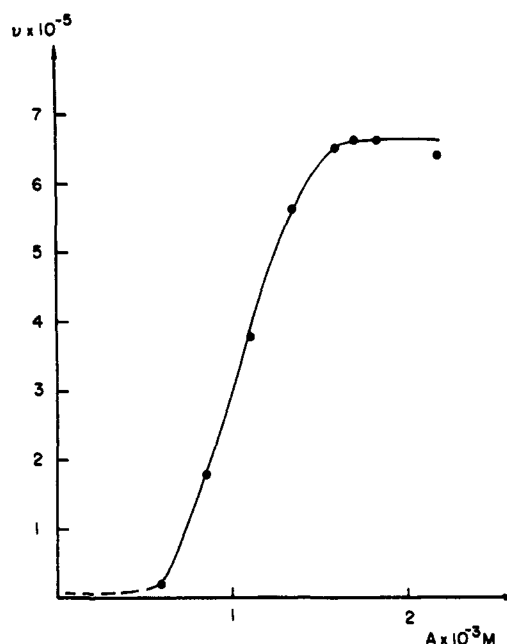

Figure 4. Spectrophotometric titration of globin solution with the complex: (A) total complex concentration; (ν) concentration of bound protein complex.

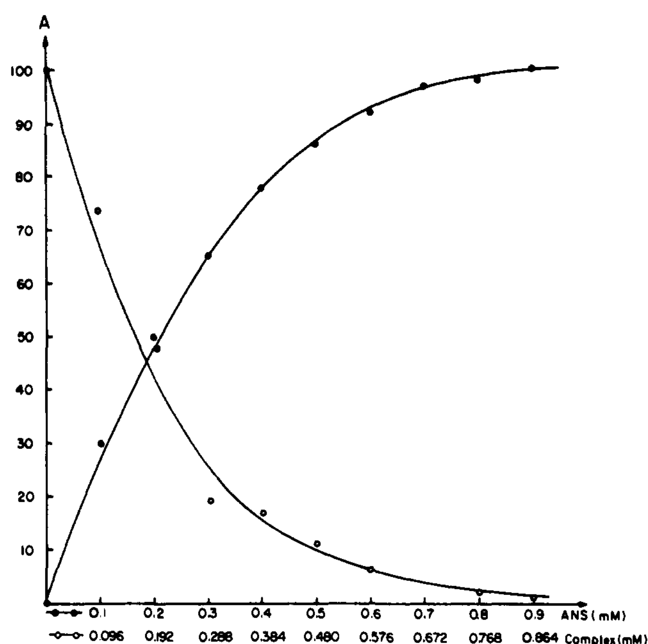

Figure 5. Fluorimetric titration of globin with ANS (●) and back titration with complex (○). Globin concentration 0.085 mM.

titration of myoglobin solution with the complex is not sigmoidal but hyperbolic.

Of particular interest is the point of attachment of the complex to the globin. It was shown by Stryer[13] that 1-anilino-8-naphthalene sulfonate (ANS) binds stoichiometrically to the heme site on apohemoglobin and that the displacement of the ANS molecule by hemin can be followed fluorimetrically. Thus, the ANS apohemoglobin complex was prepared and the displacement of the ANS molecule by the complex was followed fluorimetrically. As can be seen from Figure 5, the enhanced fluorescence of bound ANS decreases with an increase in the amount of added complex and is abolished at 10:1 mol of complex/mol of globin. This result, coupled with the stoichiometry of binding clearly establishes that the complex and the ANS molecule bind at the same site, which has been

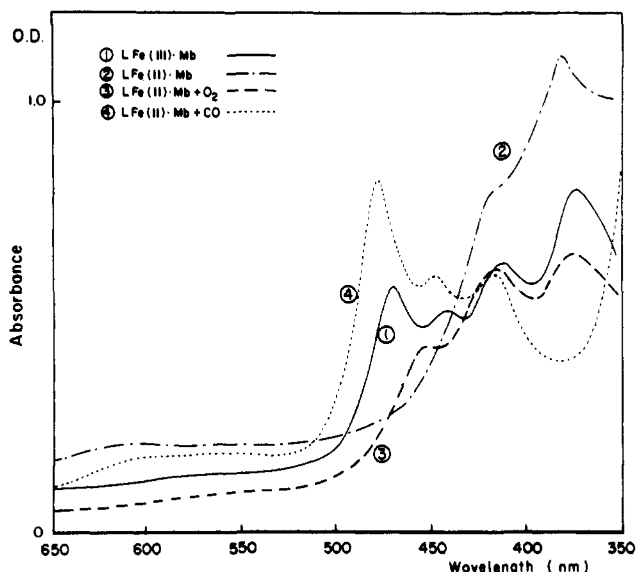

Figure 6. The visible spectra of the Fe(III) and Fe(II) complexes with apomyoglobin, and the interaction of Fe(II) bound myoglobin complex with O_2 and CO.

shown to be the heme site in hemoglobin.[13] Similar results have been obtained with apomyoglobin.

The binding of O_2 and CO to apomyoglobin bound complex was examined spectrophotometrically. The electronic absorption spectra of the bound complex after reduction with dithionite is shown in Figure 6. Exposure of the solution to air leads to an electronic absorption spectrum which differs considerably from that of the Fe(III) bound complex. It is suggested that this electronic spectrum results from binding of O_2 to Fe(II). This suggestion is strongly supported by the spectral changes which occur with carbon monoxide. When the reduction is performed in CO atmosphere, the spectrum of the CO adduct is obtained (Figure 6). On exposure of this solution to air, the electronic spectrum reverts to that of the O_2 adduct. This process is irreversible in that the dioxygen molecule can not be displaced with CO. Attempts to remove O_2 by freeze-thaw techniques were so far unsuccessful because of denaturation of the protein. However, it should be pointed out that, although the hydrophobic cavity of the protein prevents the oxidation of the iron complex, the O_2 binding to this particular complex need not be reversible, since the reversibility depends primarily on the electronic structure of the complex.

We are currently studying other iron complexes as well as their cobalt analogues in order to shed more light on this interesting problem.

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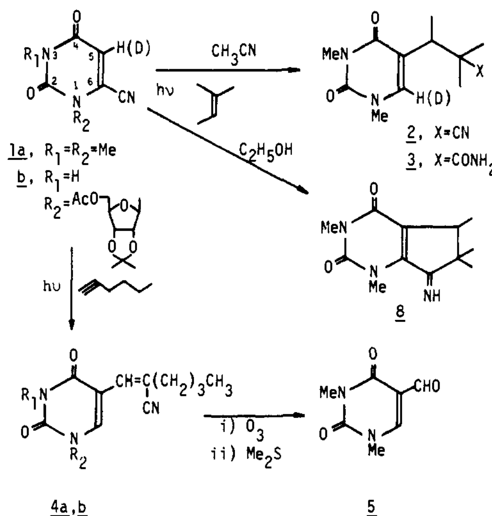
A Novel Photoaddition of 6-Cyanouracils to Alkenes and Alkynes Involving Migration of a Cyano Group[1]

Sir:

Photochemical cycloaddition of cyclic enones to olefins has been studied for many years from mechanistic and synthetic points of view.[2,3] Recently, Swenton and co-workers have described the remarkable effect of α substituents in controlling the regioselectivity of the photocycloadditions of uracils[4] and cycloalkenones[5] to olefins.[6] During our studies directed toward the photochemical synthesis of nucleic acid–amino acid adducts,[7] we have found that 6-cyanouracils undergo an unusual photoaddition to alkenes and alkynes leading directly to 5-substituted uracils via the migration of the cyano group. The present reaction provides a novel type of photoaddition that can compete with [2 + 2] cycloaddition through a biradical intermediate, and constitutes a new concept for the direct functionalization at the C-5 position of uracil and uridine derivatives.[8]

Irradiation of 6-cyano-1,3-dimethyluracil[9] (**1a**, 1 mM) in acetonitrile at 20 °C in the presence of 2-methyl-2-butene (20 mM) with a high-pressure mercury lamp (Pyrex filter) followed by preparative TLC produced a rearranged adduct, **2** (60%). The structure of **2** was assigned on the basis of spectral data[10] and by converting it into the amide **3**[10] (50%, AcOH–H_2SO_4). Irradiation of **1a** with 1-hexyne in acetonitrile under the same conditions gave a 1:1 E–Z mixture of **4a**[10] (65%).[11] Both isomers produced 5-formyl-1,3-dimethyluracil[12] (**5**, 55%) upon ozonolysis. In none of these cases was the cycloadduct detected in the reaction mixture.[13] This novel photoaddition was also successfully applied to a 6-cyanopyrimidine nucleoside. Thus, irradiation of **1b**[9] in acetonitrile in the presence of 1-hexyne followed by preparative TLC yielded **4b**[10] (37%).



Irradiation of **1a** with other olefins under similar conditions gave the corresponding 5-substituted uracils but in competition with the formation of cycloadducts (Table I) with the ratio of the products being temperature dependent. For example, ir-

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