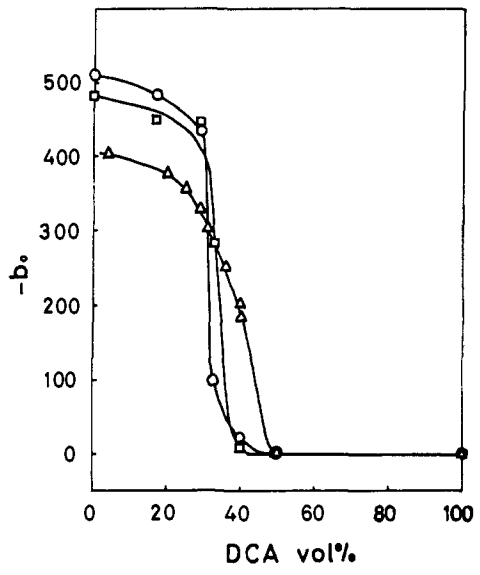

Figure 1. Moffitt's b_0 *vs.* solvent composition in a chloroform–dichloroacetic acid system: ○, $(Cbz\text{-}Lys)_n$; □, $(Cbz\text{-}Orn)_n$; Δ, poly(γ-*N*-carbobenzoxy-L-α,γ-diaminobutyric acid).

butyric acid) extrapolated at 100 vol % of chloroform is rather low. Though the difference between the curves of $(Cbz\text{-}Lys)_n$ and $(Cbz\text{-}Orn)_n$ is small, the helix content of $(Cbz\text{-}Orn)_n$ is, for example, about three times that of $(Cbz\text{-}Lys)_n$ at 33 vol % of dichloroacetic acid in spite of the DP of $(Cbz\text{-}Orn)_n$ being lower than that of $(Cbz\text{-}Lys)_n$. This seems, in turn, to show that the helical structure of $(Cbz\text{-}Orn)_n$ is a little more stable than that of $(Cbz\text{-}Lys)_n$. It can be considered that, in the case of the polymers studied here, the urethan linkages in the side chains, which can combine with one another through hydrogen bonds, may serve to stabilize the helical structures of the polymers in the helix solvent, and that in the helical structure of poly(γ-N-carbobenzoxy-L-α,γ-diaminobutyric acid), which has shorter side chains, interaction between the urethan linkages in the side chains may be relatively large.

The samples of $(Orn)_n$ and poly(L-α,γ-diaminobutyric acid) were obtained by the decarbobenzoxylation of $(Cbz\text{-}Orn)_n$ (DP = 190) and poly(γ-N-carbobenzoxy-L-α,γ-diaminobutyric acid) (DP = 110), respectively. The ORD and circular dichroism measurements were carried out in aqueous solution at various pH values at 20° with the same recorder as mentioned above. The concentration of the polymers was about 0.2 g/dl. From the spectra, Moffitt's b_0, reduced mean residue rotation at 233 mμ, $[m']_{233}$, in deg cm²/dmol, and residue ellipticity at 222 mμ, $[\theta]_{222}$, in deg cm²/dmol, were calculated. The estimation of fraction of helix, f_H, was made with the following empirical equations:[5,8] $f_H = -b_0/630$, $f_H = -([m']_{233} + 2000)/13{,}000$, and $f_H = (4000 - [\theta]_{222}))/42{,}000$.

The helix content of $(Orn)_n$ was estimated to be about 60% at pH 12 using any of these three methods. Our estimate of the helix content of $(Orn)_n$ agrees well with that obtained from the CD measurement by Grourke and Gibbs.[4] However, the estimate of the helix content of $(Orn)_n$ by Blauer and Alfassi[3] based on b_0 was about 20% at pH 11.4 and that by Chaudhuri and Yang[5] based on b_0 and $[m']_{233}$ was about 25% at pH 12. These two estimates do not agree with ours.

The helix content of poly(L-α,γ-diaminobutyric acid) was found to be practically zero even at pH 12 from any of the three methods mentioned above. This extraordinary instability of the helical structure of poly(L-α,γ-diaminobutyric acid) may be due to the stronger interaction between the amino groups in the side chains and the carbonyl groups in the main chain than in the cases of $(Orn)_n$ and $(Lys)_n$, though the instability may be partly due to the rather low DP of the sample of poly(L-α,γ-diaminobutyric acid) used here. It seems of interest to study the oxidation reaction of 3,4-dihydroxyphenylalanine using poly(L-α,γ-diaminobutyric acid)–copper(II) complex as a catalyst in order to elucidate whether the asymmetric selectivity by $(Lys)_n$–Cu(II) complex in the reaction is due to the helical structure of the polymer.[1]

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Reactions of Nitric Oxide with Methemoglobin[1]

Sir:

We wish to report that the reaction of nitric oxide and methemoglobin[1b] is accompanied by the reduction of methemoglobin.

Hemoglobin is known to react with molecular NO to form nitrosylhemoglobin (HbNO) having a paramagnetic susceptibility[2] of 3.07 BM. The electron paramagnetic resonance spectra (epr) of this hemoprotein has been briefly studied.[3,4] More recently, Sancier, *et al.*,[5] compared the reaction products of Hb and of Hi with NO. The authors reported that the long-wavelength absorption (α band) of HiNO was shifted slightly toward the blue and reduced in intensity as compared to the same transition in HbNO. Both hemoproteins were found to have the same spin concentrations as determined by epr; they also have the same isotropic *g* value. This similarity in paramagnetism of the two hemoproteins is surprising and

(1) (a) Much of the experimental work was carried out in the Department of Chemistry at Stanford University and was supported by the Office of Naval Research under Contract 228(88); research also benefited from facilities made available by the Advanced Research Projects Agency through the Center for Materials Research at Stanford University. The hospitality of Professor H. M. McConnell is gratefully acknowledged. (b) Hemoglobin and methemoglobin are represented by Hb and Hi, respectively.

(2) C. D. Coryell, L. Pauling, and R. W. Dodson, *J. Phys. Chem.*, **43**, 825 (1939).
(3) D. J. E. Ingram and J. E. Bennett, *Discussions Faraday Soc.*, **19**, 140 (1955).
(4) W. Gordy and H. N. Rexroad, "Free Radicals in Biological Systems," Academic Press, New York, N. Y., 1961, p 263.
(5) K. M. Sancier, G. Freeman, and J. S. Mills, *Science*, **137**, 752 (1962).

cannot be easily rationalized. Our experiments were aimed to resolve this problem. The results of these experiments are described below.

Hemoglobin was prepared from fresh defibrinated horse blood (W. T. Bennet Ranch Laboratory) according to the procedure given by Benesch and Benesch.[6] Methemoglobin was prepared by the oxidation of Hb with $K_3Fe(CN)_6$, followed by separation on a 12-in. Sephadex G-25 column. The purity of Hb and of Hi were ascertained by their absorption spectra.[6]

A 2% solution (pH 7.0 phosphate buffer) of the hemoprotein was flushed with prepurified nitrogen at 5° for 20 min while the reaction vessel was manually agitated. During this period, an appreciable portion of Hb was deoxygenated as judged by the change of color to that characteristic of deoxyhemoglobin. There was no noticeable change in the dark brown color of methemoglobin in a parallel treatment. Prepurified nitric oxide[7] was subsequently admitted and flushing continued for 20 min. An immediate change to brilliant red color occurred in both reactions; the reaction was essentially completed in less than 5 min.

Single crystals of the NO complexes from both reactions were grown by a procedure developed by Perutz[8] and slightly modified.[9] Paramagnetic resonance spectra were obtained with a Varian X-band spectrometer. Solution and polycrystalline spectra were obtained with the samples contained in a melting point capillary tube.[10] The procedure used to obtain the epr spectra of single crystals are described elsewhere.[9]

Electronic spectra were obtained with a Cary 14 spectrophotometer. The infrared spectra were obtained with a Beckman IR-7 instrument following the difference method given by Alben and Caughey.[11]

Several properties of the two reaction products were compared. The results can be summarized as the following. (1) They have identical electronic spectra; the extinction coefficients (OD/(cm % heme)) at 5730, 5400, and 4160 Å are 5.80, 6.60, and 71.0, respectively. Wavelength and intensity differences of the kind reported by Sancier, *et al.*,[5] were not observed. (2) Phosphate buffer solutions of the two products have superimposable room-temperature paramagnetic resonance spectra; both having a Lorentzian line shape, a width of 74 G, and a g value of 2.030. (3) Their asymmetric polycrystalline paramagnetic resonance spectra obtained at $-195°$ agreed in all details; the isotropic hyperfine splitting is 50.4 MHz. (4) The paramagnetic resonance spectra of single crystals of these products are characterized by the same **g** and **A** (hyperfine) tensors. The principal values of the **g** tensor are:[9] $g_{xx} = 2.0820$, $g_{yy} = 2.0254$, $g_{zz} = 1.9909$. (5) Both products crystallize in the monoclinic form; they have the same unit cell dimensions: $a = 111$ Å, $b = 63.4$ Å, $c = 48.7$ Å; $\beta = 110° 53'$; $V = 3.49 \times 10^5$ Å^3. These observations furnish rather incontrovertible evidence that the same nitrosylhemoglobin is formed in the reaction of NO with either Hb or Hi.

The reaction of paramagnetic Hi with nitric oxide should give either a spin-paired diamagnetic molecule derived from a low-spin Fe^{3+} or a $S = 2$ state derived from a high-spin Fe^{3+}. Since Kramer's theorem does not apply to the $S = 2$ even-spin system, the ligand field could remove all the degeneracies in the absence of a magnetic field. Applied magnetic fields would merely shift the relative energies of all the nondegenerate levels; paramagnetic resonance is unlikely to be observed unless two levels are fortuitously close to one another for the absorption of a microwave photon. Deoxyhemoglobin, a similar $S = 2$ molecule, has no paramagnetic resonance in the microwave region. Therefore, the paramagnetic nitrosylhemoglobin cannot be HiNO.

The reaction of the diamagnetic Hb with nitric oxide should give either a strong field, $S = {}^1/_2$, or a weak field, $S = {}^3/_2$, ground state. Both states are paramagnetic. The high-spin state should have short spin–lattice relaxation time, rendering it difficult to observe the paramagnetic resonance spectrum except at very low temperature. The observation of paramagnetic resonance spectra at room temperature with an intensity corresponding to one unpaired spin, and the known magnetic susceptibility of HbNO,[2] showed that both reaction products are the low-spin nitrosyl- (ferro) hemoglobin.

Attempts of direct displacement of the bonded NO by N_2, with the objective of showing that both reaction products in question lead to deoxyhemoglobin in which the iron is definitely in the ferrous state, were all unsuccessful. Purging with N_2 did not cause the dissociation of HbNO as it does to HbO_2. CO, which Hb has affinity for, replaces NO only very slowly (less than 10% in 10 hr in the dark). Purging with O_2 in the dark caused denaturation of the protein. These results are consistent with the known affinity of Hb for NO.[12]

It is well known that HbCO undergoes photodissociation.[14] A 2% solution of HbNO (Pyrex vessel) was irradiated at 5° with a Hanovia 480-W medium-pressure lamp. When the photolysis was carried out under a constant stream of nitrogen, there was no significant loss of the bonded NO. Photolysis in the presence of O_2 results in the increase of absorption at 6300 and 5000 Å with concomitant denaturation.

Photolysis of the NO complexes under a constant stream of CO afforded near quantitative conversion (>90% yield) to the same HbCO as judged by the increase of absorption intensity at 5670 Å (ε 8.03 OD/(cm % heme)), the disappearance of paramagnetic resonance absorption, and the appearance of carbonyl absorption at 1950 cm^{-1}. Hi was not formed in this photolysis if air is completely excluded at all times. Therefore, HbNO is formed from either Hb or Hi upon reaction with NO, and one can conclude with confidence that Hi is reduced by NO under these conditions.[15]

(6) R. E. Benesch and R. Benesch, *Biochemistry*, **1**, 735 (1962).
(7) Nitric oxide was purified by passage through a 20-in. column of solid KOH in order to remove higher oxides of nitrogen which could cause rapid denaturation of the proteins.
(8) M. F. Perutz, *J. Crystal Growth*, **2**, 57 (1968).
(9) J. C. W. Chien, manuscript in preparation.
(10) The space above the sample was flushed with N_2 delivered by a long syringe needle prior to the sealing of the capillary tube. There was no difficulty in locating the capillary tube in the null electric field region of the cavity. Polycrystalline spectra was obtained on the same sample at $-195°$.
(11) J. O. Alben and W. S. Caughey, *Biochemistry*, **7**, 175 (1968).
(12) The equilibrium constant[13] for ([HbNO][CO])/([HbCO][NO]) is 1350 at pH 6.8 and 19°.
(13) Q. H. Gibson and F. J. W. Roughton, *J. Physiol.* (London), **136**, 507 (1957).
(14) Q. H. Gibson, *Progr. Biophys. Biophys. Chem.*, **9**, 1 (1959); *Proc. Roy. Soc.* (London), **B146**, 206 (1957).
(15) The same conclusion was arrived at[16] *via* indirect chemical evidences.
(16) D. Keilin and E. F. Hartree, *Nature*, **139**, 548 (1937).

The mechanism of this reduction of Hi by NO is not known. However, the known hydrolysis[17,18] of the nitroprusside to $[Fe(CN)_5NO_2]^{4-}$ suggests the possible reaction mechanism[19,20] given by eq 1–3.

$$(\text{heme Fe})^{3+} + NO \rightarrow (\text{heme FeNO})^{3+} \quad (1)$$

$$(\text{heme FeNO})^{3+} + 2OH^- \rightarrow (\text{heme FeNO}_2)^{+} + H_2O \quad (2)$$

$$(\text{heme FeNO}_2)^{+} + NO \rightarrow (\text{heme FeNO})^{2+} + NO_2^- \quad (3)$$

The relative photostability of HbNO as compared to HbCO can be attributed to the dissimilar nature of the metal–ligand bonds. The ligand in HbCO is axially bonded.[11] Photodissociation could result from the excitation of an electron in the e_g ($d\pi$) to the strongly metal–ligand antibonding $A_{1g}(d_{z^2})$ orbital.[21] However, HbNO has a bent Fe–N–O bond with an angle of 110°.[9] Therefore, this d–d transition in HbNO is shifted to the infrared region and has much lower oscillator strength. Instead there is expected to be a $d_{xz} \rightarrow NO(\pi^*)$ charge-transfer band in the visible wavelengths. The photoinitiated ligand exchange observed here may be represented by eq 4, where HbNO* could be the excited charge-transfer doublet state.

$$HbNO \xrightarrow{h\nu} HbNO^* \xrightarrow{CO} HbCO + NO \quad (4)$$

(17) L. Cambi and L. Szego, *Atti Accad. Naz. Lincei, Rend. Classe Sci. Fis. Mat. Nat.*, **5**, 737 (1927).
(18) K. A. Hofmann, *Ann.*, **312**, 1 (1900).
(19) We thank the referee for calling our attention to the paper on cytochrome *c*.[20]
(20) A. Ehrenberg and T. W. Szczepkowski, *Acta Chem. Scand.*, **14**, 1684 (1960). These authors proposed a similar mechanism for the autoreduction of the stable ferricytochrome *c*–NO complex in neutral and alkaline media. They postulated a dissociation equilibrium which could also be involved in our system, *i.e.*

$$(\text{heme Fe NO})^{3+} \rightleftharpoons (\text{heme Fe})^{2+} + NO^+$$

(21) This transition has been estimated[22] to lie between 13,600 and 23,200 cm^{-1}.
(22) M. Zerner, M. Gouterman, and H. Kobayashi, *Theoret. Chim. Acta*, **6**, 363 (1966).

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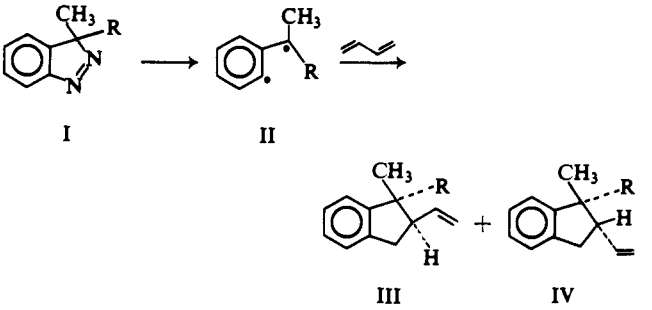
Electron Spin Resonance Identification of Diradicals Generated by Addition of Triplet Ground State Molecules to Olefins[1]

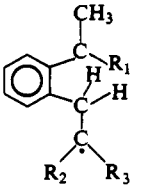
Sir:

In a previous communication we reported that irradiation of dilute solutions of 3H-indazoles (I) at 77°K produced molecules identified by esr spectroscopy to have triplet ground states.[2,3] Based on the magnitude of the zero-field splitting parameters, *D* and *E*, and on the observed chemistry, these paramagnetic species were assigned the 1,3-diradical structures II. The intermediates were trapped in solution by reaction with butadiene, giving the stereoisomeric indan derivatives III and IV. This trapping reaction is of mechanistic interest because II as a triplet molecule can be expected to react with the diene in a two-step reaction, if spin conservation is assumed in the initial addition process. Therefore, a new diradical V, also in a triplet state, should be the reaction intermediate in the formation of the indan derivatives. In this communication we wish to report the successful identification of diradicals of structure V by esr spectroscopy.



I II III IV

a, R = CN; b, R = $COOCH_3$; c, R = OCH_3



Va, R_1 = CN; R_2 = R_3 = C_6H_5
b, R_1 = CN; R_2 = H; R_3 = C_6H_5
c, R_1 = CN; R_2 = H; R_3 = $-CH{=}CH_2$
d, R_1 = $COOCH_3$; R_2 = R_3 = C_6H_5
e, R_1 = OCH_3; R_2 = R_3 = C_6H_5

Ultraviolet irradiation (3–20 sec) of a 0.1–1.0% solution of Ia in 1,1-diphenylethylene frozen to a glass at 77°K and subsequent examination by esr gave a superposition of two triplet-state spectra with approximately equal intensities. The first spectrum was identical with that obtained from IIa as previously reported, while the second extended over a much narrower range of the external magnetic field. The observed $\Delta m = 1$ transitions were fitted to the usual triplet-state spin Hamiltonian, $\mathcal{H} = \beta H \cdot g \cdot S + DS_z^2 + E(S_x^2 - S_y^2)$, $S = 1$, yielding the zero-field splitting parameters as listed in the first entry of Table I. The spectra persisted unchanged for several hours after irradiation was ceased, indicating a triplet ground state or a triplet state very close to the ground state and populated by kT.

Similar results were obtained upon irradiation of Ia in styrene and butadiene glasses and of Ib and Ic in 1,1-diphenylethylene. In each case, the spectrum of the corresponding triplet molecule II was obtained, in addition to new spectral features which should be attributed to triplet-state molecules of structures Va–e. The zero-

(1) Work supported by National Science Foundation Grant GP-7043.
(2) G. L. Closs, L. R. Kaplan, and V. I. Bendall, *J. Am. Chem. Soc.*, **89**, 3376 (1967).
(3) For similar intermediates see G. Baum, R. Bernard, and H. Shechter, *ibid.*, **89**, 5307 (1967).