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Electron Paramagnetic Resonance of Nitric Oxide–Protoheme Complexes with Some Nitrogenous Base. Model Systems of Nitric Oxide Hemoproteins*

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ABSTRACT: In order to help interpret the electron paramagnetic resonance spectra of the nitric oxide complex of ferrohemoglobin and ferrocytochrome *c*, more than 20 model systems were studied which consist of NO, heme (Fe^{2+}), and some nitrogenous base which coordinates the heme Fe. The observed paramagnetic resonance spectra were grouped into four types according to their shape. An assignment of the absorption peaks was proposed by which the relation between the four spectral types can be explained and the interpretation of the resonance spectra of NO–ferrohemoglobin and NO–ferrocytochrome *c* became possible. The ^{14}NO hyperfine splitting in each is 26.8 and 23.7 gauss, respectively, along the heme normal. Also, the hyperfine splittings of 6.4–6.8 gauss which are due to the ^{14}N in the imidazole ring of the proximal histidine residue are now resolved. Evidence for the strong interaction between the base π and the Fe d_π electrons was presented in these hemoproteins. At least 50% delocalization of the unpaired electron to the Fe d_{z^2} orbital was demonstrated by ^{57}Fe experiments with two of the model systems.

In a previous publication (Kon, 1968), the results of an electron paramagnetic resonance study of NO–Hb[1] and related systems have been reported. The ^{14}N hyperfine splitting due to the NO nitrogen was manifest only when the NO–Hb structure was modified by interaction with small denaturing molecules such as SDS or when the protein was dehydrated. For such "perturbed" systems, it was possible to make the assumption that the well-resolved, three-line absorption represents the so-called parallel absorption in which the external magnetic field is parallel to the heme normal. The unpaired electron can, then, be assigned to the antibonding σ orbital consisting of an Fe d_{z^2} orbital rather than being completely localized in a π^* orbital of the NO group. In the "unperturbed" NO–Hb, however, because of poor spectral resolution, the absorption peaks could not be identified clearly, although there is an indication of a doublet when ^{15}NO is used. Consequently, the relation between the spectra of NO–Hb itself and of its perturbed form could not be clarified and the *g* values assigned in the NO–Hb spectrum were considered only tentative.

Since that time we have carried out further studies in order to more completely interpret the electron paramagnetic resonance spectra of NO–Hb and related systems by studying the similar spectra of a number of model systems. The model compounds consist of protoheme–NO and various nitrogen-containing bases which are known to coordinate to the heme Fe in one of the axial sites.

The present paper is designed to show that it is possible, in the light of such electron paramagnetic resonance observations, to interpret the spectrum of NO–Hb consistently with those of the perturbed systems. Estimation of the unpaired electron distribution in the Fe d orbital was made with some of the model systems by incorporating ^{57}Fe. The result ($\gtrsim$50% delocalization) supports the qualitative statement in the previous report (Kon, 1968) that the unpaired electron may not be entirely localized on the NO groups, but spends a substantial fraction of the time in the Fe d orbital.

Experimental Section

Materials. Bovine hemin chloride (Pentex, Inc., BV2362) and horse heart cytochrome *c* (98% purity, Seravac Laboratories, and 97% purity, Sigma type VI) were used without further purification. Most nitrogenous bases from commercial sources were distilled at least once and the purity was checked by gas chromatography. Imidazole (Eastman Organic Chemicals) was used without further purification. NO gas (98.5% minimum, Matheson Co., Inc.) and ^{15}NO gas (96.8 atom % in ^{15}N, Isomet Corp.) were purified by fractional condensation. ^{57}Fe metal foil (95 atom %, Nuclear Science & Engineering Corp.) was converted into $FeSO_4$ as described below.

Procedure. When the base is liquid at room temperature, the base itself was generally used as solvent. Otherwise the base was dissolved in some solvent (*e.g.*, H_2O and Me_2SO) to near saturation. Typically, 0.5–1 mg of hemin dissolved in 0.5 ml of base in a modified Thunberg-type container was allowed to react with NO gas at approximately 1 atm after being thoroughly deaerated by repeated freeze-thawing. When the color change (from brown to bright red) was complete (~0.5 hr), excess NO gas was expelled by evacuation in some cases. In others, however complete degassing caused the loss of electron paramagnetic resonance signal. The solution was then transferred to another part of the container for electron paramagnetic resonance observation either at room temperature or at 77°K.

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[1] Abbreviations used are: Hb, ferrohemoglobin; SDS, sodium dodecyl sulfate; Mb, ferromyoglobin.

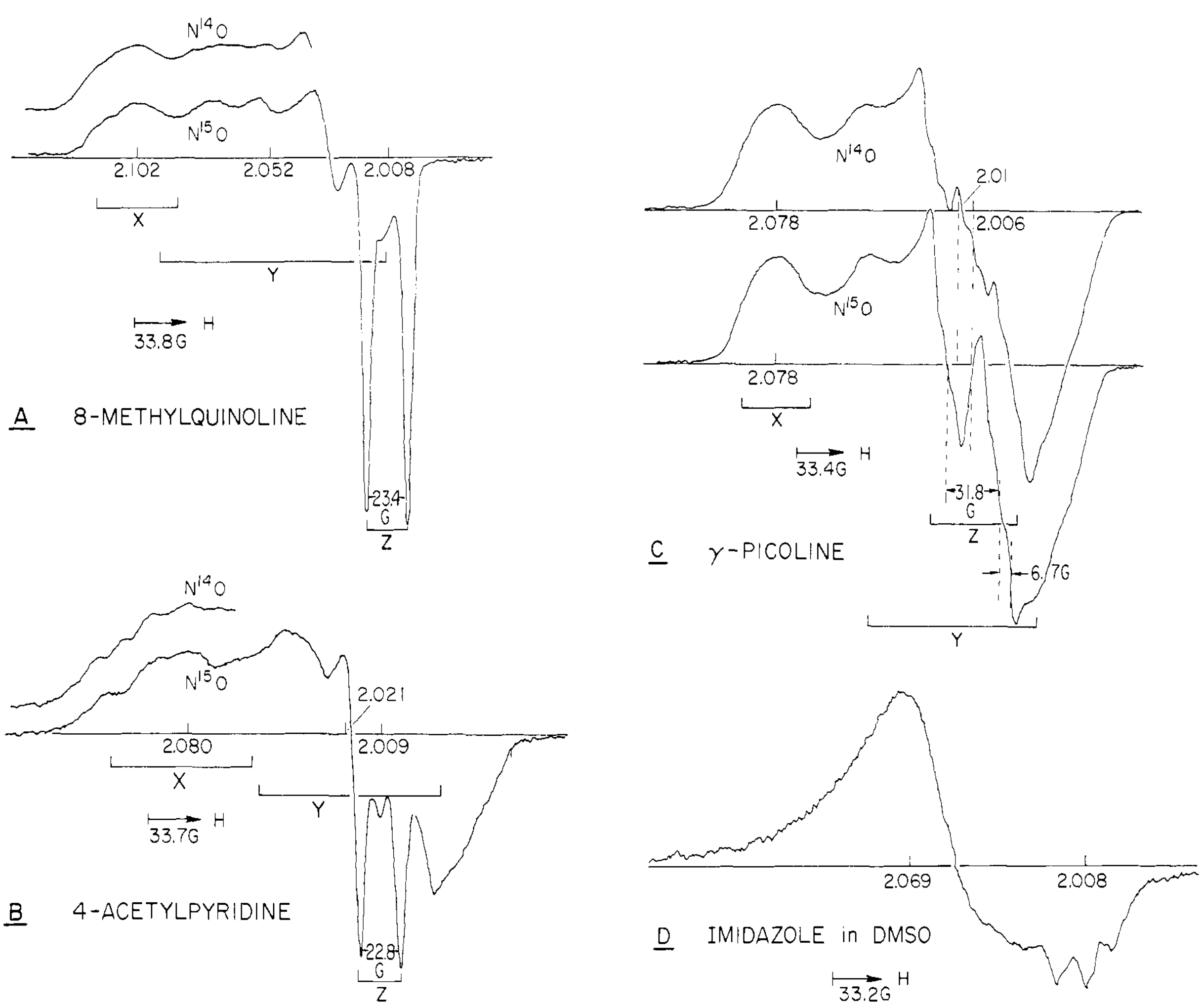

FIGURE 1: Representative electron paramagnetic resonance spectrum of NO–heme(Fe^{2+}) complex with nitrogenous base in frozen solution at 77°K. Hemin chloride (0.5 mg) was dissolved in 0.5 ml of 8-methylquinoline (A), 4-acetylpyridine (B), γ-picoline (C), and in 2 ml of Me_2SO saturated with imidazole (D). Magnetic field sweep: 500 gauss in 5 min with the amplifier time constant of 1 sec. Modulation amplitude 1–3 gauss. The g_y values were calculated from the g_x, g_z, and g_{soln} (in solution at room temperature).

NO–ferrocytochrome *c* solution was made by dissolving 4.8 mg of lyophilized ferricytochrome *c* in 4 ml of 0.01 N NaOH (pH ~ 12). After the solution was degassed by repeated evacuation and equilibration with pure nitrogen gas (99.99% minimum), NO gas was introduced for 15 min for complex formation and reduction. For observation at neutral pH, an appropriate amount of $NaH_2PO_4 \cdot H_2O$ powder was added under a nitrogen stream. NO–Hb solution was prepared as in the previous report (Kon, 1968).

^{57}Fe-labeled hemin was prepared by removing Fe from bovine hemin and then inserting ^{57}Fe, both by the $FeSO_4$–glacial acetic acid method (Morell and Stewart, 1956) which was modified for the present purpose. For a typical electron paramagnetic resonance observation 0.7 mg of bovine hemin was first converted into protoporphyrin with 25 mg of $FeSO_4 \cdot 7H_2O$ in glacial acetic acid and then recovered as roughly 0.5 mg of ^{57}Fe–hemin by treating in hot glacial acetic acid with 8 mg of $^{57}FeSO_4$ dissolved in 0.5 ml of deaerated water. $^{57}FeSO_4$ was prepared by dissolving 3 mg of ^{57}Fe foil in 2 N H_2SO_4, then percipitating and washing the precipitate with cold, dry ethanol. Visible absorption spectra of protoporphyrin (in 5% HCl) and of the recovered hemin (in ether) were in agreement with the published results (Falk, 1964).

Electron paramagnetic resonance observations were made with a Varian 4500 X-band electron paramagnetic resonance spectrometer with 100-KHz field modulation. The magnetic field (Varian V-3400 9-in. magnet) was regulated and scanned by a Fieldial (Varian).

Results and Discussion

In systems consisting of hemin, NO, and base there may be in general more than one complex species in equilibrium. Before NO is introduced, hemin (Fe^{3+}) may be coordinated by one or two molecules of base at the axial sites. Fe^{3+} may be in a low- or a high-spin state since the color of the solution is either brown or red depending upon the kind of base, or there can be an equilibrium between the two spin states, since in a few cases the color changes reversibly with varying the temperature. Nitric oxide introduced would then substitute for either one or for both of the axial ligands. In the presence of excess base and NO the complex species base–hemin-

TABLE I: The Electron Paramagnetic Resonance Parameters of NO–Heme–Nitrogenous Base Complexes.

Type	Base Molecule	g_{soln}	g_x	g_y[a]	g_z	A_N (G)
A	Quinoline	2.053	2.100	(2.051)	2.008	16.0
	8-Methylquinoline	2.054	2.102	(2.052)	2.009	16.7
B	3-Acetylpyridine	2.036	2.081	(2.017)	2.009	16.5
	4-Acetylpyridine	2.037	2.080	(2.021)	2.009	16.4
C	Pyridine	2.032	2.082	(2.007)	2.006	21.4[b]
	γ-Picoline	2.030	2.080	(2.007)	2.005	23.5[b]
D	Imidazole[c]	2.049	(2.069)		2.008	16.6
	Triethylamine[d]	2.060				

[a] Calculated from g_{soln}, g_x, and g_z. [b] Super hyperfine splitting of base ^{14}N observed (see text). [c] In Me$_2$SO. [d] In H$_2$O.

(Fe^{3+})–NO undergoes, at least partly, one electron reduction resulting in the paramagnetic complex base–heme(Fe^{2+})–NO,[2] the mechanism of which is not fully investigated. In any event it is the latter paramagnetic complex molecule that is of interest and is observed by electron paramagnetic resonance although it may not always be the major component in the solution. This conclusion is based on the observation using ^{15}NO which demonstrates that there is always one and only one NO molecule involved in the paramagnetic complex. (The possibility of heme(Fe^{2+})–$\dot{\mathrm{N}}$O without base cannot be strictly ruled out although it is very unlikely to occur in the presence of the great excess of base.) The spectra we observe would not represent the low-spin Fe^{3+} species because of the range of the observed *g* values (Kon, 1968).

Types of Electron Paramagnetic Resonance Spectrum. The electron paramagnetic resonance spectra of heme–NO–base system in liquid solution consist of mostly single, broad bands with peak-to-peak width of 45–50 gauss. In some cases, the hyperfine splitting of NO is resolved (*e.g.*, with pyridine the splitting of ^{14}NO is about 17 gauss). In frozen solution (77°K) the spectra can be conveniently divided into roughly four types by their shape. A representative spectrum of each is shown in Figure 1 and the electron paramagnetic resonance parameters are summarized in Table I.

Type A spectrum (Figure 1A), which includes all the quinoline derivatives observed (quinoline, 2-methyl-, 6-methyl-, 8-methyl-, and 8-nitroquinoline) and some aniline derivatives (*N*,*N*-dimethylaniline and 2,6-xylidine) is characterized by the well-resolved NO hyperfine splitting on the high-field end, the narrow line width of which is quite in contrast with the other parts of the absorption, indicating that the latter comprises other hyperfine splitting than the one due to NO. The pattern of NO–Hb modified by SDS (Kon, 1968) also belongs to this group.

Type B spectrum (Figure 1B) as represented by that of 4-acetylpyridine also shows fairly well-resolved NO hyperfine splitting, but there are other, usually broader absorptions on both sides. Again the difference in line width, mentioned above, is conspicuous. This type includes: 3-acetyl-, 4-acetyl-, and 4-cyanopyridine, aniline, and some of its derivatives (*o*-toluidine and *N*-methylaniline).

In the type C spectrum (Figure 1C) the hyperfine splitting due to the NO nitrogen is generally less resolved than in the other two types and is apparently superimposed on the slope of another broad absorption. In some cases this ^{14}NO hyperfine splitting can be further resolved into three-line superhyperfine splitting which is most reasonably ascribed to the nitrogen atom of the base. This observation not only demonstrates the delocalization of the unpaired electron to the other side of heme plane, but also suggests that its orbital may be extended along the axial direction. Pyridine and the derivatives (γ-picoline ,2,4,6-trimethyl, and *p*-amino), isoquinoline, and some anilines (*m*- and *p*-toluidine) give rise to spectra of this type. The NO–ferrocytochrome *c* (Kon, 1969) spectrum (Figure 2A) evidently belongs to this type, too. The spectra of NO–Hb (Figure 2B) and NO–Mb, in which the peaks are considerably broader than in the other systems, can nevertheless be grouped together because of the general resemblance in shape.

It is interesting to note that imidazole, when used in less polar solvents (CH$_3$Cl and CH$_2$Cl$_2$), or when allowed to react hemin and NO above its melting point (no solvent), gives with rise to a type C spectrum, while in polar solvents (H$_2$O and Me$_2$SO) a spectrum of a different shape was obtained which we designate as type D (Figure 1D).

This spectrum appears to represent the system with approximately axial symmetry. Similar spectra were obtained with trimethyl- and triethylamine, and also with CN$^-$ as the ligand. It may be noted that an axial but somewhat better resolved spectrum was also obtained with NO–Hb in concentrated SDS solution ($\gtrsim$0.2 M) (Kon, 1968) where the protein structure around the heme may be loosened so as to allow the access of more water molecules, making the environment of the complex similar to that of NO–heme–imidazole complex in a polar solvent.

It is noted that the variety of electron paramagnetic resonance patterns observed in NO–hemoproteins can be reproduced in the present model systems by proper selections of the base molecule. This finding demonstrates that NO–heme–base complexes are indeed adequate model systems of NO hemoproteins so far as the electronic structure of heme part is concerned, and indicates that the proximal histidine may be responsible for the change of the electronic structure of NO–hemoproteins, caused by perturbing the protein structure as is manifest in the electron paramagnetic resonance spectra.

There appears to be no simple relationship between the

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[2] The dot in the formula does not imply that the unpaired electron is localized on the NO group.

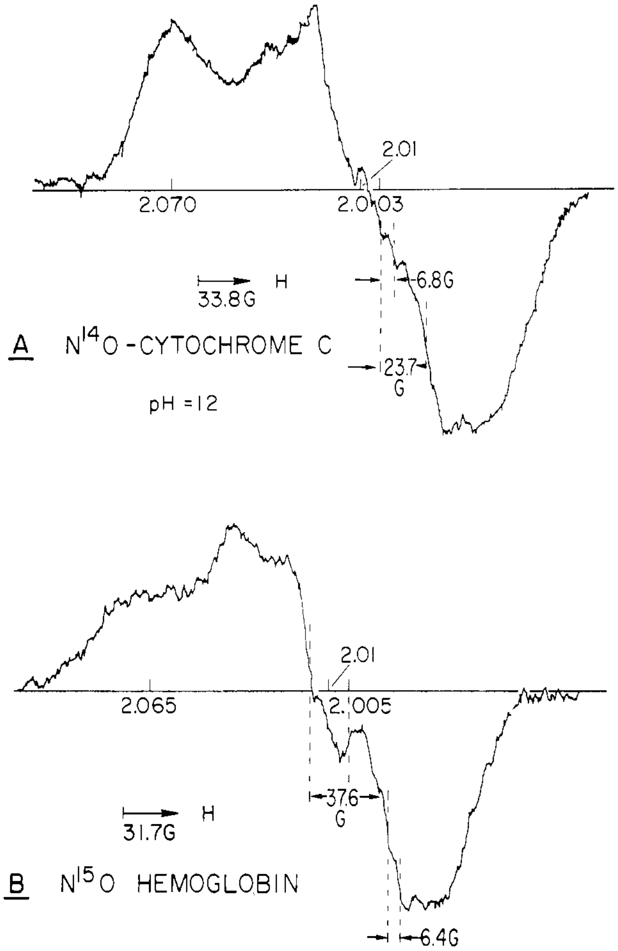

FIGURE 2: The electron paramagnetic resonance absorption at 77°K of: (A) NO–ferrocytochrome *c*, lyophilized ferricytochrome *c* (4.8 mg) in 4 ml of 0.01 N NaOH solution (pH 12), and (B) NO–Hb; Hb concentration in heme is 5×10^{-4} M in pH 7 phosphate buffer (0.05 M). NO–Mb shows an almost identical spectrum. $g_y = 2.01$ is indicated approximately at the midpoint of the *Y* absorption. Magnetic field sweep is the same as in Figure 1.

basicity of nitrogenous compound and the type of spectrum obtained (*vide infra*).

Spectral Assignment. It is now possible to arrive at a consistent interpretation of the spectra by comparing the best resolved ones in each type and by use of our knowledge of the powder electron paramagnetic resonance pattern (Sands, 1955; Kneubühl, 1960). Thus there is commonly found a well-defined absorption designated as *Z* in Figure 1 which shows the hyperfine splitting of NO nitrogen. The component line widths of this absorption are generally very narrow, in marked contrast with those of other absorptions in the spectrum. This is best explained by the "parallel" orientation of the molecule, in which the heme normal is along the direction of the external magnetic field. Since at this orientation the anisotropic contribution of the pyrrole nitrogens to the hyperfine structure would be minimal, the absorption is expected to be narrow. The extra hyperfine structure due to the base nitrogen resolved in some C-type systems is consistent with the orientation. This assignment, together with the observed *g* value of the *Z* absorption, 2.006–2.009, which is practically equal to the value for the free spin, *requires that the orbital of the unpaired electron has the symmetry of* d_{z^2} *in all the model systems* (Ballhausen, 1962).

The lowest field absorption, indicated as *X*, has the *g* values 2.08–2.10 which are relatively unaffected by change of the base molecule. The ^{15}NO experiments indicate that this absorption derives part of its width from ^{14}NO hyperfine splitting, but consists mostly of other hyperfine splitting, probably due to the porphyrin nitrogens. This *X* absorption occurs when the *x* direction in the heme plane is parallel to the external field. There is the third absorption *Y*, between *X* and *Z*, which is most clearly seen in types A and B, and less so in C. In type D, *X* and *Y* absorptions coalesce. This *Y* absorption takes place when the orientation of the external field is perpendicular to the *x* direction in the heme plane. It is important to note that the position of the *Y* absorption is, in contrast to the other two, particularly sensitive to the change of the base, the *g* value shifting between 2.00 and 2.05.

The various spectra observed in the present study can be explained as a combination of these three absorptions, especially by the location of *Y* absorption; *e.g.*, type A spectra can be obtained from type C through B by shifting the *Y* absorption down field so that there is only a little overlap between *Y* and *Z* and then diminishing the width of *Z* components, *i.e.*, smaller super hyperfine splitting.

If the NO–Hb spectrum (Figure 2B) is reexamined in this scheme, the similarity to the C-type spectra is evident in spite of the absorption bands being much broader in NO–Hb than in the model systems. The general broadness may be due to the greater hyperfine interactions which are not resolved. The broadening due to the two slightly different heme environments (α and β chain) seems to be ruled out since a similar broad signal was observed in NO–Mb. The spectrum of NO–ferrocytochrome *c* (Figure 2A) is almost indistinguishable from those of the C-type model systems. The assignment of the bands as shown in Figure 2 is made in complete analogy with the C-type spectra of the model systems. The electron paramagnetic resonance parameters thus obtained show indeed the qualitative trend of the C type (*cf.* Table 1), *i.e.*, $g_y \simeq 2.00$, the large NO hyperfine splitting as well as broader line width in *Z* absorption. The ^{14}N coupling constant in the Z orientation (26.8 gauss, obtained from the observed ^{15}N splitting) is about 3 gauss greater than in NO-cytochrome *c*. This difference may be a reflection of slightly different orientations of the NO group relative to the heme normal. Since the *Z* absorption has $g = 2.003$–2.005, *the molecular orbital containing the unpaired electron in NO–Hb and NO–cytochrome c is also the out-of-plane* σ^**-type orbital.*

Recently Shiga *et al.* (1969) found that it is difficult to simulate the electron paramagnetic resonance spectrum of NO–HB (α and β chain, separately) by using a Gaussian curve of a certain line width. In the present scheme the difficulty could be predicted, since the over-all width of *X*, *Y*, and *Z* absorption is generally not the same, as can be seen in Figure 1. This is probably due to the anisotropic hyperfine splitting from pyrrole nitrogens. Consequently, the simulation by use of a Gaussian curve with a fixed line width is considered inadequate.

d_π–p_π *Interaction.* The lack of a simple relationship, mentioned above, between the basicity and the spectral type suggests that π bonding between Fe and the base molecule plays an important role in the over-all iron-base bonding, and that the π bonding may be interplaying with the σ bond.

A somewhat more significant finding for d_π–p_π interaction would be that the observed electron paramagnetic resonance spectra always show rhombic distortions, *i.e.*, there being two distinct g values in the x–y plane, whenever the base molecule coordinated has a π electron system which can interact with the heme π orbitals so as to breakdown the fourfold symmetry axis normal to the heme plane. On the other hand, when the ligand lacks π electrons (*e.g.*, amines) or when they can interact with heme π system without lowering the fourfold symmetry around z axis (*e.g.*, NO–heme–CN$^-$ in aqueous medium (H. Kon, 1968, unpublished data) where the C–N bond is assumed to be normal to the heme plane), an electron paramagnetic resonance spectrum for an axial symmetry, *i.e.*, of D type, is observed. The only exception is with imidazole in polar media (H_2O and Me_2SO), for which we have no immediate explanation. Note, however, that in a less polar medium which is considered more like the actual environment in hemoproteins, the complex shows rhombic symmetry. These observations seem to be best understood if NO is not itself the cause of the rhombicity, but instead it is the base π orbital which, by interacting preferentially with one of the two out-of-plane d_π orbitals (d_{xz}, say), makes the two d_π orbitals inequivalent.

The perpendicular g values in the present case, where the unpaired electron is in d_{z^2}, are expressed by

$$g_y = 2.0023 + \xi\alpha^2\Sigma\beta^2\left|<d_{z^2}\middle|1_y\middle|d_{xz}>\right|^2/(E_{xz} - E_{z^2})$$

where ξ is the spin orbit coupling constant in the Fe d_{z^2} orbital; α and β the probability distribution of an electron in the d_{z^2} and d_{xz} orbital of the Fe, respectively. The summation is taken over the molecular orbitals which contain the Fe d_{xz} orbital. A similar expression applies to g_x if y is replaced by x. Interestingly, in the C type cited in Table I the g_y values are found near the free spin value; *i.e.*, the second term in g_y is rather small. This situation may be explained by large energy separation, $E_{xz} - E_{z^2}$ and/or the considerable electron delocalization out of Fe d_{xy} orbital (*i.e.*, small β^2). The small g_y value can also be the result of the cancellation of the terms with opposite signs; note that the correction term which is derived by exciting an electron from a filled d_{xz} orbital to d_{z^2} has the positive sign while the one obtained by exciting the electron in the d_{z^2} orbital to an unfilled d_{xz} carries the negative sign (McConnell and Robertson, 1957). In any case the small g_y values of the C-type spectra suggest that there is a stronger d_π–p_π interaction involving the empty π orbitals of the base than in other types.

The effect of SDS which converts NO–Hb spectrum from C to A type (*i.e.*, increasing g_y value) (Kon, 1968) can now be interpreted as decreasing d_π–p_π interaction.

A conceivable effect of such d_π–p_π interaction may be that the orientation of the Fe d orbitals relative to the porphyrin structure is in some cases determined by the orientation of the imidazole ring of the bound histidine residue. The recent study of metmyoglobin azide (Helcké *et al.*, 1968) indeed shows that the direction of the principal axes of the g tensor can be explained by such π–π interaction with the imidazole (Gibson and Ingram, 1957; Griffith, 1957, 1964).

Unpaired Electron Density. In the previous article a claim was made, on the basis of other electron paramagnetic resonance evidence (Kon, 1968), that the unpaired electron in NO–Hb and some related systems would be considerably delocalized into Fe orbitals. No quantitative statement, however, was made at that time concerning the actual spin density on Fe. It would be possible to make at least a semiquantitative estimate by using [^{57}Fe]hemin unless the spin density on Fe is in fact too low. Since, however, the nuclear g factor of ^{57}Fe is rather small (~45% of that of ^{14}N), the two systems which gave the best resolved electron paramagnetic resonance spectra were chosen for the purpose of detecting the possibly small effect of ^{57}Fe on the spectrum: 2,4,6-trimethylpyridine–[^{57}Fe]hemin–^{15}NO (I) and 8-methylquinoline–[^{57}Fe]hemin–^{15}NO (II), a model system for NO–Hb and its SDS-modified form, respectively. The effect was indeed observed as a further splitting (5.78 gauss) in II and as a broadening (4.33 gauss) in I, of ^{15}N doublets in Z absorption. In the X and Y absorptions the broadening could hardly be measured. However, by assuming the isotropic ^{57}Fe hyperfine splitting (a) being equal to the broadening due to ^{57}Fe substitution observed in solution at room temperature (3.86 and 3.13 gauss in I and II, respectively), one can calculate the anisotropic part of the hyperfine splitting due to ^{57}Fe by the following relations (McGarvey, 1967)

$$A = -K + 4\alpha^2P/7 - (g_\perp - 2.0023)P/7$$

$$a = (A + 2B)/3 = -K + 2(g_\perp - 2.0023)P/3$$

where A and B are the ^{57}Fe hyperfine splitting along the molecular z axis and in the x–y plane, respectively, and K the Fermi contact term. The average of g_x and g_y was taken as $g_\perp$. For P, which is proportional to the anisotropic part of the ^{57}Fe hyperfine interaction, two values were used: 32.9×10^{-4} cm^{-1} which was obtained by McGarvey (1967) for Fe^{3+}(3d^5) from Hartree-Fock free ion wave function, and 19.4×10^{-4} cm^{-1} calculated for Fe^0(3d^8) in this work using a similar wave function by Watson (1959). The probability density, α^2, of the unpaired electron in the d_{z^2} orbital of Fe^{3+} was then calculated to be 47 and 54% on I and II, respectively, and 76 and 86% for Fe^0. The latter set of values evidently are near the truth, since the net charge on Fe in such Fe-porphyrin complexes is shown to be almost neutral (Zerner *et al.*, 1966). The results thus corroborate the qualitative statement made in the previous work.

In I, which gives a type C spectrum, an extra ^{14}N hyperfine splitting of about 6.7 gauss due to the base nitrogen is observed. If a reasonable assumption is made that the unpaired electron is in an sp^2-hybridized orbital of the nitrogen, the super hyperfine splitting corresponds to the spin density of 3.2% (McNeil *et al.*, 1965). These estimations may be considered to give the lower limit of the unpaired electron density outside of NO group, since there could be a small but finite density in the σ orbital of porphyrin nitrogens and some s orbitals of Fe which are of the same symmetry as d_{z^2}.

Conclusion

According to a unified interpretation of the electron paramagnetic resonance spectra of NO–heme–base complexes and the NO–hemoproteins, the unpaired electron orbital in all these systems is the d_{z^2}-type σ^* orbital, not the π^* orbital of NO group. Also the importance of the d_π–p_π interaction between the Fe d_{xz}, (or d_{yz}) and the π orbitals of the base molecule in determining the electronic structure is suggested.

It is particularly worth noting that the spectra of unperturbed NO–Hb, NO–Mb, and NO–cytochrome *c* belong to the C type, in which the strong interaction between Fe and the base molecule (histidine in the hemoproteins) was found operating through π as well as σ orbitals as is manifested by the g_y value and the super hyperfine splitting, respectively. If we realize that NO–Hb molecule would perhaps be a nearest available model system of oxyhemoglobin, it seems obvious that the characteristic electronic structure of imidazole group has to be realistically taken into account, along with that of the sixth ligand, in any theoretical description of the mechanism for reversible oxygen binding of hemoglobin and myoglobin.

Furthermore, the present finding that the unpaired electron in NO–hemoproteins occupies the out-of-plane σ^* orbital, and that it is strongly delocalized out of the NO group, places severe restrictions on any theoretical model which attempts to explain the bonding of O_2 and NO to hemoglobin and myoglobin.

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Requirement of Different Sulfhydryl Groups in the Activation and Transfer Reactions of Isoleucyl Transfer Ribonucleic Acid Synthetase*

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ABSTRACT: Purified isoleucyl transfer ribonucleic acid synthetase isolated from *Escherichia coli* catalyzed two reactions: (I) the conversion of isoleucine and adenosine triphosphate into isoleucyl adenylate–enzyme complex (the activation reaction); (II) the transfer of isoleucine from the complex to transfer ribonucleic acid. The role of the sulfhydryl groups in the enzyme was studied using *p*-mercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) as inhibitors. The native enzyme contained 10–11 titratable sulfhydryl groups. One or two of these sulfhydryls reacted very rapidly with 5,5′-dithiobis(2-nitrobenzoic acid). The loss of these rapidly reacting sulfhydryl groups resulted in complete inability to catalyze reaction I. Titration of the isolated isoleucyl adenylate–enzyme complex with *p*-mercuribenzoate resulted in a loss of ability to transfer the isoleucine to transfer ribonucleic acid (reaction II), while the ability to catalyze reaction I was essentially unaffected. The results demonstrate that different sulfhydryl groups in the enzyme are involved in reactions I and II.

One of the common properties of the aminoacyl-tRNA synthetases is their requirement for intact enzyme sulfhydryl groups (McElroy *et al.*, 1967; Novelli, 1967). The only synthetase not inhibited by sulfhydryl reagents is the *Escherichia coli* lysyl-tRNA synthetase (Stern *et al.*, 1966). In the case of the tryptophanyl-tRNA synthetase from beef pancreas, it has been shown that the presence of ATP–Mg and tryptophan prevents four of eight sulfhydryls from reacting with DTNB[1] (DeLuca and McElroy, 1966),

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[1] Abbreviations used in this paper are: NEM, *N*-ethylmaleimide; PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Ile-AMP–E, the complex formed with isoleucyl-tRNA synthetase and isoleucyl adenylate; tRNA-AMP, the terminal AMP of tRNA; BSA, bovine serum albumin.