Paramagnetic Resonance Study of Nitric Oxide Hemoglobin

HIDEO KON
From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

The electron paramagnetic resonance absorption of nitric oxide hemoglobin was observed as a possible model system for oxyhemoglobin. The electron paramagnetic resonance spectrum showed three g factors indicating a rhombic symmetry around the paramagnetic center. By use of \(^{15}\text{NO}\), the unpaired electron was found very little associated with NO nitrogen, in contrast to what has been customarily assumed.

Modifying NO-hemoglobin with sodium dodecyl sulfate (SDS) shifted the whole spectrum toward a lower magnetic field retaining the rhombic symmetry. The \(^{14}\text{N}\) hyperfine structure was now resolved in one of the peaks with the splitting of 15 gauss. The number of SDS molecules to complete this transformation was found equal to the number of the basic amino acid residues. At high SDS concentrations (>0.2 M) further transformation of the spectrum took place which indicated the randomization of the structure and the change of symmetry type around the paramagnetic center from rhombic to axial.

NO-hemoglobin powdered specimen after dehydration gave a spectral pattern indistinguishable from that of the SDS-modified NO-hemoglobin. This spectral conversion to dehydrated type could be repeated completely reversibly by evacuating and replenishing H\(_2\)O vapor.

The interaction with 0.03 to 0.5 M sodium salicylate resulted in a decrease of absorption with concomitant appearance of four new peaks, tentatively interpreted as due to a low spin Fe(III) type electronic configuration. The possible implication of the rearrangement of the electronic state was discussed in connection with the known oxidase-like activity of oxyhemoglobin under similar conditions.

Since electron paramagnetic resonance was introduced for the study of molecules of biological interest, hemoglobin and myoglobin have been perhaps the most extensively studied metalloproteins. In addition to the well known successful determination of the heme orientation relative to the crystallographic axis in these two cases (1), detailed information such as the order of orbital energy levels in the d shell of iron (2), the symmetry of the electric field surrounding the iron (3, 4), and the change in electronic configuration as a result of protein denaturation (5) has been obtained. EPR can also be used as a practical means of identifying some abnormal hemoglobins (6).

These investigations, however, have been made almost exclusively with methemoglobin and its derivatives in which the iron is in the oxidized state and would no longer bind oxygen molecules. Hence little knowledge has been obtained concerning the electronic state of reduced hemoglobin or the nature of the oxygen-iron bonding.

In this paramagnetic resonance study of NO-hemoglobin our interest is focused on (a) the distribution of the unpaired electron, which has been assumed to be localized on the NO group, and (b) the effect of addition of small molecules such as sodium dodecyl sulfate or sodium salicylate upon the EPR spectrum. SDS is known to bind quantitatively to hemoglobin, causing a definite change in optical and magnetic properties (7), while sodium salicylate has been shown to change the hemoglobin function (8, 9). Also the effect of dehydration of powdered NO-hemoglobin on the EPR spectrum is discussed.

MATERIALS AND METHODS

**Hemoglobin**—Human adult hemoglobin was prepared from packed red blood cells by Drabkin's method (10). After concentrating the oxyhemoglobin solution by dialysis in 2.8 M potassium phosphate buffer, at least one recrystallization was done for each preparation. Methemoglobin was prepared according to the method of Benesch, Benesch, and Macduff, and the absence of ferrocyanide was ascertained by negative Prussian blue test (11).

**Reagents**—Fisher certified sodium salicylate and sodium dodecyl sulfate were used without further purification. Nitric oxide (98.5% minimum) and nitrogen (prepurified, 99.99% minimum) gas were obtained from Matheson Company, Inc. (East Rutherford, New Jersey). \(^{15}\text{N}\) Nitric oxide (96.8 atom % in \(^{15}\text{N}\)) obtained from Bio-Rad Laboratories and from Isomet Corporation (Palisades Park, New Jersey) were found by mass spectrometry to contain varied quantities of sulfur dioxide and nitrogen dioxide. These impurities were eliminated by condensation at liquid nitrogen temperature.

The abbreviations used are: EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate.
Preparation of NO-hemoglobin Solutions—Two to 3 ml of oxyhemoglobin solution (0.5 to 0.8 mM heme in 0.01 M phosphate buffer, pH 7) were placed in a Thunberg type container modified for EPR observation and was deoxygenated at room temperature by several alternating evacuations and equilibrations with high purity nitrogen gas. Conversion to reduced hemoglobin was confirmed spectrophotometrically. Nitric oxide gas was introduced to the cell to slightly less than 1 atm. Excess NO was pumped out after 2 min and the cell was filled with high purity nitrogen. The solution, transferred to the quartz side arm by tilting the cell, was frozen in liquid nitrogen for EPR observations.

The same results were obtained by starting with methemoglobin solution, plus nitric oxide, the latter acting as the reducing agent (12). In this case the degassing time may be shorter, but contact with NO was made longer to complete the reduction (~10 min). Slight turbidity observed in some cases during evacuation of methemoglobin solution disappeared after contact with nitric oxide.

The effect of sodium salicylate or SDS was studied in a closed cell with two compartments, one containing the hemoglobin solution and the other that of SDS or salicylate. After degassing the two solutions and forming NO-hemoglobin, as described above, the two solutions were mixed fully and incubated for 5 min at room temperature.

Within the used concentration range of SDS or salicylate, the reversal of the order of NO-hemoglobin formation and the reaction did not cause significant difference in EPR absorption. In these experiments, oxyhemoglobin solution was generally used as the starting material, although the final results in a few cases starting with methemoglobin solution were the same as with oxyhemoglobin.

Paramagnetic Resonance Absorption—Most observations were made in a 3-mm inside diameter quartz tube attached to the Thunberg type container at liquid nitrogen temperature. The EPR spectrometer used, except in experiments at liquid helium temperature, was the Varian V-4500 with 100-KHz field modulation combined with the microwave bridge circuit adopting a circulator and V-232 Klystron. The microwave power level was always attenuated by at least 10 decibels.

The Varian 6-inch magnet was regulated and scanned by a Fieldial (Varian Associates). The magnetic field strength was calibrated by a proton resonance gaussmeter combined with a frequency counter. The microwave frequency was measured with a resonant cavity frequency meter. Most observations were made at liquid nitrogen temperature, with a small Dewar flask similar to the Varian V-4546.

A special flat quartz cell was made for observations at liquid helium temperatures, 4.2° and 2°K. The appropriate solution, prepared anaerobically in a Thunberg type cell, was dispensed into this flat cell in a dry box, frozen, and sealed under vacuum. The cell was placed at the bottom of a rectangular resonant cavity for observation.

Several freeze-thaw cycles did not change the EPR spectrum of NO-hemoglobin. When the perturbator was added, freeze-thaw cycling did produce some change (see "Results and Discussion"). All EPR spectra shown in this article were recorded as the first derivative of absorption, with the ordinate the intensity of absorption in arbitrary units and the abscissa the magnetic field strength in gauss. The positions of absorption bands are designated by the spectroscopic splitting factor, or the g factor, which is proportional to the ratio of the microwave frequency used and the magnetic field strength at the particular absorption band, and therefore is independent of the microwave frequency used. All of the experiments were repeated at least three times. There were no other absorptions detected between g ~ 2 and g ~ 6 regions than those discussed in the following section.

RESULTS AND DISCUSSION

NO-Hemoglobin—The EPR spectrum of NO-hemoglobin is shown in Fig. 1 by the solid line. The spectrum was taken in frozen solution, where each NO-heme group is randomly oriented relative to the external magnetic field direction. Since, in general, the positions of the absorption, or g factors, depends upon the molecular orientation, the spectrum obtained is a superposition of spectra corresponding to each orientation. It was shown by theoretical analysis that, in spite of its apparent complexity, the random pattern can be used: (a) to distinguish the symmetry type of the electric field surrounding the paramagnetic center (octahedral, axial, or rhombic) and (b) to identify the peak corresponding to the molecular orientation in which each symmetry axis is parallel to the magnetic field direction (13, 14). Although the peaks shown here are broad and partly superimposed, the absorption curve of NO-hemoglobin shows the characteristic shape of the system with rhombic symmetry around the paramagnetic center: there are peaks on each side of the horizontal line at g = 2.000 and 1.986, respectively, and, in between, an "inverted S shape" with some structure located around g = 2.023.3 Because of the broadness of the peaks and overlapping the g values must be considered only tentative.

One intriguing feature of the spectrum is the absence of resolved nuclear hyperfine structure due e.g. to the nitrogen nucleus which has spin I = 1 and should show a set of three peaks with equal intensity. The splitting of the lines is proportional to the probability distribution of the unpaired electron on the nitrogen atom. One possible explanation for the lack of such

2 The experiments at helium temperatures were carried out at the Westinghouse Laboratories by courtesy of Dr. D. Mergerian and his co-workers.

3 Obtained at the midpoint of the inverted S shape region of 14N curve.
4352

liquid nitrogen temperature. If the correlation time of the NO absorbed on MgO...

ment of the NO group may vary from one heme group to another.

be caused by some structural inhomogeneity; i.e. the environ-

to be quenched. The spectra at both temperatures were virtu-

ally identical with that at 77°K, showing that the broadening,

yond resolution. However, this explanation was ruled out by

motion is long, the anisotropic part of the hyperfine interaction

(CR&NO").

ence observed here seems to indicate that the coupling constants

splitting would be almost 20 gauss. The much smaller differ-

known nitric oxide compounds is 60 to 70 gauss when the un-

paired electron is known to be localized mainly on the NO group

in Table I, the total spread of the 14N hyperfine structure of

structure would be that the nitric oxide group, with one end at-
tached to Fe(II) ion, may still have some random motion even at

liquid nitrogen temperature. If the correlation time of the motion

would not be averaged out and the peaks may be broadened be-
yond resolution. However, this explanation was ruled out by

experiments at 4.2° and 2°K, at which such motion is presumed
to be quenched. The spectra at both temperatures were virtu-

ally identical with that at 77°K, showing that the broadening,

if any, could not be motional. The broadening could, however,

cause by some structural inhomogeneity; i.e. the environ-

ment of the NO group may vary from one heme group to another.

It is also conceivable that the nitrogen hyperfine structure may

not be resolvable simply because the unpaired electron is coupled

too many magnetic nuclei. In any case, if the hyperfine

splitting due to the NO nitrogen is sufficiently large but unres-

olved, the use of 14NO may be helpful, since the 15N nucleus with

spin I = 1/2 would show a doublet hyperfine structure and the

total splitting would be approximately 30% less than with 14N.

Consequently, the absorption curve of 14NO-hemoglobin would

show considerable narrowing. In Fig. 1, the result of the 15NO

experiment is compared with that of 14NO, both curves being nor-
malized to the same integrated intensity. There is virtually no

difference between the two spectra at g = 2.060 and 1.986 and

the difference at the center is no more than 10 gauss. As shown

in Table I, the total spread of the 14N hyperfine structure of

known nitric oxide compounds is 60 to 70 gauss when the un-

paired electron is known to be localized mainly on the NO group

and, consequently, the decrease by 15N labeling of the over-all

splitting would be almost 20 gauss. The much smaller differ-

ence observed here seems to indicate that the coupling constants

are rather small. Coupling constants are the sum of contribu-

tions from the isotropic and anisotropic interactions. These

could have opposite signs and make some component of the hy-

perfine coupling tensor rather small. However, the fact that all

three components along the mutually perpendicular axes are

small should indicate that the hyperfine interactions, isotropic

as well as anisotropic, are indeed weak.

It may be inferred that the distribution of the unpaired electron

on the NO nitrogen is rather small. The oxygen atom in NO

may have some unpaired electron density, but it should be less

than that of nitrogen (15, 16). A reasonable assumption

would be that the unpaired electron is distributed considerably to iron

ion possibly with small probability on the porphyrin and the im-

idazole group of the proximal histidine. There is some support-

ing evidence for this interpretation. The g factors of other NO-

containing molecules are always very close to the free spin value,

2.0023, when the unpaired electron is known to be localized

mainly on the NO group, while there is a fair amount of devia-

tion when the unpaired electron is mostly on a transition metal

ion as the result of spin orbit interaction (Table I). All of three

g factors of NO-hemoglobin deviate considerably from 2.0023,

indicating that the 3d orbital of the iron ion is strongly involved.

Also, the large Mössbauer hyperfine structure of NO-hemoglobin

was interpreted as a sign of very strong covalent bonding between

iron and NO and of a large spin transfer to the iron orbital (20).

It was shown also by the same authors that the Mössbauer results

are satisfactorily explained neither by the ferric assignment (one

hole in t2g shell), nor by the ferrous assignment (the unpaired

electron in a π orbital of NO). Thus, there is a strong possibility of

Fe1-type electronic configuration, i.e. of the unpaired electron

spending a considerable amount of time in one of the antibonding

orbitals of iron as, for example, in K[Fe(CN)6]NOH (15,

17, 21).

The conclusion that unpaired electron distribution to NO is

small would clearly contradict what has been assumed for the

electron structure of NO-hemoglobin, i.e. considering the un-

paired electron as associated entirely with NO group in the inter-

pretation of the magnetic susceptibility (22), EPR absorption

(23, 24), and also in the theoretical calculation of the g factor (25).

Gordy and Rexroad (24), e.g. reported a three-line hyperfine

structure with a coupling constant of 18 gauss centered at g =

2.006 in the powdered methemoglobin-NO system at 77°K.

They concluded that the unpaired electron is associated mainly

with the NO group. As is shown below, however, reexamination

of the same system has shown that the hyperfine structure can

be observed only when the protein loses some of the bound water,

and that the spectrum discussed by Gordy and Rexroad actually

consisted of parts of spectra of two molecular species, NO-hemo-

globin and partially dehydrated derivative. Consequently, the

fact that g = 2.006 happens to be close to the free electron

value can hardly be an adequate evidence for localization of the

unpaired electron on the NO group.

This conclusion as to the spin distribution, as well as the finding

that in NO-hemoglobin the EPR absorption shows the three com-

ponents of g factor indicating rhombic symmetry, have not been

pointed out in previous EPR works on NO-hemoglobin (23, 24,

26). It would be stretching the point too far to extend the con-

clusion concerning the transfer of the unpaired electron in NO-

hemoglobin to the Fe-O2 bonding in oxyhemoglobin. The differ-

ence in the relative height of iron 3d orbitals and the antibonding

π orbitals of the ligand in the two cases could make a substantial

change of situation (27). It is interesting to note that Weiss

(28) has proposed recently that binding of oxygen by hemoglobin

is accompanied by transfer of an electron from iron to the O2

group. A similar claim was made for CO-hemoglobin, i.e.

Fe3+·CO−. This mechanism appears consistent with a theo-

retical calculation (29). Perhaps the significance of the result

a Rhombic symmetry.
presented here regarding the distribution of the unpaired electron in NO-hemoglobin is that it provides a test case for the theoretical investigation (e.g. by molecular orbital calculations) of the Fe-oxygen bonding in oxyhemoglobin. An experiment with Fe-labeled NO-hemoglobin is in progress with a view to obtaining a more quantitative measure of the electron transfer.

Finally, it may be worth noting that the \( g = 2.023 \) peak shows a barely recognizable structure which might represent a three-line hyperfine splitting due to a nitrogen nucleus. There is also a marginal indication of a possible hyperfine structure in the peak with \( g = 2.094 \).

**Effect of Sodium Dodecyl Sulfate**—This anionic detergent is known to cause a reversible change in the protein structure of myoglobin and hemoglobin as shown by optical and magnetic susceptibility measurements (7). Its effect on the EPR spectrum of NO-hemoglobin is shown in Fig. 2. At low concentrations of SDS, the peak at \( g = 1.986 \) started decreasing and a new three-line peak with its center at \( g = 2.009 \) emerged. A new peak also appeared at the low field end around \( g = 2.094 \). As SDS concentration was increased the transformation from NO-hemoglobin pattern to the one shown in Fig. 2b became complete at a certain concentration, beyond which no further change was observed until the concentration (\( \sim 0.2 \) M) was reached, above which another change of signal was observed (side infra). In the example shown in Fig. 2 in which the hemoglobin concentration was \( 2.6 \times 10^{-4} \) in heme, the changeover of the spectrum was completed at around \( 6 \times 10^{-4} \) in SDS. Thus, the number of SDS molecules needed for this transition per one functional chain is approximately equal to the number of basic amino acid residues, 24 per unit.

This number and the fact that the change appears to proceed in an all or none fashion agree with the similar observation of reduced hemoglobin and myoglobin by the magnetic susceptibility measurements. The phenomenon was explained as a “loosening” of the protein structure due to the combined effect of electrostatic and hydrophobic attractions between the detergent molecules and the protein. The shape of the EPR pattern after the complete transformation shows that the molecule retains the rhombic symmetry around the paramagnetic center. There are two broad peaks at \( g = 2.094 \) and 2.051, the latter showing a barely visible three-line structure similar to that mentioned in connection with the \( g = 2.023 \) peak of NO-hemoglobin. The third peak centered at \( g = 2.009 \) has a well resolved three-line pattern which was confirmed, by \( ^{14} \text{NO} \), to be the hyperfine structure due to \( ^{14} \text{N} \) of the NO group. There is an over-all similarity between the spectra in Fig. 1 and that of the SDS-modified NO-hemoglobin, although there are two interesting differences: (a) all three peaks are shifted by SDS modification toward lower field approximately to the same extent (increase in \( g \) factors) and (b) the peak at \( g = 2.009 \) is now very well resolved. The shift of the \( g \) factors could be understood qualitatively as a direct result of the loosening of the protein structure mentioned above. The deviation of a \( g \) factor from the free spin value (2.0023) is, in general, inversely related to the energy separation between the unpaired electron orbital and the orbitals coupled to that by spin-orbit interaction (30). This separation tends to be greater when the electric field surrounding the unpaired electron is stronger, and vice versa. Thus the observed increases in \( g \) factors may indicate lowering of the unpaired electron orbital relative to the others.

There seems to be no immediate explanation for the improved resolution of the peak at \( g = 2.094 \). It may be the result of disappearance of some other hyperfine structure which may have existed in the peak at \( g = 1.986 \) of NO-hemoglobin obscuring the hyperfine structures. The widths at half-height of the two peaks (\( g = 1.986 \) and \( g = 2.009 \)), however, are almost the same (\( \sim 42 \) gauss), whereas they would be expected to be somewhat different if originally obscured by other hyperfine structures.

It is also conceivable that the lack of resolution in NO-hemoglobin may represent some subtle structural inhomogeneity which disappears on loosening the protein structure.

---

**Fig. 2.** a, the EPR spectrum of NO-hemoglobin partly modified by sodium dodecyl sulfate (SDS). Hemoglobin concentration is 0.26 mM in heme. SDS concentration is 4 mM. The spectrum was taken at \( 77^\circ \text{K} \). b, comparison of the EPR spectrum of the completely SDS-modified \( ^{14} \text{NO} \) (---) and \( ^{15} \text{NO} \) (----) hemoglobin at \( 77^\circ \text{K} \). Both spectra were normalized to the same integrated intensity. Solvent, 0.01 M, pH 7 phosphate buffer. Heme concentration, 0.26 mM. SDS concentration, 6.7 mM. c, a typical EPR spectrum of NO-hemoglobin with concentrated SDS solution at \( 77^\circ \text{K} \). The spectrum indicates the randomization of structure around the heme-NO moiety. Similar spectrum was obtained by repeated freeze-thawing of NO-hemoglobin solution with lower SDS concentrations. Solvents, 0.01 M, pH 7 phosphate buffer. Heme concentration, 0.3 mM. SDS concentration, 200 mM.
From the difference in line shapes of the $^{14}$N and $^{15}$N spectra (Fig. 2b), we can conclude that the nitrogen hyperfine splitting in the $g = 2.094$ and 2.051 peaks is no more than that in the $g = 2.009$ peak, 17 gauss. If the hyperfine splitting is completely isotropic, the unpaired electron density on the nitrogen 2s orbital is only 3.1% and even if we assume an anisotropy of 80% as in the $\pi$ radical, RR'NO, the density on the nitrogen 2p orbital is still not greater than 20% (assuming that the three hyperfine coupling constants have the same sign, as is generally the case in NO-containing radicals). Thus, the unpaired electron appears not to be concentrated on the NO group, but associated rather with the iron ion as in native NO-hemoglobin.

At very high SDS concentration ($>0.2$ mM) or by freeze-thawing a solution of lower concentration, a further change in EPR spectrum takes place as shown in Fig. 2c. The overall EPR shape then shows the characteristics of the powder pattern of a molecule with axial symmetry around the paramagnetic center (13, 14), the rhombic character which existed before having disappeared. The $g$ factors of the parallel, 2.010, and perpendicular, 2.071, peaks are closely related, respectively, to 2.009 and to the average of 2.094 and 2.051, peaks of the previous spectrum (Fig. 2b). It would be reasonable to assume that the symmetry axis is almost parallel to the 4-fold axis ($z$ axis) perpendicular to the porphyrin ring ($x-y$ plane). Thus it appears that the effect of strong SDS solution is to eliminate the factor which distinguished the two directions in the ($x-y$) plane, resulting in merging of the two perpendicular peaks (2.004 and 2.051). There is, in the parallel band, a slight decrease in the apparent $^{14}$N splitting (15 gauss) and a remarkable change in the relative height of the hyperfine components, the side peaks being almost smeared out by broadening. Since the side peaks correspond to the $^{14}$N nuclear spin state with $I_z = \pm 1$, and the central peak to $I_z = 0$, the relative broadening of the side peaks would indicate randomization of the magnetic environment along the $z$ axis and, consequently, of the structures surrounding the nitrogen concerned.

The $g$ factors obtained here approximately satisfy the condition for the unpaired electron assignment to the 3d$_z$ orbital (30) ($g_1 \approx 2.0023$, $g_4 > 2.0023$). Thus the situation appears analogous to the pentacyanonitrosferrocene anion radical in its protonated form (15, 17, 21), in which the unpaired electron is mostly in 3d$_z$ orbital.

One unpaired electron in the nitrogen 2s and 2p orbital would give rise to the $^{14}$N isotropic and anisotropic splitting of 550 and 34.1 gauss, respectively (15).

**Effect of Sodium Salicylate**—The effect of sodium benzoate and

---

**Fig. 3.** The EPR spectrum of the powdered methemoglobin-NO system at 77K. Lyophilized methemoglobin, 3 mg, evacuated ($5 \times 10^{-4}$ mm Hg) for 12 hours, made contact with NO gas (~1 atm) for 50 min and reevacuated. All procedures at room temperature. The spectrum is interpreted as the superposition of NO-hemoglobin spectrum and that of its dehydrated form.

Methemoglobin Powder Nitric Oxide System; Effect of Dehydration—It has been reported by Gordy and Rexroad (24) that methemoglobin and cytochrome $c$ powders treated with NO gas developed a three-line pattern with a splitting of 18 gauss, plus a fourth, broader peak on the high field side. They interpreted the three-line pattern as $^{14}$N structure of the NO molecule which is complexed with methemoglobin at a distance from heme close enough to be effectively coupled with Fe$^{3+}$ ions. The fourth peak was considered to be the result of the anisotropic $g$ factor. In view of the apparent similarity of the results with a part of the spectrum of SDS-modified NO-hemoglobin described above, an effort was made to reproduce the result for close comparison.

Lyophilized methemoglobin powder kept under high vacuum ($~5 \times 10^{-5}$ mm Hg) for 12 hours showed, on contact with NO gas for 50 min and reevacuation, the spectrum shown in Fig. 3. The hyperfine splitting (16.6 gauss) is slightly smaller than the value (18 gauss) reported by Gordy and Rexroad. There is a broader high field peak at $g = 1.984$ which presumably is identical with Gordy's "fourth peak." A remarkable aspect, however, is the close resemblance of the whole spectrum to that of the NO-hemoglobin modified by SDS (Fig. 2a). A low field half of the spectrum apparently was overlooked by Gordy and Rexroad. Since the spectrum in Fig. 2a represents a mixture of native and SDS-modified NO-hemoglobin, the system under discussion should, also, be a mixture of similar nature.

Thus, iron ions, at least those represented by the spectrum in Fig. 3, are reduced in the same state as that of NO-hemoglobin rather than being Fe(III) as assumed by Gordy and Rexroad. There was a marked decrease in peak intensity at $g = 6$ characteristic of the high spin methemoglobin.

When this system was equilibrated at room temperature for more than 10 min with deoxygenated water vapor, the spectrum became identical with that of NO-hemoglobin (Fig. 1, solid line). There was simultaneously a considerable increase in overall intensity, and the peak at $g = 6$ completely disappeared. When this system was evacuated again at room temperature for 60 hours, a spectrum similar to that shown in Fig. 3 was obtained, in which total intensity remained high, but the component at $g = 1.984$ was relatively low, probably due to further dehydration by the prolonged evacuation. This reversible conversion from the hydrated to the partially dehydrated spectrum could be repeated at least several times without change in shape or intensity.

Evacuation at 70–80°C for about 3 hours eliminated the peak at $g = 1.984$, the whole spectrum now being virtually identical with the completely SDS-modified system (Fig. 2b, solid line).

A reasonable explanation for the observed change would be that initially formed NO-methemoglobin (12, 31) was reduced in the presence of the H$_2$O vapor to NO-hemoglobin, and molecules which lost some of the protein-bound water exhibited a spectrum with $^{14}$N hyperfine structure similar to that with SDS. This indicates the similarity of the changes in protein structure caused by dehydration and by SDS.

The conclusions drawn by Gordy and Rexroad (24) concerning the location of the unpaired electron and the mechanism of the spin lattice relaxation evidently do not apply in view of the fact that these authors observed only part of the spectrum of NO-hemoglobin ($g = 1.986$) superimposed on that of the dehydrated NO-hemoglobin, and assumed that this spectrum represented a single molecular species.
salicylate on reduced, oxy-, and methemoglobin, respectively, has been studied with respect to the change in visible absorption spectra (32), increase of autoxidation, and formation of choleglobin (8, 9).

According to these authors, a dilute solution \(1.5 \times 10^{-4} \text{ M}\) of equine or bovine hemoglobin starts changing its visible absorption at benzoate concentrations of about 0.3 M and 0.7 M for met- and oxyhemoglobin, respectively. Reduced hemoglobin is more resistant to the perturbator under the same conditions. Similar effects are produced by sodium salicylate at somewhat lower concentrations.

A most intriguing fact, however, is that, at much lower concentrations of these perturbators than required to change the visible absorption, there is known to occur a remarkable enhancement of \(O_2\) uptake by oxyhemoglobin solution in the presence of ascorbic acid. Specifically, at salicylate concentrations as low as approximately 0.03 M, the oxygen uptake by oxyhemoglobin was twice that in the absence of salicylate, reaching a rather flat plateau at 0.1 M and starting to decrease around 0.2 M (8). The change in visible absorption starts only above 0.2 M. Thus it appears that, for a certain limited concentration range, salicylate seems to modify the structure of oxyhemoglobin such as to activate oxygen molecule to develop oxidase-like activity. These changes are not detectable in the visible absorption bands.

Although it is not known whether an analogous phenomenon would be observed with NO-hemoglobin system, it would be interesting to see the effect of salicylate modification on the EPR absorption, since, if the primary mechanism of oxygen activation is alteration of protein structure around the heme-oxygen group, it is conceivable that the heme-NO moiety would respond in a somewhat similar manner, resulting in some change in EPR spectrum.

The effect of sodium salicylate on the EPR spectrum of NO-hemoglobin is shown in Fig. 4. All experimental conditions including the instrument settings were kept constant during the series of experiments. The change in spectral shape which started at as low a concentration as 0.01 M was almost identical with that observed during the reaction with SDS: i.e. the decrease in intensity of the \(g = 1.986\) peak, the emergence of the three-line peak at \(g = 2.009\), and so on (see the preceding section). Simultaneously, however, the over-all intensity started decreasing, in this case above 0.02 M, reached a minimum around 0.05 M, and stayed low until around 0.5 M. Above 0.7 M there was a rather abrupt increase of the signal intensity and spectral shape was now similar to that of the structure randomized around the heme group in the modification by a concentrated SDS (Fig. 2c). The visible absorption of NO-hemoglobin, with peaks at 545 and 570 m\(\mu\), showed little change by addition of salicylate up to 0.5 M.

When the magnetic field was scanned over a wider range (1000 gauss), it was found that for salicylate concentrations higher than 0.02 M the spectrum was flanked by two additional peaks on each side, as shown in Fig. 5. The apparent g factors are 2.28, 2.19, 1.93, and 1.83. No other peaks were observed outside of this region. These peaks increase their intensity concomitantly with the decrease in intensity of the peaks in the middle: their intensity was highest at approximately 0.05 M, remained almost constant up to 0.5 M, and then decreased toward higher salicylate concentration as the randomization started.

If the signal intensity of 0.01 M solution is compared with, e.g. that of 0.5 M solution, it can be clearly seen that a small fraction of the NO-hemoglobin molecules underwent a transformation similar to that in the dehydration (or in the SDS modification), but others took another course, either consecutively or simultaneously, involving some change of electronic structure of the prosthetic group.

The reason why NO-hemoglobin showed the two kinds of response to salicylate ion remains to be investigated; the exact nature of the new state caused by salicylate is also unknown, but the possibility of the additional peaks coming from the salicylate-denatured methemoglobin can be definitely ruled out; i.e. the methemoglobin solutions containing 0.05 and 0.9 M...
hemoglobin, to an antibonding \( \pi \) orbital of the NO group and, therefore, would cause greater deviations in g factors. The extreme orbitals are located energetically near each other and, there-
electron is in one of the \( t_{2g} \) orbitals part of the time. These electronic structure of NO-hemoglobin in which the unpaired

of the new peaks may be that they represent a modified elec-
tronic states of iron in low spin methemoglobin derivatives. The iron
is exemplified by low spin methemoglobin derivatives. The
case of the unpaired electron localized entirely in the \( t_{2g} \) shell
would be necessary to transfer the unpaired electron, which
was tentatively assigned to the \( e_g \) orbital of iron in the NO-
hemoglobin, to an antibonding \( \pi \) orbital of the NO group and,
in addition, a fraction, \( f \), of an electron in the \( t_{2g} \) shell would have to transfer to the NO group, resulting in Fe\(^{2+ \cdot \pi \cdot \text{NO}} \). If a more electronegative O\(_2\) molecule were in place of NO under the same condition, the electron would migrate toward O\(_2\) even more, thus approximating the structure Fe\(^{3+ \cdot \text{O}_2^-}\), which could show oxidase-like activity. This, however, is only one of the several possible explanations of the observed phenomenon.

It was recently proposed by Weiss (28) that in order to explain the oxidizing effect of oxyhemoglobin observed under suitable conditions a superoxide nature of O\(_2\) must be assumed in oxy-
hemoglobin. However, since oxidase-like activity might be brought about by a change in the nature of the Fe-O\(_2\) bond by some denaturation, the oxidizing effect exhibited under some particular conditions would not require the superoxide character of O\(_2\) in oxyhemoglobin.

The rather drastic rearrangement in electron distribution assumed above as one possibility resembles somewhat a change seen in a small metal complex by Beinert, Deravartanian, and Hemmerich (17), and van Voorst and Hemmerich (21). These authors found that the pentacyanonitrosoferrate anion radical (Fe(CN)\(_5\)NO\(^-)\) showed two distinct EPR spectra depending upon pH. The spectrum observed at pH 5 to 10 was interpreted to represent the anion radical itself, in which the unpaired electron is mostly in an antibonding \( \pi \) orbital of the NO group, while that obtained in the lower pH range was ascribed to the protonated ion with its unpaired electron mostly in the \( d_{xy} \) orbital of iron ion. It is of particular interest that the reversible change from one form to the other takes place by protonation of the NO group. The possibility of hydrogen bond formation between the \( O_2 \) group and the distal histidine in oxyhemoglobin has been suggested by Pauling (35). The existence of an analogous hydro-
gen bond in metmyoglobin azide was shown by x-ray analysis (36).

The complete elucidation of the problem in the present case, however, awaits further investigation.

REFERENCES

10. Drabkin, D. L., J. Biol. Chem., 164, 703 (1946); Arch. Bio-
chem., 21, 224 (1949).

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Derivatives} & \text{g factor}\text{a} & \text{Reference} \\
\hline
\text{Heat-denatured methemoglobin} & 2.90, 2.41 & 2.25 & 1.91 & 5 \\
\text{Methanol-denatured methemoglobin} & 2.50 & 2.14 & 1.85 & 5 \\
\text{Methemoglobin azide} & 2.80 & 2.22 & 1.72 & 33 \\
\text{Methemoglobin hydroxide} & 2.6 & 2.3 & 1.7 & 34 \\
\text{Metmyoglobin hydroxide} & 2.61 & 2.19 & 1.82 & 3 \\
\hline
\end{array}
\]

a Three principal components of g factor.

\[\text{g factors of low spin methemoglobin derivatives}\]

Fig 5. The EPR spectra of NO-hemoglobin-salicylate system at 77°K in a wider range of the magnetic field to show the extra peaks. The central portions of the spectrum of 1.0 M and 0.02 M solution are curtailed. The salicylate concentrations are given on each spectrum. The three spectra were taken under the same experimental conditions. Solvent, 0.01 M, pH 7 phosphate buffer. Heme concentration, 0.4 mM.

4356 EPR of NO-Hemoglobin Vol. 243, No. 16.
Hideo Kon

Paramagnetic Resonance Study of Nitric Oxide Hemoglobin
Hideo Kon


Access the most updated version of this article at http://www.jbc.org/content/243/16/4350

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/243/16/4350.full.html#ref-list-1