Electron Spin Resonance Studies of the Denaturation of Oxy- and Methemoglobin

SPECTRA AND SOLVENT INTERACTIONS*

(Received for publication, October 21, 1965)

THOMAS C. HOLLOCHER

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

SUMMARY

The electron spin resonance technique shows that the heat denaturation of oxyhemoglobin involves (a) oxidation to methemoglobin or related material (high spin ferric), (b) conversion to low spin ferric species in an irreversible step, and (c) further changes in these low spin species under drastic conditions. The last two occurrences are observed with methemoglobin. The electron spin resonance spectra obtained upon heat denaturation are ascribed to at least two low spin ferric complexes between hemin and denatured globin.

The technique is used to follow the irreversible heat denaturation of oxy- and methemoglobin and to obtain transition temperatures for denaturation. Transition temperatures in the presence of organic solvents, solutes, and salts and in the presence of a few inorganic salts are obtained, and from these molar changes in transition temperatures are estimated. In some cases, the transition temperature is lowered to 23° and below.

Organic compounds, except for glycerol and sucrose, all lower transition temperatures, and their effectiveness as denaturants increases as their hydrophobic character increases, with the notable exception of guanidinium chlorides. Among inorganic salts, anions which salt in proteins, such as iodide and thiocyanate, decrease heat stability whereas those which salt out proteins, such as sulfate and citrate, increase stability over that found in water. These data are discussed with regard to protein-solvent interactions.

A few organic solutes, notably methanol, formamide, phenol, and urea, can bring about a change in methemoglobin, characterized by a particular electron spin resonance spectrum. The species responsible for the spectrum appears to represent an internal complex between hemin and a slightly perturbed or denatured form of globin.

Hemoglobins represent one of the most extensively studied groups of proteins. Many of their fundamental properties and reactions are carefully characterized, and in recent years the amino acid sequence and crystallographic structure of certain hemoglobins have been determined in detail. In spite of the abundance of data about these heme proteins, little information is available at present about the forces which stabilize the tertiary structure of the polypeptide chains and about solvent interactions which perturb the stability of the protein. In general, solvent interactions trend to destabilize and denature hemoglobin (1-4), but cases will be discussed below in which the opposite occurs.

Most of the present information concerning the stability of hemoglobin relates to studies on the reversible and irreversible denaturation of hemoglobin and its several ligand derivatives (1, 5-18). The following considerations are relevant to these studies.

Denaturation of hemoglobin is a complex process. There can be different degrees of denaturation and different denaturative processes leading to a variety of denatured states of the protein. As judged by reversibility, heat denaturation, for example, leads to a set of products rather different from those resulting from denaturation by extremes of pH (1, 4, 6, 11, 12).

While hemoglobin can undergo both reversible and irreversible denaturations, the extent of denaturation and the relative portion of irreversibly denatured product are related, among other parameters, to the nature and concentration of the denaturant, to the time and temperature of incubation, and to the ligand and oxidation-reduction state of the protein. Carboxyhemoglobin, for example, appears to be much more stable toward amides than is oxy- or methemoglobin (7). Even the term "irreversible" must be taken advisedly to mean "irreversible by simple means," in view of the well known processes whereby denatured and coagulated hemoglobin or globin can be reconstituted into native (or nearly native) hemoglobin (1, 4, 19).

Most physical methods of observation, particularly optical ones, are rendered difficult or impossible by the propensity of hemoglobin to coagulate upon denaturation. This has, in general, prompted studies of the interactions of hemoglobin with those solvents in which structural perturbations are not accompanied by precipitation, such as is the case generally with aqueous solutions of amides and urea and with extremes of pH.
Even where optical methods are applicable, it is often difficult to relate heme-linked reactions, such as dissociation or ligand exchange, with changes in polypeptide configuration (9, 16). This difficulty is particularly acute in those cases in which the denaturing agent itself can become a heme ligand (20).

The susceptibility studies of Pauling and Coryell (21) and the electron spin resonance studies of Bennett and Ingram (22) established that magnetic methods are useful in delineating the oxidation-reduction state, ligand fields, and spin state of the iron in heme proteins. Magnetic data are now available on a number of heme proteins and their various complexes with common ligands (20, 23–26). These methods appear to be potentially applicable to investigations concerning the denaturation of certain heme proteins, insofar as structural changes of the protein affect those physical parameters detectable by the methods.

We are aware of at least one group of detailed experiments in which magnetic susceptibility has been used to study the reversible denaturation of myoglobin (27, 28).

While magnetic methods suffer in general from the same limitations as optical methods in studies of heme protein denaturation, that is, detecting events subsequent to or dependent upon the primary events of interest, they have the important distinction of being unperturbed by turbidity. With the ESR method used in this paper, one is unable to detect ferrous states and certain ferric states of heme proteins, and in this regard the method is inferior to susceptibility measurements. However, ESR measurements, being spectroscopic in nature, are much better able to reveal small and subtle differences among compounds that otherwise appear identical.

The ESR study reported here is based on evidence that the low spin ferrheme protein species formed following the denaturation of oxyhemoglobin is peculiar to hemoglobin and is independent of the agents or conditions used to denature, providing the denaturing agents do not themselves form complexes as ligands. The denatured products are defined commonly as ligand complexes between hemin and denatured globin. In this study we describe ESR observations on the denaturation of hemoglobin, particularly with reference to the influence of various organic compounds and cation on the stability of the protein.

In this paper, hemoglobin is used as a generic term, while specific terms oxy-, met-, carboxy-, and deoxyhemoglobin have their usual meanings (1).

METHODS AND MATERIALS

Reagents—Most organic solvents and compounds were commercial samples of high purity. The results reported here were not materially affected by further purification, except in the case of dimethyl sulfoxide and p-dioxane. Alkyl ureas and guanidinium chlorides were obtained from Drs. M. Soodak and F. Maloof.

Nearly all the organic materials used here were selected to be aprotic or weakly protic by the definition of Singer (29) in order to avoid effects associated with pH. In the case of a few materials, such as phenol, which do not provide neutral aqueous solutions, appropriate pH adjustment was made prior to use. Matters concerning the pH of solutions of weakly acidic or basic salts were ignored.

The abbreviations used are: ESR, electron spin resonance; T2, transition temperature as defined in the text under “Results.”
Heat Denaturation—When solutions of human adult oxyhemoglobin or methemoglobin are heated briefly above 75°C, denaturation and precipitation result, and a characteristic ESR spectrum, shown in Fig. 1, appears. Although the signals in and around the vicinity of \( g = 2 \) are rather broad, suggesting a possible inhomogeneous population of compounds, the spectrum clearly resembles published ESR spectra of a variety of ferriheme proteins and derivatives wherein octahedral, low spin states of the iron atoms predominate (24–26). Six signals are distinguished, although Signal II (\( g = 4.3 \)) probably represents iron in something other than an octahedral ligand environment (33). The integral of Fig. 1 represents the result of a numerical integration of 200 points. Comparisons have been made of the areas under such integrals with corresponding integrals of materials in known concentration and in known spin state, such as Cupric-EDTA, neutral metmyoglobin, and alkaline methemoglobin (pH 12) (24, 26). When integrated areas are corrected for spin state and changing \( g \) value across the integral (34), these comparisons show that between 40 and 60% of the iron originally present in oxyhemoglobin or methemoglobin is denatured.

Fig. 1. ESR spectrum of human oxyhemoglobin after incubation at 80°C for 4 min. The system contained 3 mM heme of which >95% was oxyhemoglobin. Above, first derivative presentation; below, 200-point numerical integration.

Fig. 2. ESR signal amplitudes of human oxy- and methemoglobin as a function of incubation temperature. All amplitudes are relative to an amplitude of 1.0 for the signal at \( g = 2.25 \) (\( V \) of Fig. 1) following incubation at 80°C for 4 min. Amplitudes were measured from first derivative maximum to minimum and those relating to methemoglobin were corrected for concentration. Oxyhemoglobin, 5 mM heme; methemoglobin, 2.5 mM heme. ○, methemoglobin; □, oxyhemoglobin.
the native hemoglobin can be accounted for after heating as a set of ferrirheme compounds with assumed spin of \( \frac{1}{2} \) (low spin state). Values between 70 and 90% can be obtained following denaturation by certain organic solvents, as can be seen, for example, in Fig. 5, propanol. The ESR spectra associated with denatured hemoglobin are not due to aqueous hemin (35) nor to any other low molecular weight salt or complex of hemin of which we are aware, and we have been unsuccessful in reproducing the spectra of denatured hemoglobin in systems consisting of heme and either native or denatured proteins, including heat-denatured globin. The spectra are different from those observed with heat-denatured myoglobin, as will be discussed in a later paper.² ESR spectra of the type depicted in Fig. 1 and obtained from denatured hemoglobin will be called A-type spectra.

The appearance of A-type spectra upon the denaturation of oxy- and methemoglobin is strongly and similarly temperature-dependent, as can be seen in Fig. 2. The two A-type spectra, exemplified in Fig. 2 by curves labeled \( g \equiv 5.9 \), are also closely comparable in amplitude. The strong signal at \( g = 5.9 \), characteristic of methemoglobin (22, 24, 26), falls as denaturation progresses and the signal at \( g = 2.25 \) rises in a reciprocally manner. Note, however, that in the case of oxyhemoglobin denaturation a signal at \( g = 5.9 \) (again characterizing methemoglobin or similar material) appears at a lower temperature, passes through a maximum, and then decreases as the signal at \( g = 2.25 \) grows. The first observable step, therefore, seems to be the conversion of oxy- to methemoglobin. The growth of the signal at \( g = 2.25 \) and the corresponding decrease of signals at \( g = 5.9 \) correlate closely with hemoglobin solubility as a parameter for the irreversible denaturation of the heme protein (9, 10, 15). Relative hemoglobin solubility (protein analysis) follows closely the curve in Fig. 2 labeled \( g = 5.9 \) Hb⁺. ESR observations, therefore, can be used to obtain the transition temperatures (\( T_d \)) for the irreversible denaturation of met- and oxyhemoglobin. The \( T_d \) for the purposes of this paper is taken to be the temperature, during a 4-min incubation, required for a signal at \( g = 2.25 \) to achieve half of its maximum amplitude. The \( T_d \) for neutral human adult oxyhemoglobin by this definition is 72–73°C at ionic strengths between 0.05 and 0.10 (NaCl). The \( T_d \) for the corresponding methemoglobin is 69–71°C at ionic strength of 0. The value of \( T_d \) for methemoglobin has been found to be dependent on NaCl concentration (15), and our ESR studies confirm this observation. With increasing concentration of NaCl between 0 and 0.15 M, \( T_d \) decreases some 6 to 8°C. Beyond 0.15 M, however, there is little or no further change in \( T_d \), at least to about 2 M. The \( T_d \) for oxyhemoglobin shows little apparent dependency on NaCl concentration between 0 and 0.2 M. Except for small differences in \( T_d \), the results discussed above apply also to horse and beef hemoglobin.

The data of Table I support the idea, mentioned above, that A-type spectra are produced by a group of heme-containing compounds and not by a single one. Note that Signals I and IV and Signals V and VI change amplitudes as separate pairs and not together. A-type spectra are caused by what appear to be at least two classes of ferrirheme compounds.

Fig. 3 concerns the kinetics of heat denaturation of oxyhemoglobin. In this figure, incubation temperature is 76°C, and the incubation time is varied. Qualitatively the sequence of events and the ESR spectra are the same as when the temperature is varied.

**Table I**

Relative ESR signal amplitudes of human oxyhemoglobin as a function of incubation temperature

The system contained 2.5 mM heme of which 93% was oxyhemoglobin and 7% was methemoglobin. Incubation time was 4 min in a water bath at the designated temperatures. Amplitudes of Signals I, II, and V and VI were measured from first derivative maximum to minimum; those of Signals III and VI from maximum to baseline; that of Signal IV, which is a shoulder of Signal V (see Fig. 1), from the shoulder to an extrapolation of Signal V in the region of the shoulder. The amplitude of Signal IV is intended to represent no more than a rough estimate. All amplitudes are referred to an amplitude of 100 for Signal V at 80°C, 4 min.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>26°C</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>30°C</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>40°C</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>50°C</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>60°C</td>
<td>19</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>70°C</td>
<td>40</td>
<td>3</td>
<td>1</td>
<td>1.5</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>80°C</td>
<td>4</td>
<td>9</td>
<td>4</td>
<td>6</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>90°C</td>
<td>5</td>
<td>8</td>
<td>6</td>
<td>9</td>
<td>63</td>
<td>9</td>
</tr>
</tbody>
</table>

² T. C. Hollocher, S. Shin, and E. Gill, unpublished results.

Denaturation by Organic Compounds and Salts—It is well known that many of the physical properties of hemoglobin solutions are altered by aprotic or weakly protic compounds and that denaturation can occur (1), depending upon the material, its concentration, temperature, and time of incubation. Consistent with these observations is our finding, illustrated in Fig. 4, propanol, for example, that certain solvents, organic solutes, and salts can promote the oxidation of oxyhemoglobin and can cause the appearance of A-type spectra, indicating irreversible denaturation. Certain materials, therefore, have the ability to lower \( T_d \) for
Fig. 4. The effect of several solutes on ESR spectra. Hemoglobin concentrations were from 2 to 3.5 mM of which 70 to 95% was oxyhemoglobin and 3 to 30% was methemoglobin. Initial pH before addition of solute was from 6.5 to 7.5. With each solute internal amplitude references are provided by spectra labeled 80° and 0 M. The former refers to the system incubated at 80° for 4 min in the absence of solute; the latter refers to the unperturbed system. These are provided for purposes of comparison in view of hemoglobin to room temperature and below. Spectra of the type seen in Fig. 4, methanol, are not of the A-type and will be designated B-type. The characteristics of B-type spectra and the compounds responsible for their appearance are discussed separately below. Fig. 5 provides an extension and summary of the type of data shown in Fig. 4.

In Fig. 5, the amplitudes of A-type spectra (an index of extent of denaturation) are represented by the signal at \( g = 2.25 \); B-type spectra, the weak high field lines of neutral methemoglobin, or both of these, by the signal at \( g = 2.14 \); and neutral methemoglobin or, more generally, high spin ferriheme components, by the signal at \( g = 5.9 \). In Fig. 5, propanol or N-methylformamide, the behavior of the signal at \( g = 2.25 \) is very similar to that seen in Fig. 2 during heat denaturation. A rather abrupt rise is seen, followed by a decrease of amplitude. In other cases, Fig. 5, butanol, for example, the amplitude of the signal at \( g = 2.25 \) is limited by the limited miscibility or solubility of the solute; in still others, Fig. 6, ethanol, denaturation is limited by the ineffectiveness of the solute as a denaturing agent at room temperature.

While Figs. 4 and 5 apply to systems incubated at room temperature, it is clear from Fig. 6 that \( T_2 \) is a function of solute concentration. Plots of \( T_2 \) with respect to solute concentration, the above variations and the additional gain variations provided by instrument settings. In every case, except spectra labeled 80°, samples were incubated in a water bath at room temperature for 4 min following the addition of solute. The spectra were uncorrected for changes in hemoglobin concentration incurred through dilution by the solute added. Instrument gain was constant throughout each set, but varies from set to set.

While Figs. 7 and 8, permit us to calculate the molar change in \( T_2 \) as a function of solute concentration, \( \Delta T_2/\Delta C \). Representative results are tabulated in Table II. The linear relationship between \( T_2 \) and concentration assumed in Table II is justified for our rough approximations by Figs. 7 and 8, although, as seen in Fig. 7, it is not a perfectly valid assumption. The errors estimated in Table II include considerations of instrument effects, number of data points collected, and other matters of reproducibility. The errors tend to be similar whereas the values vary widely. As a result the relative errors become quite large for small values. Note that while most values are negative, indicating a lowering of \( T_2 \) with concentration and a tendency toward denaturation, glycerol and sucrose show positive values.

B-type Spectra—B-type spectra (see Fig. 4, methanol, for example) arecharacterized by three sharp high field lines appearing at \( g = 2.50, 2.14, \) and 1.85 and a low field line (possibly two overlapping lines) at \( g = 5.9 \). At neutrality, methemoglobin exhibits a set of three very weak lines appearing at \( g = 2.53, 2.14, \) and 1.82. This spectrum, therefore, is not identical with B-type spectra (see Fig. 4 and Fig. 9). The line at \( g = 5.9 \) is broader than that shown at the same position by methemoglobin at neutrality. B-type spectra arise from methemoglobin and...
not from oxyhemoglobin. The amplitudes of the signals at 
g = 2.50, 2.14, and 1.85 with a specified solute and concentration are 
proportional to the concentration of pre-existing methemoglobin as ascertained optically or by ESR. B-type spectra occur 
in Figs. 4 and 5 by virtue of the fact that the systems contain 
between 5 and 30% methemoglobin. The occurrences of B-type 
spectra in Fig. 5 are recognized by an increase and, generally, a 
maximum in curves designating signal amplitudes at \( g = 2.14 \).

While the appearance of B-type spectra depends on the contact of methemoglobin with a suitable solute, the persistence of these 
signals does not require the continuing presence of the solute. This is shown by considering Table III and Fig. 9 together.

The change from B- to A-type spectra can be brought about by excess solvent in certain cases (Fig. 5) or by heat. This 
change appears to be irreversible. The change from methemoglobin to low spin forms with B-type spectra appears to be slowly 
reversible, at least in part.

**DISCUSSION**

Heat Denaturation—The data presented in Figs. 1 to 3 and in Table I show that a change in spin state accompanies the irreversible 
denaturation of methemoglobin by heat and that an apparently identical change occurs with oxyhemoglobin following 
an initial oxidation to methemoglobin or to a closely related derivative. Resulting ESR spectra are referred to as A-type, 
above. The spin change enables one to use the ESR technique 
to follow the process of denaturation and provides an operational 
definition of denaturation, even though the ESR information, per se, relates to the electric environment of the ferric atoms of 
haem in and not to the general shape or conformation of the protein. We observe that a large portion of the haem present, 40 
90% depending on conditions, undergoes the change in spin 
state upon denaturation at neutrality.

The transition from high to low spin state involves an increase in 
the ligand (electric) field strength about the ferric atoms. In 
general this is accomplished by an exchange of one or more original 
ligands for a set of stronger ligands (20, 36). By analogy 
with certain low spin complexes of methemoglobin and metmyoglobin, such as cyanide or azide complexes (24, 26), it would seem 
that upon denaturation the haem must coordinate with rather 
strongly basic groups. Since the latter complexes are not related 
to hydroxide ion (37) and water concentrations over wide ranges and since no other anions or bases are required for their formation 
upon denaturation, they represent internal complexes between haem and strong ligands of denatured globin. Precisely 
what groups of denatured globin serve as ligands of haem to produce the A-type ESR spectrum is not known at present. 
In the case of denatured sperm whale metmyoglobin, the ESR spectrum resembles that of haem-imidazole complex very 
closely. However, we know of no model system as yet that 
reproduces the A-type spectrum of methemoglobin. While imidazole 
may be one of the ligands (1, 38), it would seem that another 
group might also be involved. The fact that the A-type spectrum is due to the contributions of at least two compounds is a 
complication in this regard.

Since A-type spectra are detected neither with mixtures of haem and denatured globin nor with other proteins, native or 
denatured, the prior existence of native hemoglobin may be uniquely important for their formation.

**Denaturation by Organic Compounds and Salts**—As an index of irreversible denaturation, the ESR techniques can be used to 
 obtain transition temperatures \( T_D \) and, in the case of denaturation by organic compounds and salts, to obtain critical concentra-
tions and molar changes in \( T_D \).

An examination of Table II indicates the following. In general, organic compounds and those salts of the Hofmeister 
anion series (39, 40) which salt in proteins and polypeptides decrease \( T_D \) and tend to denature. Salts of anions which tend 
to salt out proteins and polypeptides, such as sodium sulfate and citrate, do not denature oxy- or methemoglobin at any concentration at room temperature; rather, such salts increase \( T_D \) and protect against denaturation. Glyceraldehyde and azide, but not ethylene glycol, represent exceptions among organic compounds since they increase \( T_D \). Certain other sugars and polyols also provide protection against thermal denaturation. The mechanism by which polyols stabilize hemoglobin is not known at present.

It is also clear from Table II that the effectiveness of organic compounds as denaturants of hemoglobin increases generally 
with their hydrophobic character within each homologous series studied. Anomalous effects are noted, however, among amides 
and amide-like compounds. Members of the N-substituted formamide series are better denaturants than are corresponding 
members of the N-substituted acetamide series, in spite of the presence of an extra methyl group in the latter series. Gua-
nidinium chloride is a better denaturant than its less soluble 
1,1,3,3,5,5-hexamethyl derivative, contrary to expectation from hydrophobic effects alone. On the other hand, alkyl-substituted 
ureas constitute a normal hydrophobic series, and the alkyl-

substituted thioureas are stronger denaturing agents than the 
corresponding ureas, as might be expected from the lower solubilities of thioureas. We find guanidinium chloride to be a 
stronger denaturant for hemoglobin than is urea, but this rank 
order seems to become reversed in their alkyl derivatives, be-
ginning at about their respective dimethyl or ethyl derivatives. The special rank of formamide with respect to acetamide and the “cross over” in the alkyl-substituted urea and guanidinium chloride series have been noted by Robinson and Jencks (41) in their 
studies of the activity coefficient of acetyltetraglycine ethyl ester and polar compounds in water. The former effect of 
formamides and acetamides, but not the latter cross over have been noted in the depolymerization of F-actin by Nagy and Jencks (42).

Our results are consistent with the idea advanced by Jencks et al. (41–45) that a major factor in the salting in or denaturing 
ability of amides and certain anions is a nonhydrophobic effect 

of these compounds toward other polar groups, such as amide and 
polyamide groups of protein. In spite of the fact that the rank 
order we find, guanidinium chloride > thiourea > urea > 
formamide > acetamide, is similar to that shown by Robinson 
and Jencks (41) and Gordon and Jencks (43), such effects appear 
not to be too important in the denaturation of hemoglobin by organic compounds. With the exception of guanidinium chloride, 
simple, unsubstituted amides are weak denaturants toward met-
and oxyhemoglobin. Nonhydrophobic effects may be quite important, however, in explaining the effectiveness of 
anions which salt in proteins, such as iodide and thiocyanate, in promoting the oxidation and denaturation of oxyhemoglobin 
(42, 44, 45). Considerations of hydrophobic and electrostatic effects seem unable to account for the results. While we observe 
nonhydrophobic effects in the denaturation of hemoglobin, an
The important outcome of this study is the finding that oxy- and methemoglobin are very sensitive to hydrophobic denaturants.

As seen in Figs. 7 and 8, the relation between concentration of organic compound and $T_s$ is roughly linear and agrees with similar findings by Schrier, Ingwall, and Scheraga (46) regarding ribonuclease. These authors consider a complexation of nonpolar groups of the protein with nonpolar ends of denaturing solutes as a possible causative mechanism. Whether this explanation applies to hemoglobin denaturation is not clear. While in most cases $\Delta T_s/\Delta C$ changes monotonically within a homologous series, the change in this function with solubility or with size of the alkyl group seems quite variable. In addition, the increases in $T_s$ effected by glycerol (Fig. 8, Table II) and sucrose (Table II) are roughly linear in concentration of the solute.

Sequence of Events—In Figs. 2 and 3 and Table I one can discern three events in the denaturation of oxyhemoglobin: (a)
oxidation to methemoglobin or closely related material, (b) the appearance of low spin species in which Signals V and VI predominate, and (c) the decline of these species with the continuing increase of species in which Signals III and IV predominate. During the last step, the total concentration of low spin species decreases.

These three steps can be dissociated kinetically from each other rather effectively, but not completely, by performing experiments of the type illustrated in Fig. 3 at a variety of temperatures. Note in Fig. 3 that the third step above is separated from the first two by virtue, apparently, of a much larger activation energy for the third step. Compare Fig. 3 with Fig. 2. The first step is partly reversible, although some of the methemoglobin formed is unable to form stable complexes with oxygen following reduction. The second and third steps are irreversible. The same considerations apply to methemoglobin except, of course, that the first step is missing.

The first two steps are often difficult to distinguish when denaturation is accomplished by organic compounds. Notice, for example, in Fig. 5, propanol, that the signal at $g = 5.9$, characteristic of methemoglobin, fails to pass through a maximum in amplitude and immediately declines. With Fig. 5, ethanol, however, the curve at $g = 5.9$ passes through a maximum just...
compounds have the ability, even at relatively low concentrations, to convert methemoglobin to a low spin complex showing spectra designated as B-type. Solubility of the protein seems not to be impaired. With the exception of phenol, these compounds represent weak denaturing agents which show little or no ability to promote the oxidation of oxy- to methemoglobin (see Fig. 4, methanol, for example, and Fig. 5). In Fig. 5, the appearance of B-type spectra is indicated wherever the amplitude of the signal $g = 2.14$ initially increases and passes through a maximum. With methanol and formamide, at least, the change from high to low spin states is nearly quantitative. Of the compounds studied, the following are found to induce the appearance of B-type spectra in methemoglobin at room temperatures. The order concerns extent of conversion of methemoglobin into species giving B-type spectra: methanol $>$ formamide $>$ urea $>$ phenol $>$ ethyl urea $\cong$ N-methylacetamide $>$ ethanol $\cong$ dimethyl sulfoxide. The position of phenol may be incorrect since denaturation begins in the same range of concentrations over which B-type spectra appear.

Fig. 9 and Table III show that the low spin species exhibiting B-type spectra cannot be defined as ligand complexes between methemoglobin and the organic compound. The species survive dialysis, whereas the organic compound (here methanol) responsible for the ESR spectral change is lost quickly. As with the appearance of A-type spectra, these low spin forms must represent internal ligand complexes. Here, however, the complexes involve a perturbed but nearly native globin rather than a grossly denatured globin. By implication these complexes arise as a result of some change in protein configuration, however slight. Preliminary experiments suggest that the process leading to B-type spectra may be partly but very slowly reversible after the inducer is removed. The three characteristic signals of B-type spectra are quite narrow and of line widths comparable to those shown by well defined complexes between strong ligands as seen in Fig. 2. The relative rates of the first and second steps apparently determine whether or not the signal amplitude at $g = 5.9$ passes through a maximum.

B-type Electron Spin Resonance Spectra—Certain organic

![Fig. 6. Influence of solutes on ESR signal amplitudes of human oxyhemoglobin as a function of incubation temperature. The systems contained 2 mM heme initially of which $>95\%$ was oxyhemoglobin. All amplitudes refer to the signal at $g = 2.25$ (V of Fig. 1) and are relative to an amplitude of 1.0 for the signal following incubation at $80^\circ$ for 4 min in the absence of solute. This is the $80^\circ$ point in the curve labeled Water. The data are corrected for changes in hemoglobin concentration incurred through dilution by the solute added.](image)

![Fig. 7. Lowering of transition temperature for human hemoglobin denaturation by dioxane. The systems contained 2.5 mM heme of which 70% was oxyhemoglobin and 30% was methemoglobin. See the text for the definition of transition temperature.](image)

![Fig. 8. Changes in transition temperature for human hemoglobin denaturation by several organic solutes. The systems contained 1.5 to 2.7 mM heme of which 70 to 95% was oxyhemoglobin and 5 to 30% was methemoglobin. See the text for definition of transition temperature.](image)
and metmyoglobin (24). A degree of chemical homogeneity is thus implied.

Since they seem to be caused by internal ligand complexes, it is understandable that B-type spectra are independent in form.

TABLE II
Molar changes in transition temperature of human hemoglobin produced by solutes

The systems contained 1 to 4 mM heme of which 70 to 95% was oxyhemoglobin and 5 to 30% was methemoglobin. The transition temperature in water was 73°C, determined as described in the text. Negative values of $\Delta T_0/\Delta C$ indicate a lowering of the transition temperature and a destabilization of hemoglobin.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$\Delta T_0/\Delta C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohols</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>$-2 \pm 0.5$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>$-3 \pm 0.5$</td>
</tr>
<tr>
<td>1-Propional</td>
<td>$-12 \pm 0.5$</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>$-33 \pm 2$</td>
</tr>
<tr>
<td>Phenol</td>
<td>$-22 \pm 3$</td>
</tr>
<tr>
<td>Benzy alcohol</td>
<td>$-50 \pm 3$</td>
</tr>
<tr>
<td>Polyols</td>
<td></td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>$-2 \pm 0.5$</td>
</tr>
<tr>
<td>Glycerol</td>
<td>$+1 \pm 0.5$</td>
</tr>
<tr>
<td>Sucrose</td>
<td>$+4 \pm 0.3$</td>
</tr>
<tr>
<td>Amides</td>
<td></td>
</tr>
<tr>
<td>Formamide</td>
<td>$-3.0 \pm 0.5$</td>
</tr>
<tr>
<td>N,M-Dimethylformamide</td>
<td>$-8 \pm 0.5$</td>
</tr>
<tr>
<td>N,N-Dimethylvformamide</td>
<td>$-10 \pm 0.5$</td>
</tr>
<tr>
<td>Acetamide</td>
<td>$-1 \pm 0.5$</td>
</tr>
<tr>
<td>N-Methylecetamide</td>
<td>$-6 \pm 1$</td>
</tr>
<tr>
<td>N,N-Dimethylecetamide</td>
<td>$-7.5 \pm 1$</td>
</tr>
<tr>
<td>Ketones</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>$-5 \pm 2^*$</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>$-25 \pm 1$</td>
</tr>
<tr>
<td>3-Pentanone</td>
<td>$-29 \pm 1$</td>
</tr>
<tr>
<td>Nitriles</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>$-11 \pm 1$</td>
</tr>
<tr>
<td>Proponitrile</td>
<td>$-22 \pm 2$</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>$-4.2 \pm 0.5$</td>
</tr>
<tr>
<td>Ethylurea</td>
<td>$-9.5 \pm 1$</td>
</tr>
<tr>
<td>Thiourea</td>
<td>$-5.5 \pm 0.6$</td>
</tr>
<tr>
<td>Trimethylthiourea</td>
<td>$-10 \pm 1.5$</td>
</tr>
<tr>
<td>Guanidinium salts</td>
<td></td>
</tr>
<tr>
<td>Guanidinium chloride</td>
<td>$-8.4 \pm 1$</td>
</tr>
<tr>
<td>1,1,3,3-Tetramethylguanidinium chloride</td>
<td>$-2.8 \pm 0.3$</td>
</tr>
<tr>
<td>Salts</td>
<td></td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>$-12 \pm 2$</td>
</tr>
<tr>
<td>Potassium thiocyanate</td>
<td>$-12 \pm 2$</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>$(+)^*$</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>$(+)^*$</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>$+2.1 \pm 0.2$</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>$-8 \pm 0.5$</td>
</tr>
<tr>
<td>p-Dioxane</td>
<td>$-15.8 \pm 0.2$</td>
</tr>
<tr>
<td>Ethylene carbonate</td>
<td>$-8 \pm 1$</td>
</tr>
</tbody>
</table>

$^*$ The determination is assigned a high error due to the cavity dielectric loss effects associated with aqueous acetone systems at the temperature of liquid nitrogen.
$^*$ Concentration given in moles per liter.
$^*$ Based on single concentration of salt. $\Delta T_0/\Delta C$ is not estimated. The plus sign indicates a protective effect and an increase in transition temperature.

TABLE III
Loss of 14C-methanol from methemoglobin upon dialysis

The system consisted of an aqueous solution containing 3.5 mM methemoglobin heme and 250 mM 14C-methanol with an estimated specific activity of 0.50 μCi per mg. About 1 ml of this system was placed in dialysis tubing ½ inch in diameter and dialyzed with stirring against several 1-liter changes of water at 4°C. At the times indicated 0.1-ml samples were taken and counted. Counting efficiency was estimated at 2% as opposed to the usual efficiency of about 40% in the absence of hemoglobin. Background was 18 cpm.

<table>
<thead>
<tr>
<th>Time of dialysis</th>
<th>Activity of 14C-methanol$^a$ (cpm)</th>
<th>Methanol concentration</th>
<th>Methanol to heme mole ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25,600</td>
<td>250</td>
<td>71</td>
</tr>
<tr>
<td>1</td>
<td>14,780</td>
<td>144</td>
<td>41</td>
</tr>
<tr>
<td>20</td>
<td>310</td>
<td>3</td>
<td>0.9</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>120</td>
<td>12</td>
<td>0.1</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$^a$ Corrected for background.

Fig. 9. The failure of dialysis to abolish B-type spectra. The system contained 3.2 mM heme of which >95% was methemoglobin (Methb.). Above, reference spectra as in Fig. 4; middle, following addition of methanol; below, during the course of dialysis. The experiment was done in parallel with the one described in Table III.

from most properties of the inducers. It is difficult to relate the commonality of action of inducers to a unique property or set of properties. They all seem to be small relatively polar compounds and represent the first one or two in an aliphatic series.
General Considerations—A striking aspect of this study is the constancy of the ESR spectral types observed and their reproducibility over a wide range of conditions. This implies that the denaturative changes associated with the changes in spin states of hemoglobin are dependent on the structure of the native protein and not much on the circumstances of denaturation. The denatured state appears to be a rather specific and definite group of states. The ESR spectra observed on the denaturation of hemoglobin from man, horse, and beef are virtually identical but much different from those seen with denatured myoglobins. It is interesting that the dissociation of hemoglobin into dimers or monomers or both (4, 7, 47) seems to have little or no bearing on our results. Heat denaturation of oxyhemoglobin in concentrated urea gives ESR spectra indistinguishable from those seen without urea. Human and horse hemoglobin differ only slightly, if at all, in these experiments. Further studies on this point and other points concerning hemoglobin dissociation are in progress.

For the most part, the appearance of A-type spectra concerns irreversible denaturation. However, in the case of the incubation of oxy- or methemoglobin with urea and certain amides at room temperature or below for short periods of time, some degree of reversibility can be seen. Upon dialysis in the cold, the amplitude of A-type spectra in these systems can be seen to decrease while a methemoglobin signal at $g = 5.9$ intensifies. This effect, an apparent reversal of denaturation, seems never to go very far toward completion and has not yet been observed with hydrophobic denaturants, such as propanol, or following heat denaturation. However, in the case of the incubation of hemoglobin with urea to completion and has not yet been observed with propanol, or following heat denaturation. These results find a parallel in the well known reversible and irreversible effects of amides and urea on hemoglobin (4, 7, 47).

The fact that the protein remains quite soluble in the above amide systems, particularly with urea, shows that the appearance of A-type signals are not necessarily related to coagulation, which is a consequence of heat denaturation in any of our systems.

Acknowledgments—I wish to acknowledge the able technical assistance of Miss Linda Buckley. Contributions by the following undergraduate and graduate trainees are also acknowledged: Miss JoAnn Winog and Messrs. Edward Gill, William Stryczarz, Joel Kowit, and Robert Brundage. I thank Professors William Jencks and Gerald Fasman for their interest and suggestions in regard to this work.

REFERENCES
Electron Spin Resonance Studies of the Denaturation of Oxy- and Methemoglobin: SPECTRA AND SOLVENT INTERACTIONS
Thomas C. Hollocher


Access the most updated version of this article at http://www.jbc.org/content/241/9/1958

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/241/9/1958.full.html#ref-list-1