The Combining Power of Normal Human Hemoglobin for Nitrosobenzene

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The nitrosobenzene complex of hemoglobin is a violet pigment which is associated with nitrobenzene poisoning. FicATone (1) reported in 1878 that “nitrobenzol” (nitrobenzene) exerts its toxic effect by making blood unable to transport oxygen. This took place because nitrobenzene apparently underwent a reduction to form a hemoglobin complex with a characteristic spectrosopic absorption which did not correspond to any of the ordinary hemoglobin products. The violet pigment was also observed by Loeb et al. (2) in 1921 while investigating nitrobenzene poisoning. These workers reported that the violet hemoglobin compound had a markedly diminished oxygen capacity.

Jung (3) and Keilin and Hartree (4) reported on the reaction of hemoglobin with nitrosobenzene; they showed that a well characterized violet compound was formed with hemoglobin; the same compound was formed from oxyhemoglobin and from carbonmonoxyhemoglobin with the evolution of stoichiometric amounts of O₂ and CO, respectively. The latter reaction was reversed by high CO pressure; the compound was quite stable, and had a broad absorption band like that of reduced hemoglobin but with two weak maxima at approximately 567 and 543 μm. No quantitative measurements of apparent equilibrium constants were reported.

During the course of investigations on heavy metal binding sites (predominantly mercapto groups) of hemoglobin and on the nature of the mercapto-mercapto interactions (5) it became necessary to study the influence on the oxygen affinity of human hemoglobin of a large ligand bound to the heme groups, since such ligands as p-chloromercuribenzoate and phenylmercuric nitrate (5) could form a hemoglobin complex with a characteristic spectroscopic absorption which did not correspond to any of the ordinary hemoglobin products. The violet pigment was also observed by Loeb et al. (2) in 1921 while investigating nitrobenzene poisoning. These workers reported that the violet hemoglobin compound had a markedly diminished oxygen capacity.

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The human hemoglobin solution was prepared as previously described (5); the solution was dialyzed against a large volume of deionized water and then the last traces of electrolyte were removed by electrodialysis.

Nitrosobenzene and o-nitrosotoluene (2-methyl nitrosobenzene) were purchased from K & K Laboratories, Inc., Jamaica, New York. The standard solution was prepared fresh by dissolving the crystals with a minimal amount of ethanol and then made up to volume with a buffer in a 100-ml graduated mixing cylinder. The concentration of the ligand was 5 × 10⁻⁴ M. The pH of the buffer was determined at the temperature designated for the experiment. From pH 7.0 to 9.0, 0.1 M tris(hydroxy-methyl)aminomethane (Tris) buffer was used. For the pH range below 7.0, 0.1 M phosphate buffer was used.

A set of data for a typical experiment of nitrosobenzene and o-nitrosotoluene binding by oxyhemoglobin were obtained as follows. Into each of twelve 10.0-ml volumetric flasks in each set of experiments was added 1.0 ml of oxyhemoglobin solution (whose concentration was adjusted to about 65 μmoles of Fe per ml). No ligand was added to Flask 0. Into the other 10 flasks the ligand (5 × 10⁻⁴ M) was added from a microburette in geometric progression, i.e., into Flask 1, 10 μl, Flask 2, 20 μl, Flask 3, 40 μl, and so forth, so that the flasks contained 0.05, 0.10, 0.20, 0.40, . . . μmoles of the ligand in ascending order. Then the flasks were filled to the mark with buffer which had been maintained at the temperature of the experiment. In the meantime another set of 10.0-ml volumetric flasks was prepared for the reagent blanks in order to subtract absorbancies due to the ligand; into these flasks, no hemoglobin, only ligand, was added. The contents of the corresponding flask were used to fill the absorption cell for the blank. Three milliliters of solution were pipetted into a 1-cm absorption cell of a model 14 Cary automatic recording spectrophotometer. The compartment for the absorption cell was maintained at the temperature of the experiment. The absorption spectra were recorded between the wave lengths of 450
FIG. 1. This is a typical set of spectrophotometric recordings for determining the affinity constant of oxyhemoglobin for nitroso-
benzene. Ordinates, absorbancy (or optical density); abscissae, wave length 440 to 640 mp; the concentration of hemoglobin is 0.576 μM. The oxyhemoglobin curve is the double peaked one to 700 mp. The successive absorption spectra were carefully superimposed. From recorded absorption spectra similar to those shown in Fig. 1, the degree of saturation of hemoglobin with ligand was calculated as described below.

In these calculations the symbol Hb signifies a quarter of a hemoglobin molecule, containing but one heme per molecular weight of 16,500. For the present study the molecular extinction coefficient of 11.5 x 10³ at wave length of 540 mp (7) was adopted for Hb in the form of cyanmethemoglobin. From a set of curves such as in Fig. 1 the ratios of absorbancy at each isosbestic point to that at the wave length of maximal absorption of the complex was calculated for each of the spectra recorded. The percentage saturation (or the fractional saturation, y) was calculated from the ratio of absorbancies observed to the total change on saturation. Finally, the average y was taken from values obtained from the isosbestic points at 483 and 585 mp. The amount of unbound ligand could be determined from the amount of total ligand (i.e. the amount added) minus the amount bound (calculated from y). The heme-heme interaction (Hill’s constant n) was calculated from the well known expression

\[ y = \frac{Kc^n}{1 + Kc^n} \]  

where \( y \) = the fractional saturation; \( c \) = molar concentration of unbound ligand (\( \phi \)NO or o-nitrosotoluene); \( K \) = the apparent equilibrium constant and \( n \) = the heme-heme interaction (Hill’s) constant or the sigmoid coefficient (\( n = 1 \), for no heme-heme interaction). By rearrangement

\[ \frac{y}{1 - y} = Kc^n \]  

or on taking logarithm of both sides of Equation 2:

\[ \log \left( \frac{y}{1 - y} \right) = n \log c + \log K \]  

A plot of \( \log \left( \frac{y}{1 - y} \right) \) against \( \log c \) yielded a curve with an

A due to the heme-heme interactions a single equilibrium constant cannot be used; accordingly, the overall average affinity constant pK is used; it is defined as the negative logarithm of concentration of unbound ligand when the hemoglobin is half-saturated.
The temperature of the experiments was 0°, unless otherwise indicated; in comparison, the o-nitrosotoluene (NT) binding curve is included. The others are binding curves for oxyhemoglobin; nitrosobenzene is for the binding of +NO by deoxygenated human hemoglobin.

The combining constant (Hill's) constant for deoxygenated hemoglobin is 0.7; the slope of 0° curve for oxyhemoglobin is essentially 1.0; for 25° curve, two constants are needed; 1.30 and 0.56 for the "toe" and the "shoulder" sigmoid coefficients, respectively.

Because o-nitrosotoluene (2-methyl nitrosobenzene) readily denatured the hemoglobin, the study with this compound was limited. Since complete saturation with o-nitrosotoluene (NT) was not obtained, it was assumed in the calculation that the ratios of the complex were the same as those obtained for nitrosobenzene (+NO) hemoglobin complex or with nitrosobenzene (+NO).


d and 385 μm (Table I and Fig. 1). Each of the absorption maxima and isosbestic points is essentially the same for normal as well as for sickle cell and homozygous C hemoglobins. These wave lengths are essentially the same either with the o-nitrosotoluene (NT) hemoglobin complex or with nitrosobenzene (+NO) complex.

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**RESULTS**

The absorption maxima are 562 and 542 μm for the major and minor peaks, respectively (Table I). Isosbestic points are at 483

### Table I

**Human hemoglobin-nitrosobenzene complexes—absorption maxima of pure substances and isosbestic points in mixtures with oxyhemoglobin**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Absorption maxima (wave length, μm)</th>
<th>Isosbestic points (wave length, μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosobenzene</td>
<td>562 542</td>
<td>483 585</td>
</tr>
<tr>
<td>o-Nitrosotoluene (2-methyl nitrosobenzene)</td>
<td>542 562</td>
<td>483 585</td>
</tr>
</tbody>
</table>

* The combining power for the ligand is expressed as pK; (see text, footnote 2).
† n is Hill's (heme-heme interaction) constant; n1 and n2 (see text).

<table>
<thead>
<tr>
<th>Hb-A</th>
<th>0</th>
<th>5.0</th>
<th>0.7</th>
<th>0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25</td>
<td>4.1</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>3.6</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4.8</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>4.2</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4.43</td>
<td>1.06</td>
<td>0.98</td>
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<td></td>
<td>25</td>
<td>4.38</td>
<td>1.30</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>3.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Hb-S</td>
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<td>5.4</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>4.6</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Hb-C</td>
<td>0</td>
<td>4.7</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.4</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>4.4</td>
<td>0.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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The combining constant of deoxygenated hemoglobin for nitrosobenzene is about 10 times greater than that for 2-methyl nitrosobenzene (Table II). The combining constant is given as pK, which is the negative logarithm of concentration of unbound ligand in the presence of half-saturated hemoglobin. It should be noted that if the heme-heme interaction did not exist, pK and Keq would be the same.

The combining constant of deoxygenated hemoglobin for nitrosobenzene is about 10 times greater than that for oxyhemoglobin under the same conditions (pH 7.4, 0°); this is to be expected from mass law considerations.

The combining constants obtained for the sickle cell and homozygous C hemoglobins are essentially the same as those found

There are other apparent isosbestic points as can be seen from Fig. 1, e.g. 536 and 571 μm; these points become diffused when the equilibration takes place at 38°. At the present time this observation is taken to mean that the configuration of the molecule changes on heating and that the change is reflected in the absorption spectra.

**Table II**

<table>
<thead>
<tr>
<th>ligand</th>
<th>Temperature</th>
<th>Nitrosobenzene</th>
<th>2-Methyl nitrosobenzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK</td>
<td>n1</td>
<td>n2</td>
<td>pK</td>
</tr>
<tr>
<td>Hb-A</td>
<td>0</td>
<td>5.0</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>3.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Hb-S</td>
<td>0</td>
<td>5.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Hb-C</td>
<td>0</td>
<td>4.7</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.4</td>
<td>0.8</td>
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</tbody>
</table>

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for normal human hemoglobin (see Table II). The heme-heme interaction constants are also essentially the same for all human hemoglobins investigated (Table II).

**DISCUSSION**

St. George and Pauling (11) who studied the combining power of hemoglobin for alkyl isocyanides found that, although the combining powers of formyl with ethyl isocyanide, isopropyl isocyanide and tertiary butyl isocyanide were essentially the same, hemoglobin combined far more strongly with ethyl isocyanide than with tert-butyl isocyanide. The former was favored by a factor of 200 in equilibrium constants, the isopropyl compound having an intermediate value. It was concluded that this large effect arose from steric hindrance and not from a change in the process of oxygenation; i.e. the molecule tends toward more order.

The heme-heme interaction constants, \( n_1 \) and \( n_2 \), are the sigmoid coefficients for the "toe" and the "shoulder," respectively, of the binding curve. From Fig. 2 it can be seen that there is a similarity between these slopes and the mercapto-mercapto interaction constants in reference (5). However, there is a difference with respect to the oxygen dissociation curve; for instance, Roughton et al. (15) have presented a plot of \( \log \frac{[y]}{[1 - y]} \) against \( \log p \) giving a sigmoid rather than linear curve. This was first noted by Adair (16) and later by many investigators.

Preliminary studies indicate that the nitrosobenzene "wedge" appears to decrease the energy barrier due to steric hindrance with respect to mercapto groups and their ligands (19); the wedge also appears to influence the mercapto-mercapto interaction constants (19). Cullis and Perutz squared the crystals of horse hemoglobin-nitrosobenzene complex by x-ray diffraction. Nitrosobenzene-hemoglobin complex was first prepared, then HgCl\(_2\) was added to the complex while in solution and finally it was allowed to crystallize. It was found that \( \phi\text{NO} \) acted to "open up" the Hg binding sites of the molecule and that heat was equally effective. It may be concluded that nitrosobenzene is a useful probe in the study of structure and function of heme protein. Accordingly, additional work is in progress to reveal details of its effect on the conformation of the mercapto groups and on the mercapto-mercapto interaction constants.

**SUMMARY**

1. The combining constant of deoxygenated human hemoglobin for nitrosobenzene has been determined at \( 0^\circ \), pH 7.4 in 0.1 m tris(hydroxymethyl)aminomethane buffer; \( pK \) (the negative logarithm of concentration of unbound ligand in the presence of half-saturated hemoglobin) is 5.5.

2. Increase of temperature lowers \( pK \) for nitrosobenzene reacting with human oxyhemoglobin; at pH 7.4 it is 4.42, 4.39, and 3.70 at 0°, 25°, and 38°, respectively.

3. The combining power of human oxyhemoglobin for nitrosobenzene increases with hydrogen ion concentration; at pH 6.6, \( pK \) amounts to about 5.0 at 0°, in contrast \( pK \) is 4.2 at pH 9.0 at the same temperature.

4. For 2-methyl nitrosobenzene binding to oxyhemoglobin \( pK \) is 3.47 at pH 7.4, at 0°.

5. The presence of a methyl group \textit{ortho} to the binding site seems to increase the energy barrier, presumably arising from steric hindrance, by about 1.2 kilocalories mole\(^{-1}\); the affinity of 2-methyl nitrosobenzene is about one-tenth as much as that of nitrosobenzene.

6. The data presented support the steric hindrance theory which postulates that the heme groups are buried below the surface of hemoglobin molecule.

**REFERENCES**


\[ \text{Hb} + \text{O}_2 = \text{HbO}_2 \quad \Delta H = -9.4 \text{ kilocalories} \]

\[ \text{HbO}_2 + \phi\text{NO} = \phi\text{NOHb} + \text{O}_2 \quad \Delta H = -14.5 \text{ kilocalories} \]

\[ \text{Hb} + \phi\text{NO} = \phi\text{NOHb} \quad \Delta H = -23.9 \text{ kilocalories} \]

\[ \frac{\text{Hb} + \phi\text{NO} \to \phi\text{NOHb}}{\text{Hb} + \phi\text{NO} \to \phi\text{NOHb}} \]

From the equation \( \Delta F = -RT \ln K \), it follows that \( \Delta F \) amounts to about -5.9 kilocalories mole\(^{-1}\). The entropy change in this reaction is in the same direction as in the process of oxygenation; i.e. the molecule tends toward more order.

For a more detailed discussion on the heme-heme interactions the reader is referred to George and Lyster (17) and Wyman (18).

Dr. Max F. Perutz, personal communication.
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