Titratable Sulfhydryl Groups of Horse, Sheep, Dog, and Cow Hemoglobins at 0° and 38°*

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(Received for publication, March 5, 1958)

In a previous paper (1) it was reported that normal hemoglobin (Hb-A) and sickle cell hemoglobin (Hb-S) have eight argentometrically titratable sulphydryl groups per molecule at 38°. The mercurimetric-amperometric titration data suggested that there are two $-S\text{Hg}--S-$ linkages possible in the Hb-A molecule, whereas three such bridges are possible in the Hb-S molecule. A subsequent report (2) indicated that there are eight argentometrically titratable sulphydryl groups in the hemoglobin C (Hb-C) molecule; since 8 mercury atoms are also bound per molecule, it was suggested that these are “lone” $-SH$ groups. It was also reported that fetal hemoglobin (Hb-F) contains six titratable sulphydryl groups per molecule which occur as three close, neighboring groups so that three $S\text{Hg}--S-$ linkages are possible in this molecule.

Ingram (3) has reviewed the literature on sulphydryl groups of hemoglobins and made a comprehensive study of the $-SH$ groups of hemoglobins from man, horse, ox, and sheep. He reported that native horse and ox hemoglobins have four sulphydryl groups in two pairs of closely neighboring groups. Native sheep hemoglobin has eight $-SH$ groups in four such pairs. According to Ingram, horse hemoglobin denatured with dodecyl sodium sulfate has six $-SH$ groups probably situated symmetrically with respect to a diad axis in two clusters of three. Benesch et al. (4) also reported that there are eight argentometrically titratable sulphydryl groups per molecule of sheep hemoglobin. They reported ten sulphydryl groups per molecule of dog hemoglobin. These investigators also noted that the sulphydryl groups of sheep hemoglobin are fully re-active while most of those of canine and human hemoglobins are highly inaccessible.

The present study is an extension of the previous reports (1, 2, 5) on the conformational differences of sulphydryl groups in hemoglobins of horse, sheep, dog, and cow. The maximal number of titratable $-SH$ groups in horse and cow hemoglobins is the same; there are six sulphydryl groups per molecule in both instances. However, the cow hemoglobin has a negative temperature coefficient of binding of heavy metals. Sheep and dog hemoglobins have eight and ten titratable $-SH$ groups per molecule, respectively. From the analysis of the data reported here, a discussion on the nature of mercapto-mercapto (or sulphydryl-sulphydryl) interactions in hemoglobins is presented.

**EXPERIMENTAL**

The diazylated hemoglobin solutions were prepared as previously described (2). The last trace of electrolytes was removed by electrodialysis.

The hemoglobin concentration was determined spectrophotometrically. An aliquot is converted to cyanmethemoglobin and its optical density determined in a 1 cm. Corex Beckman cell at 540 mp and a molecular weight of 16,700 per heme was adopted for the calculations (6).

The apparatus and technique used for the amperometric titrations were essentially the same as previously described (2). The argentometric-amperometric titration method of Benesch et al. (4) was utilized. The titrations were performed in 30 ml of tris(hydroxymethyl)aminomethane buffer, pH 7.4. The temperature of the solution was controlled in a thermostat bath.

The apparatus used for the amperometric titration was essentially the same as previously described (2). The argentometric-amperometric titration method of Kolthoff et al. (8) was utilized. The apparatus was controlled in a thermostat bath, and the temperature of the solution was monitored with a Beckman light-scattering densimeter, pH 7.4. The temperature of the solution was controlled in a thermostat bath.

The method of Kolthoff et al. (8) was utilized for the mercurimetric-amperometric titration. After the phosphate buffer was deoxygenated, the hemoglobin solution was added from a self-adjusting micropipette (9). The titrating agents were added from a syringe microburette as previously described (2). In all titrations, 20 μl of 5 × 10^-4 M titrating agent were added at 1 minute intervals. A total of 400 μl of the titrating agent were used in each titration.

**RESULTS**

Horse Hemoglobin—At 0° the steric hindrance effect on titratable mercapto groups appears to be slightly increased so that the maximal number of titratable $-SH$ groups are not available to the titrating agents; on the average, 5 silver and about 2

An automatic amperometric titration apparatus is available although it was not used in this investigation. It is described by M. Murayama (7).

Potassium nitrate was used in place of KCl.

To minimize protein denaturation the buffer was deoxygenated before the hemoglobin solution was added into the titrating vessel. Pure nitrogen was washed through a solution of the same composition as that used in the buffer. Ddeoxygenated hemoglobin was being titrated in all instances.
mercury atoms are bound per molecule. At 38°, however, 6 silver and 3 mercury atoms are bound per molecule (Table I). The data suggest that sulfhydryl groups are so arranged that three \(-S-Hg-S\) linkages are possible at the equivalence point of the mercurimetric-amperometric titration. In \(-S-Hg-S\) — the centers of the sulfur atoms are separated by twice the covalent Hg-S bond distance, giving S-Hg-S = 5.60 Å.

**Sheep Hemoglobin**—The argentometric-amperometric titration data indicate that there are eight titratable sulfhydryl groups per molecule of sheep hemoglobin at 0° as well as at 38° (Table I). Since 4 Hg atoms are bound per molecule, the data suggest that four \(-S-Hg-S\) linkages are possible for this hemoglobin molecule at 0° as well as at 38°.

**Dog Hemoglobin**—About 10 silver atoms and about 5 mercury atoms are bound per molecule of dog hemoglobin at 0°. At 38° the values found are about the same within the 1st hour of incubation of the hemoglobin solution. However, as the hemoglobin solution is incubated longer than an hour, the number of mercury atoms bound per molecule increases asymptotically up to about 9 per molecule, i.e., during the 1st hour of incubation, about 5 mercury atoms are bound per molecule, during the second hour 6, and so on. The number, however, does not rise above 9 at 38°. This suggests that the molecule undergoes a slow architectural alteration which brings about conformational changes of sulfhydryl groups so that more mercury atoms are bound (Table I). It appears that at 0°, five \(-S-Hg-S\) linkages are possible; however only one bridge remains when 9 mercury atoms are bound per molecule.

**Cone Hemoglobin**—At 0° about 6 silver atoms are bound per molecule of cow hemoglobin, and about 3 mercury atoms are bound per molecule (Table I). The data suggest that about three \(-S-Hg-S\) linkages are possible at 0° in the molecule. But in contrast to the other hemoglobins investigated thus far, the cow hemoglobin is different in that it has a negative temperature coefficient of binding of heavy metals. It appears that the molecule undergoes an architectural alteration when the temperature of the solution is changed from 0° to 38° in such a manner that some of the \(-SH\) groups become more sterically hindered by the protein part of the molecule. On the average, about 4 silver atoms and 2 mercury atoms are bound per molecule (Table I) at 38°.

**DISCUSSION**

The present study of the titratable sulfhydryl groups of horse, sheep, dog, and cow hemoglobin gives additional support to the views previously presented (1, 2, 5) that there is a close relationship between the genetics of an individual organism and the kind of protein it produces; and, further, that the heterogeneity of hemoglobins may, in part, be due to the conformational differences of the peptide chains which are reflected in the conformational differences of \(-SH\) groups. These particular differences are made apparent by the number of \(-S-Hg-S\) linkages which are possible in a mercurimetric-amperometric titration.

The data reported here agree well with those reported by Ingram (3) on \(-SH\) groups of hemoglobins of horse, sheep, and ox, as well as those reported by Benesch et al. (4) on sheep and canine hemoglobin.4

In the discussion on the steric hindrance theory of heme-heme interaction, St. George and Pauling (10) showed that the affinity of hemoglobin for the ligand depended upon the size of the alkyl groups; the larger the alkyl groups, the lower the affinity. The situation described here involves a converse relation. Since the ligand remains the same, the affinity may be inferred to depend on the difference in the “looseness” of the polypeptide chains with respect to \(-SH\) groups; i.e., the more tightly the chains are coiled or folded in the region of the sulfhydryl group, the lower the affinity for the heavy metal ions.

In a previous paper (1) it was reported that at 0° four \(-SH\)

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4 Although the specificity of the determination was adequately evaluated by Benesch, et al. (4) as well as by Kolthoff, et al. (8) for their respective methods, it was interesting to find that no heavy metal binding takes place when the following samples are tested: horse myoglobin, insulin, o-chymotrypsin, and ribonuclease. The amperometric titration methods appear to be unequivocal as shown also by Ingram (3) who found no heavy metal uptake by samples of ribonuclease, chymotrypsinogen, insulin or myoglobin of horse, sperm whale, and elephant seal. Nevertheless, it cannot be overemphasized that the sulfhydryl group is defined operationally.
groups of hemoglobins A and B are sterically hindered, but that
they become accessible to the titrating agents at 38°. In hemo-
globin C the four groups which become accessible at 38° have a
lower affinity for the titrating agents (2) than the —SH groups
denoted as 1, 2, 3, and 4. These designations are used for conve-
ience to denote those —SH groups at the periphery of
the diagrams in reference (2), whereas the —SH groups which
are titratable at 38° are designated by the numbers 5, 6, 7, and
8. Sulfhydryl groups numbers 5, 6, 7, and 8 appear to behave
between them. The uptake of the mercuric ion by —SH
number 5 appears to make it easier for the next to come onto
as a single set of binding sites with cooperative interaction
in the denatured molecule. The mercapto-mercapto interaction
is evaluated from the equation

\[ y = \frac{Km^y}{(1 + Km^y)} \]  

where \( y \) = the fractional saturation of —SH groups numbers 5,
6, 7, and 8 with respect to the mercuric ions; \( m \) = the molar
concentration of unbound mercuric ions; \( K \) = the association
constant; and \( n \) = the interaction constant (where \( n = 1 \), this
corresponds to no mercapto-mercapto interaction). By re-
arrangement

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

or on taking logarithms of both sides of equation (2):

\[ \log \frac{y}{1 - y} = n \log m + \log K \]

A plot of \( \log(\frac{y}{1 - y}) \) against \( \log m \) gives a straight line with
a slope \( n \) of about 3 for those sulfhydryl groups which become
accessible at 38°. However, upon the addition of dodecyl sodium
sulfate (400 molecules per 4 of Fe), the interaction constant \( n \)
becomes about 1, and the interaction then vanishes. Similar
observations are made with dog hemoglobin and will be elabo-
rated below.

The mercurimetric-amperometric titration curves for dog hemoglobin are essentially the same as those shown for hemoglo-
bin C (2). Plots of \( \log(\frac{y}{1 - y})^y \) against the log of concen-
tration of unbound mercureic ions is shown in Fig. 1 where it can
be seen that the slope is about 2.9 for the native molecule and
about 1 for the denatured molecule. Furthermore, the affinity
for the heavy metal ion is increased on denaturation, and an
increase of about 4-fold in the affinity constant is observed
when the dog hemoglobin is denatured with dodecyl sodium
sulfate (400 molecules to 4 of Fe). This difference in the com-
bining power of mercuric ion for mercapto-mercury complex
formation requires that the energy barrier due to steric hindrance
be about 0.9 kilocalorie mole⁻¹ greater for the native than for
the denatured molecule. The mercapto-mercapto interaction of
hemoglobins appears to arise from steric hindrance.

**SUMMARY**

1. Amperometrically titratable sulfhydryl groups of electro-
dialyzed hemoglobin C of horse, sheep, dog, and cow have been
investigated with argentometric and mercurimetric methods at
0° and 38°.
2. Horse hemoglobin binds about 5 silver and about 2 mercury
atoms per molecule at 0°; at 38°, 6 silver and 3 mercury atoms
are bound per molecule.
3. Sheep hemoglobin binds either 8 silver atoms or 4 mercury
atoms per molecule at the ice point; at room temperature, and
at body temperature.
4. Dog hemoglobin binds either 10 silver atoms per molecule
at 0° or about 5 mercury atoms per molecule. However, at
38° the number of mercury atoms bound per molecule gradually
increases as a function of incubation time, up to about 9. The
number of silver atoms bound per molecule remains essentially
unchanged.
5. Cow hemoglobin resembles horse hemoglobin with respect
to the maximal number of sulfhydryl groups titratable; how-
ever, it has a negative temperature coefficient of binding of
heavy metals. At 0° it binds 6 silver atoms per molecule and 3
mercury atoms per molecule. At 38° only about 4 silver atoms
are bound per molecule and about 2 mercury atoms.
6. A discussion on the nature of mercapto-mercapto (or
sulfhydryl-sulfhydryl) interaction is presented.

**Acknowledgments**—The author wishes to express his indebted-
ness to Dr. Frederick Leaver and Dr. Harry M. Vars who
generously provided the blood specimens.
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