A STUDY OF SULFHEMOGLOBIN*

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The hemoglobin derivative produced by the action of hydrogen sulfide on oxyhemoglobin, now known as sulfhemoglobin, was first studied with the aid of the spectroscope by Hoppe-Seyler (1) who described the characteristic absorption band in the red region of the spectrum, slightly displaced toward the blue from the band for methemoglobin. The compound was named sulfmethylhemoglobin by Hoppe-Seyler, who found that it could not be formed in the absence of oxygen, nor could it be obtained in the crystalline state. Harnack (2) claimed to have prepared the compound in the absence of oxygen, but the experimental conditions described by him did not insure complete absence of air. Clarke and Hurtley (3) found that reducing agents such as sodium hydrosulfite or phenylhydrazine greatly catalyzed the formation of sulfhemoglobin from oxyhemoglobin and traces of hydrogen sulfide. They discovered also that carbon monoxide caused a shift in the absorption maximum of sulfhemoglobin of about 5 μμ toward the violet end of the spectrum. Haurowitz (4) attempted to prepare pure, crystalline sulfhemoglobin by treating horse oxyhemoglobin for a period of 1 week with hydrogen sulfide and air, but the analyses reported by him indicate that he did not obtain a pure product. Haurowitz did not believe that sulfhemoglobin bound carbon monoxide, but that the shift in the

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absorption spectrum caused by this gas was due to the superimposed spectra occurring in the complex solution of pigments obtained by treating oxyhemoglobin with hydrogen sulfide. Keilin (5) showed definitely that sulfhemoglobin is not a methemoglobin compound by preparing a compound of methemoglobin with hydrogen sulfide, which gave a spectrum entirely different from that of sulfhemoglobin. Drabkin and Austin (6) were able to deduce the spectrum of sulfhemoglobin from solutions of oxyhemoglobin partially converted to sulfhemoglobin. A fairly complete recent review of the chemical and clinical work done on sulfhemoglobin is given by Giordano and Vigliani (7).

In view of the conflicting opinions in the literature with regard to the chemical mechanism of formation of sulfhemoglobin this problem was reinvestigated in general. In particular, experiments were designed in an attempt to establish the mechanism of formation and the stoichiometric relations of sulfur to iron in the sulfhemoglobin molecule, and to test the validity of the questionable carbon monoxide-sulfhemoglobin complex. This study also included further observations of the chemical and physical properties of sulfhemoglobin.

EXPERIMENTAL

The quantitative determination of sulfhemoglobin was carried out by use of the spectrophotometer, according to the technique suggested by Williamson (8), Heilmeyer (9), and Drabkin and Austin (10) and with the extinction coefficients for hemoglobin and sulfhemoglobin given by Drabkin and Austin (6). This was supplemented by gasometric and iron analyses. Further details on quantitative measurements and methods of preparing pigments are given in the thesis by the author (11).

Role of Peroxides in Sulfhemoglobin Formation

Crystalline dog hemoglobin was dissolved in 0.1 M phosphate buffer, pH 7.4, to a concentration of 0.2 per cent. To 5 cc. of this solution were added about 20 mg. of Na$_2$S$_2$O$_4$, after which only the absorption spectrum of reduced hemoglobin could be observed. To the solution was then added 1 drop of 0.1 M (NH$_4$)$_2$S. No change in the visible spectrum occurred. 1 drop of 3 per cent hydrogen peroxide was finally added, giving instantly
a strong sulfhemoglobin band at 620 mµ. This experiment could be repeated at will with either dog or human hemoglobin.

The above experiment was repeated with the substitution of 1 mg. of sodium perborate for hydrogen peroxide, giving the same result. The spectrum was observed continuously during the addition of sodium perborate, and at no time did the spectrum of oxyhemoglobin appear.

The effect of an old sample of benzoyl peroxide was tried and this caused the formation of a very weak sulfhemoglobin band.

Hemoglobin was reduced with excess sodium hydrosulfite and then 1 mg. of sodium perborate was added. No change in the spectrum occurred. A drop of dilute ammonium sulfide was added to the solution, with the spectrum continuing to remain unchanged. Finally to this solution was added another mg. of sodium perborate. Sulfhemoglobin formed at once.

To determine whether or not sodium hydrosulfite played a direct part in the peroxide effect, human hemoglobin in 0.2 per cent solution was deaerated in the Van Slyke manometric apparatus. A drop of 0.1 M (NH₄)₂S was added to the reduced hemoglobin with precautions to exclude air. This did not cause any change in the spectrum. Sodium perborate, dissolved in air-free water, was then added, bringing about a rapid formation of sulfhemoglobin. Clarke and Hurtley (3) showed that although dilute solutions of oxyhemoglobin and H₂S form sulfhemoglobin very slowly, the addition of sodium hydrosulfite or phenylhydrazine caused the immediate formation of sulfhemoglobin. Van den Bergh and Wieringa (12) showed that no sulfhemoglobin is formed if sodium hydrosulfite is first added to oxyhemoglobin, followed by H₂S.

In an attempt to elucidate the Na₂S₃O₄ effect, the following experiments were performed. Human oxyhemoglobin, 0.2 per cent solution buffered at pH 7.0 with phosphate, was reduced with a large excess of Na₂S₃O₄. To 5 cc. of this solution were added 2 mg. of KCN. A drop of (NH₄)₂S, 0.1 M, produced no effect. The addition of 2 mg. of sodium perborate then produced sulfhemoglobin immediately. The experiment shows that potassium cyanide has no inhibiting effect on the formation of sulfhemoglobin.

To a dilute oxyhemoglobin solution, prepared as described in
the preceding experiment, were added a few mg. of KCN. Then the minimum quantity of Na₂S₂O₄ necessary to cause the disappearance of the oxyhemoglobin absorption bands was added. Immediately following this a drop of dilute ammonium sulfide was introduced into the solution, resulting in the formation of a weak but plain sulfhemoglobin band.

The above experiment was repeated with the omission of potassium cyanide. In this no sulfhemoglobin was formed.

From these experiments one may conclude that in the presence of cyanide, sodium hydrosulfite reacts with oxyhemoglobin to form hydrogen peroxide and hemoglobin. The subsequent addition of sulfide causes sulfhemoglobin to form. In the absence of cyanide, any peroxide which is formed is immediately decomposed by catalase, which is always present in hemoglobin solutions. Potassium cyanide poisons catalase and prevents the rapid destruction of peroxide.

The following experiments were performed in an attempt to find, from the amount of sulfhemoglobin formed and the amount of H₂S oxidized, just how much H₂S was used to form a given amount of sulfhemoglobin.

The oxygen uptake was measured in the Warburg respirometer. Buffered oxyhemoglobin solution was placed in the main vessel and the sulfide solution was added from the side arm. In general, sulfide solutions were freshly prepared by bubbling H₂S gas through 1 N or 0.1 N NaOH to the desired concentration. Fig. 1 shows the results of a typical experiment.

Preliminary experiments showed that the oxidation of H₂S in buffer solution alone in the region from pH 6 to 8 was very slow, increasing in rate with increase in pH. In the presence of oxyhemoglobin, the oxygen uptake is very rapid, the rate of oxidation increasing with hemoglobin concentration and with temperature. With smaller quantities of oxyhemoglobin, the final oxygen uptake is greater, owing to the fact that less sulfhemoglobin is formed, leaving more sulfide available for oxidation.

The total oxygen uptake shows that for every molecule of H₂S oxidized to sulfur 1 molecule of oxygen is used up.

In Fig. 1 is shown also the rate of formation of sulfhemoglobin plotted with the oxygen uptake curve. This experiment was performed by setting up a series of Warburg vessels, all containing
the same concentrations of oxyhemoglobin and sulfide. At the intervals noted, the contents of one Warburg vessel were removed and diluted 100 times with half saturated (NH₄)₂SO₄. This procedure reduces the rate of formation of more sulfhemoglobin to a negligible quantity, and also yields, after filtration, a clear solution which may be read in the spectrophotometer.

Effect of Various Oxidizing Agents on Sulfhemoglobin Formation

Having found that sulfhemoglobin can be formed from reduced hemoglobin and sulfide by adding peroxides, the effect of other oxidizing reagents was studied. Beef hemoglobin, 0.2 per cent in phosphate buffer of pH 7.4, was extracted in the Van Slyke apparatus until the oxyhemoglobin absorption bands disappeared. Air-free 1 per cent potassium sulfide was added, 0.3 cc. being used. No change in spectrum occurred. Then 1 cc. of air-free 0.3 per cent potassium ferricyanide was added. No sulfhemoglobin formed. In the presence of sulfide, no methemoglobin band forms at 634 m. After 2 hours there was still no sulfhemoglobin band visible. At this time, air was admitted, and sulfhemoglobin formed rapidly. The experiment was repeated, with air-free solutions of 8 per cent KClO₃, 0.1 N KMnO₄, 1 per cent NaClO, and 0.1 per cent p-aminophenol each in place of ferri-
cyanide, but in no case was sulfhemoglobin formed. Hemoglobin prepared as above was reduced with excess sodium hydrosulfite and then dilute potassium sulfide was added. To this solution were added several drops of saturated bromine water. No sulfhemoglobin formed. Finally on addition of 1 mg. of sodium perborate, sulfhemoglobin formed at once. The experiment demonstrates that of a large number of oxidizing reagents, only hydrogen peroxide, or substances yielding this substance in water, will cause the formation of sulfhemoglobin from reduced hemoglobin and sulfide.

Fig. 2. Effect of sulfide concentration upon the fraction of Hb converted to SHb. \( \text{HbO}_2 = 0.0118 \text{ M} \). pH 7.0, 25°C.

Effect of Sulfide Concentration on Sulfhemoglobin Formation

In this experiment, varying amounts of sodium sulfide were added to a constant quantity of 0.012 M human oxyhemoglobin solution in a series of flasks. The solutions were buffered to pH 7.0, and the stoppered flasks were shaken slowly for approximately 30 minutes at 30°C. The amount of sulfhemoglobin formed was then determined spectrophotometrically. The results are shown in Fig. 2. The log of the sulfide concentration is used so that the points for small concentrations would not be crowded together. The sulfide concentration, which was varied, is divided by the oxyhemoglobin concentration which was kept...
constant, so that the zero point on the abscissa of this curve represents an equimolecular solution of sulfide and oxyhemoglobin.

Along the ordinate is plotted the ratio of the moles of sulfide required to give 1 mole of sulfhemoglobin, 1 mole being assumed for each heme iron. Fig. 2 shows that at low sulfide concentrations a theoretical ratio of 1 atom of sulfur for each atom of sulfhemoglobin iron is approached. This is confirmed by the sulfur analyses as given in a subsequent part of this paper. With higher sulfide concentrations a greater ratio is indicated, owing to oxidation of a large part of the sulfide to elemental sulfur.

Effect of pH on Formation of Sulfhemoglobin

To 1 cc. portions of 8.1 per cent human oxyhemoglobin were added 0.5 cc. portions of McIlvaine's phosphate-citrate buffer, 1 M, at pH 6.0, 7.0, and 8.0. To each of the vessels was then added 0.5 cc. of 0.013 M K₂S, after which they were closed and shaken for 50 minutes at 37°. The solutions were then removed, diluted 100 times with half saturated ammonium sulfate, filtered, and read in the spectrophotometer. The solution at pH 6.0 formed 20 per cent sulfhemoglobin, that at pH 7.0 formed 12 per cent, and that at pH 8.0 formed 9 per cent. The experiment shows that other things being equal, the effect of increasing the pH is to decrease the amount of sulfhemoglobin formation.

Effects of Some Reagents on Sulfhemoglobin and Its Formation

A large number of qualitative experiments were carried out with sulfhemoglobin and on its formation in which most of the observations recorded in the literature were repeated, and some additional properties of the compound were noted.

In conformity with older observations, it was found that other forms of sulfur, such as those occurring in thiourea, cystine, cysteine, thioglycolic acid, sodium thiosulfate, sodium sulfite, and elemental sulfur, could not be substituted for sulfide sulfur in the formation of sulfhemoglobin. It was observed that 1 per cent formaldehyde did not prevent the formation of sulfhemoglobin and did not have any immediate action on it after formation. Lead acetate and iodoacetate, which react with the —SH group, were without appreciable action on sulfhemoglobin.

It was found that in the presence of a fairly large amount of
330 **Sulfhemoglobin**

potassium cyanide, approximately 1 M, strongly buffered with phosphate to keep the pH below 8, oxidized sulfhemoglobin, upon being reduced with sodium hydrosulfite, gave rise to a new absorption band at 644 m\(\mu\), which could not be accounted for on the basis of any known hemoglobin derivative. When the solution was stirred, so that it became saturated with oxygen, the typical band of sulfhemoglobin at 620 m\(\mu\) returned, replacing the band at 644 m\(\mu\). The compound is presumably a very unstable cyanide complex of reduced sulfhemoglobin, which only forms if a large amount of cyanide is present and easily reverts to sulfhemoglobin.

In general these experiments present evidence that sulfhemoglobin can be formed only from soluble sulfides as the source of sulfur, and that the compound formed is not a sulfhydryl type, or at least not like any known sulfhydryl compound.

**Stability of Oxidized and Reduced Sulfhemoglobin**

If a solution containing a mixture of sulfhemoglobin and oxyhemoglobin is permitted to stand in contact with air, at pH 7.0, either at 0° or room temperature, a brownish precipitate forms gradually. In the course of a week at room temperature, two-thirds of the pigment may be precipitated. The precipitate dissolves in dilute ammonium hydroxide and gives a typical denatured globin hemochromogen spectrum on reduction with sodium hydrosulfite. The iron content of the precipitate was found to be 0.30 per cent.

In contrast to the fairly rapid decomposition of sulfhemoglobin in contact with oxygen, reduced sulfhemoglobin is very stable. A solution of human hemoglobin containing 60 per cent of the total pigment as sulfhemoglobin at pH 7.0 was kept reduced with excess sodium hydrosulfite and left at room temperature. After 3 months there was no apparent decrease in the intensity of the sulfhemoglobin absorption band. This is in conformity with the clinical observations of the extreme stability of sulfhemoglobin in the blood as contrasted with other abnormal blood pigments.

**Alkaline Denaturation of Sulfhemoglobin**

In this experiment a study was made of the stabilities of the hemoglobins from two different species and of their corresponding
sulfhemoglobins, 0.05 N NaOH being used as a denaturing agent. In the usual method of measuring the rate of alkaline denaturation, as used extensively by Haurowitz (4), the oxygenated form of hemoglobin is used. The reaction proceeds in two steps, reduced hemochromogen being first formed, followed by a rapid oxidation to the oxidized form of the hemochromogen by the oxygen present in the solution. To avoid the necessity of measuring two simultaneously proceeding reactions, all solutions were reduced with excess sodium hydrosulfite. When reduced hemochromogen is formed, an intense absorption band appears at

![Graph showing the rate of denaturation of human and beef Hb and SHb in 0.05 N NaOH, 25°C.](http://www.jbc.org/)

Fig. 3. Rate of denaturation of human and beef Hb and SHb in 0.05 N NaOH, 25°C.

558.6 m\(\mu\). By measuring the rate of increase of light absorption in the reduced alkaline hemoglobin solution with a spectrophotometer, a measure of the rate of denaturation of hemoglobin was obtained. A similar procedure has been employed by Drabkin and Austin (13), who used dog hemoglobin and a lower alkalinity than employed here.

Since sulfhemoglobin in alkaline solutions is converted to denatured globin hemochromogen, with the disappearance of the characteristic absorption band at 620 m\(\mu\), a measure of the rate of denaturation of sulfhemoglobin was obtained by determining the decrease in light absorption at 620 m\(\mu\) with the spectrophotometer.
The rates of denaturation of human and beef hemoglobin and of their corresponding sulfhemoglobins in 0.05 N NaOH are shown in Fig. 3. The percentage decomposition was calculated on the basis of the fractions of the initial or final absorption remaining at any time. The results show that the resistance to alkaline denaturation of sulfhemoglobin is practically identical to that of the hemoglobin from which it was derived.

Sulfhemoglobin from Hematoporphyrin

In considering the nature of sulfhemoglobin, it is important to know whether the formation of this compound is a special property of protoporphyrin, the natural porphyrin occurring in hemoglobin, or whether it can be formed from artificial hemoglobins containing other porphyrins.

The most readily obtainable porphyrin is hematoporphyrin, which never occurs naturally but may be easily obtained by the action of concentrated sulfuric acid on hemoglobin. The method described by Kaplan (14) was used for the preparation of hematoporphyrin.

Hill (15) has shown that an iron derivative of hematoporphyrin can be prepared, and Hill and Holden (16) have shown that the compound so produced will combine with native globin.

Hematoheme was prepared by heating hematoporphyrin with iron filings in glacial acetic acid for 4 hours on the steam bath and then precipitating the compound by dilution with water. To be certain that a heme compound had been formed, the pyridine hemochromogen was prepared by dissolving hematoheme in 40 per cent pyridine and reducing with sodium hydrosulfite. A typical hemochromogen spectrum was obtained, with bands at 548 and 519 μμ.

Hematoheme was dissolved in dilute sodium carbonate and then added to native beef globin, buffered to pH 7.4 with phosphate buffer. The globin was prepared according to the method of Roche and Combette (17). It was found very difficult to prepare any appreciable amount of native globin from human or dog blood, but beef blood gave good yields.

The hematohemoglobin, on being reduced by sodium hydrosulfite, gave a single broad absorption band, very similar to re-
duced hemoglobin. After oxygenation of the solution, two bands appeared, similar to oxyhemoglobin, at 573 and 537 mp. Upon addition of a few drops of saturated H2S water, and reduction with sodium hydrosulfite, an absorption band formed at 618 mp. The band was very poorly defined, but had the properties of a sulfhemoglobin band, being destroyed by alkali. The denatured globin hemochromogen from hematoporphyrin which formed in 0.1 N NaOH had absorption bands at 552 and 522 mp. The corresponding bands for ordinary denatured globin hemochromogen are at 558 and 528 mp.

On the basis of these results, it may be concluded that protoporphyrin possesses no special structure which is essential to the formation of sulfhemoglobin, and also that sulfhemoglobins may be prepared from reconstituted hemoglobins.

**Molecular Weight of Sulfhemoglobin**

The molecular weight of sulfhemoglobin was estimated by means of its osmotic pressure. An approximately 3 per cent solution of human oxyhemoglobin buffered to pH 6.7 gave an osmotic pressure by means of an osmometer corresponding to a molecular weight of 68,000. After conversion of the oxyhemoglobin to 83 per cent sulfhemoglobin, the average molecular weight was 66,000, indicating that no significant aggregation or dissociation occurs when oxyhemoglobin is transformed to sulfhemoglobin.

**Solubility of Sulfhemoglobin**

Sulfhemoglobinemia was produced in rats by mixing powdered sulfur and phenacetin with their food. To twelve rats, averaging 75 gm. in weight, were given 3 gm. of sulfur and 1 gm. of phenacetin daily. After 5 days, one of the animals was killed and the blood diluted with 1 volume of water. To the diluted blood was added ½ volume of toluene, and the mixture was thoroughly shaken and placed in the refrigerator to allow crystallization to occur.

The crystals and mother liquor were separated by centrifugation, and, after the crystals were dissolved with the aid of sodium carbonate, both solutions were analyzed with the spectrophotometer. By means of the hand spectroscope, a very plain sulfhemoglobin absorption band could be seen.
In the crystals was found 5 per cent SHb and in the supernatant, 4 per cent, showing, within the experimental error, that the solubility of the sulfhemoglobin and oxyhemoglobin was approximately the same. The sulfhemoglobin-containing crystals were examined under the high power field of a microscope. The crystals were sharply defined, 4- and 5-sided, thin plates, all of which appeared to be of a single type indistinguishable from the normal hemoglobin crystals, making it unlikely that sulfhemoglobin had formed in separate crystals from oxyhemoglobin, but rather a solid solution had formed.

Washed dog red blood cells were treated with H2S, washed again with physiological saline, and then hemolyzed by mixing with $\frac{1}{4}$ volume of toluene. After the mixture had stood in the cold for 24 hours, the crystals which had formed in very small amount were separated from the supernatant fluid, dissolved with the minimum quantity of sodium carbonate, and analyzed spectrophotometrically, as was the supernatant fluid. In the crystals 23 per cent and in the supernatant 22 per cent sulfhemoglobin was found.

Sulfhemoglobinemia was produced in a dog by giving daily doses of 1 gm. of powdered sulfur and 1 gm. of phenacetin mixed with ground beef. Crystals and supernatant fluid were obtained from the blood of this animal after 30 days of the above treatment, the procedure described above for rat blood being followed. Spectrophotometric analysis showed 8.5 per cent sulfhemoglobin in the crystals and 9 per cent in the supernatant fluid.

As the solubilities of rat and dog oxyhemoglobins were found to be the same as the corresponding sulfhemoglobins, an attempt to separate dog sulfhemoglobin from reduced hemoglobin was made, since it is known that reduced dog hemoglobin is very much more soluble than the oxyhemoglobin from the same species. Crystalline dog oxyhemoglobin was converted partially to sulfhemoglobin (40 per cent) in the usual way. The total pigment concentration was 14 gm. per cent. Phosphate buffer, pH 6.7, was added to a concentration of 0.1 M. The solution was deaerated with an oil pump, and was left under nitrogen at 2°. No crystallization occurred during a period of 1 week.

The separation of sulfhemoglobin from oxyhemoglobin was attempted by means of the salting-out procedure. Human oxyhemoglobin containing 70 per cent sulfhemoglobin was half
saturated with ammonium sulfate and left overnight in the refrigerator. The salted-out protein and supernatant were separated by filtration and analyzed spectrophotometrically. The percentage of sulfhemoglobin in the total pigment of the solid and liquid phases was the same.

The relative solubilities of reduced human hemoglobin and sulfhemoglobin were examined in strong phosphate buffer at pH 6.7. Reduction was brought about by addition of excess Na₂S₂O₄. Phosphate buffer, 2.8 M, was added until precipitation commenced. The mixture was left for 8 hours at 25° in a stoppered centrifuge tube. After centrifugation the ratio of sulf-}

**Fig. 4.** Solubilities of beef HbO₂ and SHb in phosphate buffer, pH 7.0, 25°. The concentration of SHb was 67 per cent of the total pigment.

hemoglobin to total pigment in the supernatant fluid and precipitate was found to be the same.

The solubility of beef sulfhemoglobin and oxyhemoglobin was investigated at different concentrations of phosphate buffer. The results are shown in Fig. 4. These results show that under the conditions employed hemoglobin and sulfhemoglobin do not differ measurably with regard to their solubilities.

**Formation of CO-Sulfhemoglobin**

The formation of a carbon monoxide compound of sulfhemoglobin was first suggested by Clarke and Hurtley (3) who discovered that passing CO gas into solutions containing sulf-
hemoglobin caused a shift in the characteristic absorption band at 620 \textmu m, of approximately 5 to 7 \textmu m, toward the violet end of the spectrum. On the basis of experiments performed by him, Haurowitz (4) denied the possible existence of a compound between CO and sulfhemoglobin.

The shift in the spectrum of sulfhemoglobin which occurs when CO is passed into the solution of the pigment is shown in Fig. 5. The formation of a new compound is indicated by this shift, since it is not possible to construct any mixture of pigments which might be present and would give the observed effect. The same shift in the spectrum is observed when mixtures of oxyhemoglobin and sulfhemoglobin are completely reduced with \textit{Na}_2\textit{S}_4\textit{O}_6 before addition of CO gas.

Since no evidence of a gasometric study of the reaction between sulfhemoglobin and carbon monoxide could be found in the literature, the reaction was studied gasometrically, with the Van Slyke manometric apparatus and the Warburg respirometer.

In the Warburg procedure, sulfhemoglobin was freshly prepared from oxyhemoglobin which was mixed with sulfide in the closed
vessel and shaken until the oxygen uptake came to a stop. The vessels were then opened and acid ferricyanide was added to the side arm and 40 per cent KOH to the central inset vessel. The Warburg vessels were then saturated with air in those cases in which the oxygen capacity was being determined, and with CO for the carbon monoxide capacity. The addition of acid ferricyanide from the side arm releases oxygen from oxyhemoglobin and carbon monoxide from its combinations with hemoglobin and sulfhemoglobin and the amount of gas formed was determined manometrically.

**TABLE I**

*Combination of SHb with Carbon Monoxide*

<table>
<thead>
<tr>
<th>Original Hb binding CO or O₂</th>
<th>SHb, spectrophotometric</th>
<th>Binding O₂</th>
<th>Binding CO*</th>
<th>Extinction coefficient of globin hemochromogen, original Hb = 1.93</th>
</tr>
</thead>
<tbody>
<tr>
<td>gm. per cent</td>
<td>gm. per cent</td>
<td>gm. per cent</td>
<td>gm. per cent</td>
<td></td>
</tr>
<tr>
<td>15.9</td>
<td>68</td>
<td>6.0</td>
<td>12.2</td>
<td>1.55</td>
</tr>
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<td>18.1</td>
<td>51</td>
<td>8.2</td>
<td>16.0</td>
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<tr>
<td>17.7</td>
<td>47</td>
<td>6.8</td>
<td>15.6</td>
<td></td>
</tr>
<tr>
<td>16.9</td>
<td>88</td>
<td></td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>18.2</td>
<td>87</td>
<td>75</td>
<td>10.8</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td></td>
<td>1.86</td>
</tr>
</tbody>
</table>

* Represents total intact Hb and SHb.

The results of these gasometric analyses are shown in Table I. The hemochromogen absorption coefficients shown in Table I give a measure of the total amount of heme pigment left after oxyhemoglobin is partially converted to sulfhemoglobin. The absorption coefficient as used in this paper is defined as the negative log of the fraction of light transmitted by a layer of solution 1 cm. in thickness and containing 0.1 per cent pigment.

The possibility existed that the extra carbon monoxide capacity of the sulfhemoglobin solutions examined was due to hemochromogen which might have formed during the preparation of sulfhemoglobin. However, at no time was there any spectroscopic evidence for the presence of hemochromogen in freshly prepared
solutions of sulfhemoglobin, after reduction with sodium hydro-
sulfite. Owing to the great intensity and sharpness of the spec-
trum of reduced hemochromogen, it is possible to detect very 
small amounts of this pigment in the presence of other pigments. 

In the absence of active reducing agents, such as Na$_2$S$_2$O$_3$, or 
alkaline sulfides, hemochromogen is oxidized by air practically 
instantaneously to its ferric state, in which form it does not bind 
CO. In the experiments with the Warburg respirometer no 
CO was added to the oxygenated solution of sulfhemoglobin until 
all the sulfide had been oxidized, so that any hemochromogen 
which might have been present would have been in the oxidized 
form and so incapable of combination with CO.

The results indicate that probably 1 molecule of CO is bound 
for every atom of iron in reduced sulfhemoglobin.

**Cataphoresis of Sulfhemoglobin and Oxyhemoglobin**

The cataphoresis of sulfhemoglobin and oxyhemoglobin was 
carried out in a U-tube type of apparatus, with reversible copper-
copper sulfate electrodes, according to the procedure described 
by Michaelis (18). An oxyhemoglobin solution containing 1.49 
gm. per cent total pigment, of which 81 per cent was sulfhe-
modin, was buffered to various pH values with 0.05 M phosphate 
buffer. At pH 6.8 no movement of pigment occurred in 4 hours. 
At pH 7.8 the pigment moved 3 cm. toward the anode in 6 hours.

The pigment column was separated into four parts, Part 1 
being nearest the anode and Part 4 nearest the cathode. The 
ratio of sulfhemoglobin to total pigment was determined in each 
section by means of the spectrophotometer. The results were 
as follows: Part 1, 82 per cent of the total was sulfhemoglobin; 
Part 2, 84 per cent; Part 3, 80 per cent; Part 4, 83 per cent.

From these results it is deduced that the isoelectric points and 
electrophoretic mobilities of sulfhemoglobin and oxyhemoglobin 
are not significantly different.

**Sulfur Content of Sulfhemoglobin**

The only quantitative data on the sulfur content of sulfhe-
modin are those reported by Haurowitz (4). The values obtained 
were very variable and quite high, making it appear unlikely that 
Haurowitz was working with a pure compound.
In various attempts to purify sulfhemoglobin it was found very
difficult to separate extraneous sulfur by extraction or precipita-
tion methods, so that excessively high sulfur values were obtained,
similar to those found by Haurowitz. It was finally decided that
cataphoresis offered the best possibility of accomplishing the
purification of sulfhemoglobin.

Good descriptions of the cataphoretic method are given in the
works of Tiselius (19) and Theorell (20). However, in working
with sulfhemoglobin a sufficient quantity of material must be
obtained in the shortest time possible because of the instability
of the compound in oxygenated solutions. The methods already
described did not meet these requirements, so a procedure worked
out by Dr. T. B. Coolidge1 of this laboratory was tried out and
found to be suitable. With this apparatus a yield of over 1 gm.
of total pigment could be obtained in about 12 hours.

In order to dry the purified sulfhemoglobin with a minimum
amount of denaturation the solution was placed in a porcelain
dish, and a stream of air, which had been passed over calcium
chloride, was blown over the surface. After 12 hours the pigment
was sufficiently dried so that it could be ground to a fine powder
in a mortar. Further drying was carried out in a desiccator over
sulfuric acid.

The sulfur was determined by fusing the sample in a micro-
Parr bomb, precipitating the sulfate formed with barium, and
weighing on the microbalance.

The following averaged figures for total sulfur were obtained.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Per cent S</th>
</tr>
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<tbody>
<tr>
<td>Human HbO₂</td>
<td>0.614 (0.601-0.631)</td>
</tr>
<tr>
<td>&quot; SHb 38% + HbO₂ 62%</td>
<td>0.700 (0.685-0.711)</td>
</tr>
<tr>
<td>&quot; &quot; 45% + &quot; 55%</td>
<td>0.717 (0.710-0.725)</td>
</tr>
<tr>
<td>Extra sulfur due to sulfhemoglobin</td>
<td>0.228</td>
</tr>
<tr>
<td>1 sulfur atom per sulfhemoglobin iron atom requires</td>
<td>0.192% S</td>
</tr>
</tbody>
</table>

Labile Sulfur Split from Sulfhemoglobin by Bromine

Hemoglobin solutions partially converted to sulfhemoglobin,
and purified by cataphoresis, were saturated with bromine vapor,
according to the method of Blumenthal and Clarke (21). After
standing an hour, protein was precipitated with 20 per cent tri-

1 Coolidge, T. B., personal communication, to be published.
chloroacetic acid, and the solutions were filtered and analyzed for sulfate gravimetrically. By this treatment only a negligible quantity of sulfate was obtained from oxyhemoglobin.

The labile sulfur found in three different samples is shown.

<table>
<thead>
<tr>
<th>Human SHb, per cent of total pigment</th>
<th>Per cent S</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.238 (0.220-0.255)</td>
</tr>
<tr>
<td>42</td>
<td>0.272 (0.267-0.277)</td>
</tr>
<tr>
<td>28</td>
<td>0.264 (0.254-0.281)</td>
</tr>
</tbody>
</table>

Average amount of labile S: 0.258
Expected, on basis of Fe:S = 1: 0.192

From these results and from the analyses of the total sulfur in sulfhemoglobin it may be concluded that in the formation of sulfhemoglobin 1 atom of sulfur is introduced into the hemoglobin molecule for each iron atom, in a state which is easily oxidized to sulfate by bromine.

*Myosulfhemoglobin*

Beef muscle hemoglobin or myohemoglobin was prepared from fresh, lean beef according to the method described by Theorell (22) for beef heart. During the final stages of purification the myohemoglobin rapidly oxidized to the ferric form and was thus preserved and used in this state.

When dilute hydrogen sulfide solution was added to a solution of myohemoglobin at pH 7.2 and followed by a little sodium hydrosulfite, myosulfhemoglobin formed at once, as indicated by the appearance of a strong absorption band at 617 to 618 m\(\mu\).

The addition of dilute potassium ferricyanide to myosulfhemoglobin caused the instant disappearance of the band at 617 m\(\mu\). Then the addition of a few crystals of Na\(_2\)S\(_2\)O\(_4\) brought the band back at once to its original intensity.

Owing to the rapid and reversible oxidation of myosulfhemoglobin, it was found possible to determine the approximate number of equivalents of oxidizing reagent necessary to convert sulfhemoglobin to its ferric form.

The solution of myosulfhemoglobin used in this experiment contained a small amount of oxymyohemoglobin, but this is readily oxidized by potassium ferricyanide, so that it does not interfere with the interpretation of the results.

The total pigment concentration was determined by converting
all the hemoglobin derivatives to pyridine hemochromogen and reading in the spectrophotometer at \( \lambda = 525 \text{m} \mu \), according to the procedure described by Drabkin and Austin (6). More recently, Drabkin (23) has applied this procedure to the determination of total pigment in mixtures containing sulphhemoglobin. The concentration of pigment was 0.498 per cent or 0.30 mm.

The myosulphhemoglobin solution was titrated with 0.01 m \( \text{K}_3\text{Fe(CN)}_6 \), the end-point in the reaction being determined as that point at which further addition of ferricyanide caused no further decrease in absorption at 617 m\( \mu \). For 6.15 cc. of sulphhemoglobin solution was required 0.15 cc. of 0.01 m \( \text{K}_3\text{Fe(CN)}_6 \).

The absorption coefficient of the myosulphhemoglobin solution at the start of the titration was \( E_{617} = 4.03 \) and at the end \( E_{617} = 1.38 \). At the end of the titration excess sodium hydrosulphite was added to the solution to convert completely the oxidised myosulphhemoglobin back to the reduced form. The absorption coefficient was then found to be \( E_{617} = 4.40 \), indicating that the myosulphhemoglobin was partially oxidised by atmospheric oxygen at the beginning of the titration. The titration result is corrected by the factor \((4.40 - 1.38)/(4.03 - 1.38)\), giving the corrected titration value of 0.17 cc. of 0.01 m \( \text{K}_3\text{Fe(CN)}_6 \). Theoretically to oxidize 6.5 cc. of 0.30 mm sulphhemoglobin, assuming a ferrous-ferric couple, there would be required 0.195 cc. of 0.01 m \( \text{K}_3\text{Fe(CN)}_6 \), which is sufficiently close to the experimental value.

In agreement with Theorell (22) it was found that beef myohemoglobin is extremely stable toward alkali, being slowly denatured over the course of 24 hours in 0.1 n NaOH. The interesting observation was made that myosulphhemoglobin was equally stable toward alkaline denaturation, and led to a study of the relative resistances of blood hemoglobin and sulphhemoglobin to the action of alkali, described above.

The action of 1 per cent \( \text{HgCl}_2 \) was tested on a solution of myosulphhemoglobin. A few drops of mercury solution caused the band at 617m\( \mu \) to disappear. On addition of sodium hydrosulphite to the solution the band at 617m\( \mu \) returned, suggesting that the action of mercuric chloride is one of oxidation. No formation of hemochromogen occurred, such as one obtains in the corresponding experiment with sulphhemoglobin from blood.

The spectrum of myosulphhemoglobin is shown in Fig. 6.
The data obtained in this experiment indicate that a very stable myosulfhemoglobin can be formed and that the resulting compound can be reversibly oxidized. The spectrum shows that the absorption maximum of myosulfhemoglobin is shifted slightly toward the blue end of the spectrum as compared with that of blood sulfhemoglobin and is almost twice as intense. The approximate specific absorption coefficient of myosulfhemoglobin is $E_{617} = 1.03$ and for blood sulfhemoglobin $E_{560} = 0.64$. The broken line curve in Fig. 6 shows the effect produced by CO on the spectrum of myosulfhemoglobin. It can be seen that CO causes a slight shift toward the blue in the maximum at 617 m\(\mu\). The two new maxima at 540 and 579 m\(\mu\) indicate the slight contamination of myosulfhemoglobin with myohemoglobin.

**DISCUSSION**

From the results shown in Fig. 2 it can be seen that sulfhemoglobin involves 1 atom of sulfide sulfur for each Fe atom of sulfhemoglobin produced. Fig. 2 shows that when a large excess of sulfide is used, most of it is oxidized. With a molar ratio of sul-
fide to oxyhemoglobin of 1, only one-third of the theoretically possible sulfhemoglobin is formed. By reducing the sulfide concentration to a low value the theoretical ratio is approached but never attained, owing to the fact that there is always a small fraction of the sulfide oxidized and also because it is experimentally impossible to determine with any accuracy the very small amounts of sulfhemoglobin which are formed.

The experiments in which sulfate was formed on treating sulfhemoglobin with bromine confirm the results described in the preceding paragraph. It was found that hemoglobin does not give sulfate on being treated with bromine. This was to be expected since Blumenthal and Clarke (21) have shown that cystine, cysteine, and methionine, which apparently account for all the sulfur in hemoglobin, do not yield sulfate on bromine oxidation. These workers found, however, that such compounds as thiourea, thioamides, and thiolhistidine do give sulfate when oxidized with bromine. It is not to be expected that any stable mercaptan type of bonding would occur upon treating a solution of oxyhemoglobin with sulfide at room temperature, but rather a relatively unstable type of linkage which would be susceptible to bromine oxidation such as was found experimentally. The formation of methylene blue, which involves the linking of 2 molecules of \( p \)-aminodimethylaniline by hydrogen sulfide in the presence of ferric chloride to form a thiazine ring, is a well known type of reaction to which the reaction to form sulfhemoglobin might be related.

It is less reliable to draw conclusions from the results of the total sulfur content in hemoglobin before and after partial conversion to sulfhemoglobin, the theoretical increase in the sulfur content being in these experiments of the order of less than 0.1 per cent. However, it was found that in the purest samples of sulfhemoglobin obtained by cataphoresis, the increase in sulfur content due to the formation of sulfhemoglobin corresponded closely to 1 sulfur atom for each atom of iron.

With few exceptions the workers in this field have shown that sulfhemoglobin can be formed from hemoglobin and sulfide only in the presence of oxygen. Clarke and Hurtley (3) observed that the addition of a reducing agent to oxyhemoglobin and a trace of sulfide greatly catalyze the formation of sulfhemoglobin. This apparently anomalous result, which has been con-
firmed by other workers, and also in these experiments, seems inexplicable at first sight, because it leads to the conclusion that the formation of reduced hemoglobin speeds the formation of sulfhemoglobin, whereas it has been definitely shown that sulfide has no action on reduced hemoglobin.

All the known facts regarding the formation of sulfhemoglobin are correlated by introducing hydrogen peroxide as a necessary component in the reaction. The most active catalyst for the formation of sulfhemoglobin from oxyhemoglobin and sulfide is sodium hydrosulfite. This compound also reacts faster with molecular oxygen to form hydrogen peroxide as one of its products than most other reducing agents, as stated by Mellor (24) and others. Phenylhydrazine also reacts quite rapidly with oxygen, very probably forming hydrogen peroxide as a product. It also catalyzes the formation of sulfhemoglobin.

The data on the oxidation of sulfide by oxygen in the presence of hemoglobin indicate the involvement of hydrogen peroxide. The oxygen uptake, when relatively small amounts of oxyhemoglobin were used, corresponded to 1 mole of oxygen for each mole of sulfide used. This corresponds to the oxidation of the sulfide to elementary sulfur with the formation of hydrogen peroxide. In the absence of hemoglobin the oxidation of hydrogen sulfide to sulfur is very slow. The oxidation of hydrogen sulfide by oxyhemoglobin and the subsequent formation of sulfhemoglobin can be represented by the following equations.

\[ \text{HbO}_2 + \text{H}_2\text{S} = \text{Hb} + \text{S} + \text{H}_2\text{O}_2 \]

\[ \text{Hb} + \text{H}_2\text{S} + \text{H}_2\text{O}_2 = \text{SHb} + \text{unknown products} \]

Only a small part of the hydrogen peroxide can conceivably be utilized in the formation of sulfhemoglobin. The remainder must be dissipated by directly attacking the globin or heme of hemoglobin. That this can occur with hemoglobin has been shown by Keilin (25) and Bingold (26) if the catalase is destroyed by heat or inhibited by cyanide or sulfide ion. Evidence for the destruction of heme pigment on conversion of hemoglobin to sulfhemoglobin is shown in Table I. The total pigment which was convertible to denatured globin hemochromogen had been decreased by 4 per cent in a solution containing 30 per cent sulfhemoglobin,
but in a solution containing 75 per cent of the total pigment as sulfhemoglobin formed by using a large excess of \( \text{H}_2\text{S} \) and thus presumably involving a large amount of hydrogen peroxide, the destruction of heme pigment amounted to 30 per cent.

In sulfhemoglobinemia, there is a great deal of evidence indicating that acetanilide or related derivatives are very important or necessary factors along with an intestinal condition which is conducive to the production of hydrogen sulfide. In the absence of these drugs, or similar factors, hydrogen sulfide rarely, if ever, has been shown to produce sulfhemoglobin in the intact animal. It has been shown by Michel, Bernheim, and Bernheim (27) that \( p \)-aminophenol is readily produced in tissues from acetanilide. *In vitro* acetanilide or aniline does not catalyze the formation of sulfhemoglobin from oxyhemoglobin and sulfide. Ivens and van Vollenhoven (28) have shown that \( p \)-aminophenol does bring about this catalysis. Since \( p \)-aminophenol is fairly rapidly autoxidizable to a quinone form with the probable formation of hydrogen peroxide as a result of its reaction with oxygen, it is very likely that it catalyzes the formation of sulfhemoglobin by causing the production of hydrogen peroxide.

The formation of sulfhemoglobin does not involve any detectable change in the globin, or protein part of the hemoglobin molecule. The rates of alkaline denaturation of human and beef hemoglobin, which differ greatly, remain unchanged on conversion of these compounds to their corresponding sulfhemoglobins. Since it is the globin part of the hemoglobin molecule which determines the alkaline denaturation rate, these experiments indicate that apparently no important change in the globin has occurred.

From the solubility measurements it can be seen also that no major change in the hemoglobin molecule takes place upon conversion to sulfhemoglobin.

The cataphoretic measurements and the molecular weight determination show that no aggregation or dissociation has occurred.

Apparently no special porphyrin is necessary for the formation of sulfhemoglobin, since hemoglobins prepared from protoporphyrin and hematoporphyrin both gave absorption bands in the region of 620 m\( \mu \), having the properties ascribed to sulfhemoglobin.

In forming sulfhemoglobin from hemoglobin there is no irrevers-
Sulfhemoglobin

ible change in the heme. This is shown by the fact that hemochromogens may be prepared from sulfhemoglobin which do not appear to differ from the corresponding compounds prepared from hemoglobin. List (29) showed that the porphyrin obtained from sulfhemoglobin was spectroscopically identical with protoporphyrin obtained from hemin or hemoglobin.

The sulfhemoglobin derivative of myohemoglobin was prepared very easily, giving an absorption band at 618 mμ which had almost double the intensity of the corresponding band in blood sulfhemoglobin. Theorell (20) has pointed out that the molecular weight of muscle hemoglobin is only half that of blood hemoglobin, so that each molecule contains only two heme groups, the iron content being the same as in blood hemoglobin. The formation of myosulfhemoglobin indicates that the four-heme structure possessed by blood hemoglobin is not a necessary factor in the formation of sulfhemoglobin.

One of the interesting difficulties encountered in working with sulfhemoglobin was the failure to convert completely hemoglobin to sulfhemoglobin when the compound was treated with an excess of sulfide. This fact seems to indicate that an equilibrium is reached. However, this is unlikely, because it is impossible to reconvert sulfhemoglobin to hemoglobin either by exposing it to a vacuum for several days or by prolonged dialysis. The answer apparently resides in the complexity of the reaction involving hydrogen peroxide. In order to get a good yield of sulfhemoglobin just the proper amount of hydrogen peroxide must be formed, and most of this must presumably be used in forming sulfhemoglobin. If an excessive amount of peroxide is formed, it will directly attack the pigment, causing some heme destruction. Also it will oxidize hemoglobin to methemoglobin, with which it combines to form a very slightly dissociated compound having a spectrum qualitatively similar to that of oxyhemoglobin.

From the major change which occurs in the visible spectrum on conversion of oxyhemoglobin to sulfhemoglobin it seems that the heme iron must be involved. Major changes in the globin, involving marked differences in composition and in resistance to denaturation, as shown by human and beef hemoglobin, do not cause any detectable differences in the absorption spectra of the oxyhemoglobin compounds. However, any change involving the
heme iron in any hemoglobin compound results in a marked change in the absorption spectrum, such as the change from the reduced to the oxygenated form.

The formation of a carbon monoxide compound of sulfhemoglobin indicates that the sulfur cannot occupy the place normally occupied by oxygen or carbon monoxide. The gasometric determinations of the carbon monoxide capacity of sulfhemoglobin indicate that slightly less than 1 mole of carbon monoxide is found for each iron atom, but this can be correlated with the fact that pigment destruction had occurred, as shown by the absorption coefficients of the denatured globin hemochromogens from the corresponding sulfhemoglobin solutions being diminished below that for the original hemoglobin.

From the evidence it seems possible that the formation of sulfhemoglobin may involve the globin heme linkage, which has never been definitely explained but is apparently through the iron. The instability of the oxidized form of sulfhemoglobin would probably support this hypothesis. A clearer insight to this question will probably have to involve newer approaches, such as the magnetic method used by Coryell, Stitt, and Pauling (30) in working out the structures of hemoglobin and some of its derivatives.

**SUMMARY**

1. Sulfhemoglobin is formed from hemoglobin and in the presence of any soluble inorganic sulfide by the action of hydrogen peroxide.

2. Hydrogen sulfide is catalytically oxidized by oxygen in the presence of hemoglobin to sulfur and hydrogen peroxide.

3. The heme of hemoglobin is not irreversibly changed by the formation of sulfhemoglobin.

4. The solubility, resistance to alkali, molecular weight, and cataphoretic mobility of hemoglobin and its corresponding sulfhemoglobin are the same.

5. Sulfhemoglobin forms a compound with CO.

6. 1 atom of sulfur for each atom of hemoglobin iron is necessary in the conversion of hemoglobin to sulfhemoglobin. Sulfhemoglobin contains 1 atom of labile sulfur, which is not in the form of a free sulfhydryl group.

7. Reduced sulfhemoglobin is very stable, but the oxidized form is unstable.
Sulfhemoglobin

8. A sulfhemoglobin can be formed from muscle hemoglobin. Its stability, properties, and CO compound are described.

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