Sulfhemoglobin (Sulf-Hb) is a nonfunctional form of Hb occurring in man under certain pathological conditions (2, 3). Many investigators have studied this derivative of hemoglobin and its analog sulfomyoglobin (sulf-Mb) (4-7) without firmly establishing the structural alteration in the molecule responsible for its functional abnormality.

In 1863, Hoppe-Seyler (8) found that the green spectral species which he called "sulphemoglobin" could be produced by passing H2S through a solution of HbO2. Chemical analysis of a mixture of sulf-Hb and Hb was carried out by Michel (9), who concluded that sulf-Hb contains one additional sulfur atom per heme equivalent as compared with Hb. Sulf-Hb was shown by Kellin (4) to be distinct from ferric Hb sulfide, the compound produced by the reversible addition of sulfide as a ligand to ferric Hb. From the fact that CO could bind to the ferrous forms of both sulf-Hb and sulf-Mb, Michel (5) also concluded that the additional sulfur atom was not bound as a ligand to the heme iron. Using a modification of the method of Nicholls (6), Morell et al. (7) prepared 35S-labeled sulf-Mb from MbIV and a 100-fold molar excess of (NH4)2S and found 1.5 moles of radioactive sulfur incorporated per mole of sulf-Mb produced. However, recalculation of the data on the basis of the purity of their protein preparation, as determined from the extinction coefficient (9), suggests approximately 2 g atoms of 35S incorporated per mole of sulf-Mb formed, since only about 75% of the Mb was converted. Moreover, on extraction of the prosthetic group into acetone-HCl, Morell et al. (7) found that only 50% of the radioactive sulfur was coextracted, whereas 30% remained with the precipitated globin.

In the first paper of this series (9), we described a modification of the preparative procedure for sulf-Mb which consistently gives the product in about 90% purity, with the remainder present as MbO2, using new criteria for purity based on EPR and optical spectral analysis. In the present paper we have re-examined the incorporation of radioactive sulfur into sulf-Mb and find that exactly one g atom of 35S is bound per mole of sulf-Mb formed. Moreover, this sulfur atom is extracted with the prosthetic group into butane. The optical spectral properties of the prosthetic group are presented for the first time. This prosthetic group, which we call "sulphemin," is not stable but undergoes an oxidative decomposition to protophenin. In order to explore the mechanism involved, we have studied the kinetics of this decomposition under various conditions.

**EXPERIMENTAL PROCEDURE**

**Materials**

Sulf-Mb was prepared as previously described (9) by the addition of a 1.6-fold molar excess of (NH4)2S or Na2S to MbIV at pH 8.0 followed by passage through Sephadex G-20 to remove
excess reagent. Sulf-Mb concentrations were determined as described previously (9).

Radioactive Na, 35S was obtained from New England Nuclear. Two milliliter aliquots of unlabeled NaS, analytical reagent, obtained from Mallinckrodt and stored in unopened bottles in an evacuated desiccator over dry silica until use. The final solution was 0.20 M in NaS and had an activity of 2.57 x 106 cpm per mole by scintillation counting (see below). The concentration of sulfide was verified using a method described previously (9).

Methyl ethyl ketone (2-butanol) was Fisher Certified Reagent Lot 703052-1 7. 2-Butanone was obtained from Eastman Organic Chemicals or from Aldrich Chemical Company. That obtained from Aldrich had n20 = 1.3897, b.p. 100.5-101.5°, and was judged 99% pure by vapor phase chromatography.

Argon was ultrahigh purity, iongrade, purchased from the Matheson Company, and contained less than 1 ppm of O2 and about 0.5 ppm of CO. The argon tank was equipped with a Matheson high-purity regulator, model 3500-580, tested to have a leak rate of less than 2 x 10-10 atmospheric ml per s of helium.

Protohemin IX, recrystallized, was a gift of Dr. Alan D. Adler (New England Institute, Ridgefield, Conn.). It was determined to be 98 to 99% pure, with adsorbed acetic acid as the only impurity, as described in detail elsewhere (10). Crystallized and lyophilized sperm whale Mb was the product of Seravac Laboratories, Colnbrook, England. This specific lot (Batch 10) had an optical absorbance ratio E~000:E~900 of 4.9 and an iron analysis of 0.31% iron, and it was stated to show a single homogeneous peak on Sephadex G-75 chromatography. Stock solutions of 3.45 mM ferric Mb in 0.01 M phosphate buffer (pH 7.1) were prepared by dissolving the dry protein in the buffer and centrifuging to remove the small amount of undissolved material. Concentrations were measured as described previously (9) using the extinction coefficients of Hapner et al. (11). Stock solutions were quick-frozen in C02-acetone and stored at -20°. t-Butyl hydroperoxide was obtained from the Lucid Division of Wallace and Tiernan, Inc., Buffalo, New York.

All other reagents were of the highest grade commercially available.

Methods

Optical Spectra—Optical spectra in the visible region were recorded at 4° or at room temperature (25°) on a Cary model 14R recording spectrophotometer using a high intensity source, 0 to 1.0 and 1.0 to 2.0 optical density slide wires, and 1-cm light path silica cells.

Spectra at 4° were obtained using a jacketed cell holder cooled by circulating either water from a large reservoir of ice and water, as previously described (12), or a 1:1 methanol-water mixture from a Brinkmann Lauda K-2/R refrigerated circulating bath. In either case, the temperature of the solution in the cuvette was 4°. The Cary sample compartment was flushed continuously with dry N2 to prevent fogging due to condensation of water vapor.

Anaerobic experiments were performed in a silica Thunberg cuvette under argon as described previously (9).

Kinetic studies were performed at a scan rate of 2.5 nm per s so that optical spectra extending from 800 nm to 450 nm could be determined in 2.3 min. Spectra of heme protein samples recorded at this speed were superimposable with spectra of the same samples recorded at 1.0 nm per s.

Scintillation Counting—Samples of aqueous solutions (20 n1 to 0.5 ml) and solutions in 2-butanone and 2-pentanone (up to 0.2 ml) were counted in 10 ml of Bray's solution (13) for 1.0 or 10 min in a refrigerated Nuclear Chicago Mark II scintillation counter. Ten milliliters of dry Cab-0-Sil silica gelling agent (Packard Instrument Company) were added when precipitates were counted.

Samples of 2-pentanone, hemin, aqueous buffers at the pH values used, aqueous 0.01 N KOH, 0.1 N KOH, and diluted solutions (less than 0.15 mM) of unlabeled sulf-Mb, each in the volume used in the experiments, were checked for quenching of aliquots of Na, 35S stock solution. None were found to quench more than 2 to 3% in these quantities, and small corrections for quenching were made where appropriate. However, CS2 was found to quench considerably (72 to 94% in the volumes 0.1 to 0.2 ml used).

Samples used for counting were usually greater than 10,000 cpm, except in experiments involving multiple successive extraction steps where counts were in the range of 300 to 1000 cpm. These samples were counted for 10 min.

Paper Chromatography—Samples of hemin and related compounds were chromatographed by ascending chromatography on Whatman No. 3MM paper in 1:3 pyridine-methanol solvent, according to the method of Adler and Harris (10). Aliquots of 10 to 50 ~1, with hemin concentrations varying from 0.1 to 0.5 mM, were examined. Chromatograms were observed under broad spectrum ultraviolet light. Paper chromatograms of radioactive 35S-labeled compounds were scanned on a Nuclear Chicago Actigraph III at 325 cm per hour.

Hemin Concentrations—A weighed sample of the protohemin IX, as used as a primary standard, was dissolved in 2-butanol containing 0.01 M HCl, to a final concentration of 0.49 mM. This solution was stable optically for at least a day, and triplicate 1:0 dilutions of this solution in 2-pentanone were used to determine the following optical millimolar extinction coefficients: 740 = 5.50, 400 = 10.1, and 270 = 10.0 (14). These values were used in computing hemin concentrations in experimental solutions in 2-butanol and 2-pentanone.

Pyridine Hemochromogens—Pyridine hemochromogens of solutions of hemin or sulfhemin in 2-butanol were prepared with hydrazine as the reducing agent as suggested by Adler.2 Pyridine (1 ml) was added to 1.2 ml of a 75 ~1 solution of hemin in 2-butanol at room temperature in a 1-cm path cuvette. After brief stirring followed by a 2-min incubation, 10 ~1 of a 20% solution of KOH in ethanol was added. The solution, which had now turned from orange to green, was allowed to stand for 5 min more before the addition of a drop (about 0.1 ml) of 97% hydrazine on the tip of a Teflon "plunger" (Calbiochem). The solution, which was stirred with the plunger with care to minimize the disturbance of the surface and the introduction of air, turned to the bright red of the pyridine hemochromogen. The optical spectrum was determined within 2 min, and it was stable for at least 10 min. The extinction coefficients for protohemin IX pyridine hemochromogen so prepared were determined to be 238 = 34.8 at Amax 556.5 nm; 400 = 16.6 at Amax 525.5 nm.

Several observations are noteworthy. First, with 2-pentanone as solvent instead of 2-butanol, the pyridine hemochromogen precipitated from solution. Second, it was observed that addition of 0.1 ml of 97% hydrazine alone to the butanol solution

2 A. D. Adler, personal communication.
of hemin in the absence of pyridine or KOH produced the same spectral species, almost identical in the positions and relative intensities of the two maxima to the pyridine hemochromogen prepared by the method above. Apparently, NH₂NH₂ can replace pyridine as the nitrogenous ligand (15) in the hemochromogen with little effect on the visible spectrum.

Gas-trapping Experiments—Attempts to trap volatile radioactive ³⁵S compounds such as H₂S or SO₂ from the decomposition of the isolated prosthetic group of sulf-Mb were performed using a gas bubbler system (14) designed for efficient gas-liquid equilibration, with traps containing 3.5 ml of 0.01 M aqueous KOH. Aliquots (0.5 ml) of the solutions from these KOH traps were then used for scintillation counting.

Barium Precipitation of Sulfate and Sulfite—Aqueous extracts were tested for radioactive SO₄²⁻ and SO₃²⁻ by collecting the Ba²⁺ salts after addition of a large excess of carrier SO₄²⁻ and SO₃²⁻. The aqueous solution (3 ml) was divided into two equal portions. One, for measurements of both sulfate and sulfite, was adjusted to pH 2 and stored capped at 0°C. The other, for determination of sulfite by difference, was acidified with 0.2 ml of concentrated HCl and heated to 76°C for 70 min on a steam bath to drive off SO₂. The pH of this solution was adjusted to 2 with 0.24 ml of 10 M NaOH. Barium sulfate was precipitated from both solutions with a 15-fold excess of BaCl₂, care being taken to minimize co-precipitation of other anions (16). Aliquots were taken for scintillation counting after each step.

RESULTS

Extraction of Prosthetic Group into 2-Butanone—The method of Falk (17) was modified to determine the highest pH at which extraction of hemin from Mb into 2-butanol was nearly quantitative. Butanol has an advantage over the classic acid-acetone procedure (18) because it is not miscible with water and so can be used for extraction of hemin from the aqueous solution containing the globin.

With this procedure, a rather sharp transition in the amount of color extracted occurred between pH 3.35 and pH 3.75. Below pH 3.4, extraction of the chromophore was nearly quantitative. Above pH 4.1, most of the color remained in the aqueous phase.

Two independent factors were observed to determine this transition. First, the color change produced upon acidification of the protein accurately reflected the success of the subsequent extraction. Native high spin ferric Mb is brown in solution. If the pH was lowered sufficiently to denature the protein and produce a bright red hemichrome (19), then the hemin was extractable. If, however, the pH was not lowered enough to denature the protein to a hemichrome, contact with organic solvent denatured the protein in a different fashion, producing another type of red hemichrome from which the prosthetic group could not be extracted.

The second factor involved is the solubility of hemin in 2-butanol; this is increased at low pH and high HCl concentration, because the form which is soluble in 2-butanol is the hemin chloride (λₘₐₓ 640, 540, 510 nm). Thus, at higher pH, its partition into the organic phase is decreased. Therefore, hemin could be extracted from butanone into aqueous buffers at pH 8 to 8.5. At this pH, the form soluble in water is hematin (λₘₐₓ 600, 570 nm, both broad).

Since the pH-dependence for the extraction of the prosthetic group from sulf-Mb was the same as that for Mb and the observations of denaturation to a low spin form, green in this case (9), were also true for sulf-Mb, a standard procedure for the extraction of the prosthetic group at ice temperature was developed. To 0.6 volume of Mb or sulf-Mb in 0.1 M potassium phosphate (pH 8.0), 1.0 volume of 0.1 M glycyglycine-HCl, pH 2.0, was added to give a final pH of 3.2. Then 1.6 volumes of 2-butanol previously chilled to -20°C were added for extraction. After removal of most of the butanone extract, more sulfhemin could be recovered by re-extraction with 2-pentanol. However, 2-pentanol alone was not as good a solvent for extraction of sulfhemin or hemin. The product of the extraction was the same regardless of the oxidation state (II or III) of the iron in the protein starting material. The spectrum of protohemin extracted from Mb by this technique was identical to that of the protohemin IX standard described under "Experimental Procedure."

Optical Properties of Prosthetic Group—The optical spectrum in the visible region of the butanone-pentanone extract of sulf-Mb and successive spectra recorded at brief intervals after extraction are shown in Fig. 1. The prosthetic group was found to be unstable when extracted and to decompose into a species having the
optical spectrum of hemin. The decomposition was first order with a half life of about 22 min under these conditions.

It should be noted that the spectrum of sulfhemin in acidified butanone-pentanone, with maxima at 740, 605, and 490 nm, closely resembles that of high spin acid ferric sulf-Mb, which has maxima at 717, 585, and 490 nm (9). The same resemblance is seen between protohemin IX with maxima at 640, 540, and 510 nm and the parent protein high spin acid ferri Mb with maxima at 633 and 503 nm (11). These resemblances and the common mode of extraction justify the name "sulfhemin" applied to the species extracted from sulf-Mb. By analogy with Mb, it is believed that the prosthetic group extracted from sulf-Mb is always in the ferric form, regardless of the oxidation state of the protein extracted.

**Optical Spectra of Derivatives of Sulfhemin—Pyridine Hemochromogen**—The pyridine hemochromogen of a sample of protohemin IX freshly extracted from ferri Mb, of a sample of fresh sulfhemin in 2-butanol (after negligible decomposition, since the time elapsed was less than 0.05 of the half-life), and of the same solution after decomposition to hemin were prepared. The spectra of the first two are shown in Fig. 2. There was no measurable difference in the positions of the two major maxima at 556.5 and 525.5 nm (± 0.3 nm) among the three spectra even when scanned on an expanded scale (6 nm per inch). Moreover, the ratio of the absorbance at 556.5 nm to that at 525.5 nm was 2 ± 0.1 in all three spectra. These results held true as well when the pyridine hemochromogen of sulfhemin was prepared anaerobically or when the hydrazine was added first. We must conclude that the three pyridine hemochromogens were the same and that sulfhemin is converted to hemin under the conditions of preparation and spectral observation of the hemochromogen. The very weak absorption around 610 to 620 nm in the lower trace of Fig. 2 is not identified. It appeared in two out of three preparations of the pyridine hemochromogen of sulfhemin but never in that of hemin. These results are consistent with the identification of the major sulfhemin decomposition product as protohemin.

**Sulfhematin—**On exposure of sulfhemin to alkaline conditions, a compound with the properties of "sulfhematin," i.e. the hydroxide derivative of sulfhemin, was found (Fig. 3), having a maximum at 665 nm as well as the broad maxima at 600 and 570 nm corresponding to hematin (17). At 4°C, the 665 nm peak slowly decreased in intensity concurrently with increasing intensity in the hematin peaks.

**Sulfhemin-Imidazole**—An imidazole derivative was prepared by the addition of a few crystals of solid imidazole to an extract of sulfhemin in butanone. An optical absorption maximum at 592 nm was observed for this solution (Fig. 4) together with shoulders near 560 and 540 nm attributed to the imidazole derivative of hemin (λmax 560, 538 nm). The absorption at 592 nm, which did not decrease significantly in 20 min at 4°C, is ascribed to an imidazole derivative of sulfhemin.

No cyanide derivative of sulfhemin could be prepared, as all attempts led to materials having spectral features of hematin.

**Titration of Myoglobin with Sulfide—**These experiments were performed to test the conclusion (6, 9) that addition of less than 1 mole of sulfide per mole of Mb IV produced ferric sulf-Mb, which was reduced to ferrous only by subsequent addition of excess sulfide. Following the procedure employed in the prep-
The retention of slightly more % than prosthetic group in the hemin before the phases were separated.

The assumption that the product is hemin will be justified further by the optical criteria described previously. Thus, the amount of ferrous sulf-Mb produced was nearly equimolar with the quantity of sulfide added, although no excess of sulfide was present for the proposed reduction of ferric sulf-Mb to ferrous.

In a second experiment, an identical aliquot containing 1.43 pmoles of Mb' was treated with 0.60 pmoles of Na2S (0.42 mole per mole of heme). The product found contained approximately 30% of ferrous sulf-Mb and very little ferrous sulf-Mb, with the remainder a mixture of ferric Mb and other Mb species absorbing near 545 and 580 nm, possibly including unreacted Mb'. Under these conditions, therefore, most of the sulfide reacted to form sulf-Mb, but the major product was the ferric form. However, since there was initially a 2.4-fold excess of Mb' over added Na2S, the ferric sulf-Mb found can be attributed to oxidation of the initial ferrous sulf-Mb product by this excess Mb' prepared under identical conditions, led to immediate oxidation of the ferrous sulf-Mb to ferric with concomitant reduction of the Mb' to ferrous Mb.

Incorporation of 35S in Preparation of Sulphemyoglobin—Ferric Mb (3 pmoles) was reacted with 5 pmoles of Na2S. After removal of excess reagents on a column of Sephadex G-25, the mixture was reconstituted with the balance MbO2 (9). The radioactivity was 1.45 × 10^6 cpm per ml, corresponding to 0.57 μmole of 35S per ml or 0.98 g atom of 35S incorporated per mole of Mb extracted. Control experiments demonstrated that addition of ferrous Mb to an equimolar quantity of Mb' prepared under identical conditions led to immediate oxidation of the ferrous sulf-Mb to ferric with concomitant reduction of the Mb' to ferrous Mb.

Location of Incorporated Sulfur.—To distinguish between sulfur bound to the prosthetic group of sulf-Mb and sulfur bound to the globin protein moiety, the prosthetic group of the [35S]sulf-Mb was extracted from the protein into a 2-butanol-2-pentanone mixture as described above. In two separate experiments, in which aliquots of radioactive sulf-Mb were extracted, three-quarters of the radioactivity was recovered in the organic phase (Table I). Aliquots of the extract were taken for scintillation counting immediately after extraction and after incubation at 40° to allow complete decomposition. In that period, the concentration of radioactivity in the solution had decreased 7% and 10% in the two experiments. We have determined the recovery of hemin from the optical spectrum of the extract after complete conversion to hemin based on the extinction coefficients of hemin. The assumption that the product is hemin will be justified further below. Correcting the concentration of hemin recovered in the extract for the 10% derived from unlabelled Mb, we calculate that about 0.87 μmole of radioactive 35S was extracted with each micromole of prosthetic group. We conclude that the atom of sulfur incorporated into sulf-Mb is bound to the prosthetic group.

Nature of Sulfhemin Decomposition Products—The Chromophore.—The chromophore produced in the decomposition of sulfhemin has the optical spectrum of hemin (Fig. 1), identical in both the positions of the maxima (640, 540, and 510 nm) and their relative intensities. Moreover, addition of KOH converted the spectrum to that of hematin (λmax 600, 570 nm). Also, the pyridine hemochromogen (Fig. 2) of the sulfhemin decomposition product was identical with that of protohemin. The infrared spectra of the sulfhemin decomposition product and of protohemin, shown for comparison as the dashed trace. The sample was a 62-μM solution of protohemin, freshly extracted from Mb at pH 3.2, in 2-butanol, to which were added a few milligrams of solid imidazole.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total radioactivity in sulf-Mb extracted (cpm)</td>
<td>303,000</td>
<td>787,000</td>
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<tr>
<td>Total radioactivity found in organic phase after extraction (cpm)</td>
<td>230,000</td>
<td>565,000</td>
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<tr>
<td>Percent of total input hemin (from both Mb and sulf-Mb) recovered in organic phase</td>
<td>72</td>
<td>84</td>
</tr>
<tr>
<td>Molar ratio of 35S to hemin in organic phase, corrected for the 10% of the hemin derived from unlabeled Mb (9)</td>
<td>0.95</td>
<td>0.80</td>
</tr>
</tbody>
</table>

The decomposed product of sulfhemin was further identified as hemin by paper chromatography (see "Methods"). The decomposition product of sulfhemin from 35S-labeled sulf-Mb co-chromatographed with the protohemin at an Rf of 0.9 relative to the solvent front, along with 20% of the radioactivity, whereas 80% of the radioactivity remained at the origin. This result suggests that the radioactive label was no longer attached to the hemin product, consistent with the identification of the product as protohemin. The sulfur...
The small amount of radioactivity which did travel with the hemin may have been present as a ligand to the iron. Of the radioactivity remaining in the aqueous phase, 60% of the radioactivity (26,000 cpm) remained in the aqueous phase. After extraction with acidified 2-butanone to eliminate the hemin, 80% of the radioactivity (30,000 cpm) remained in the butanone phase. This was confirmed by extraction of the sulfhemin decomposition product from an ammonium bicarbonate solution, at pH 8.4, into acidified butanone. The radioactive species contained 3% which was no longer covalently attached to the hemin after the decomposition of sulfhemin was complete. Therefore the radioactive species was not SO$_4^{2-}$, which would have been largely lost as SO$_2$. After Ba$^{2+}$ precipitation of the SO$_4^{2-}$ (and some SO$_3^{2-}$), the supernatant in the two samples contained 45% and 49% of the radioactivity, respectively, while the precipitates also contained 41% and 39.5% of the radioactivity. Thus, almost half of the radioactive $^{35}$S species was precipitable with Ba$^{2+}$. The evidence suggests that more than one sulfur-containing species is produced in the decomposition of sulfhemin, with approximately one-half ultimately appearing as sulfate.

Both sulfide and elemental sulfur were excluded as products of the sulfhemin decomposition by the following evidence. No radiotracer H$_2$S could be trapped in the gas-trapping experiments described under "Methods," even when the extract containing the sulfhemin decomposition product was acidified with a large excess of HCl or when the original extraction from the protein was performed in the presence of a large excess of H$_2$S as carrier. In the latter case, the decomposition to hemin occurred as rapidly as in the absence of H$_2$S. Control experiments with added radioactive H$_2$S demonstrated that two-thirds of the H$_2$S would have been trapped in these experiments. In all such experiments, 300,000 to 800,000 cpm of radioactivity were used. These results also support the conclusion that sulfide is not a final product of the decomposition. Elemental sulfur was not detected by extraction with CS$_2$ of a bicarbonate solution of sulfhemin decomposition products containing 128,000 cpm. All of the radioactivity remained in the aqueous phase.

**Kinetics of Spontaneous Decomposition of Sulfhemin**

The change in the optical spectrum of sulfhemin as it proceeds to hemin is shown in Fig. 1. The change in absorbance at the maximum at 605 nm as a function of time is shown in Fig. 5. The reaction follows first order kinetics under these conditions, and the half-life of the reaction is about 22 min.

The decomposition of sulfhemin was studied under varying conditions in the same fashion as in the experiment shown in Figs. 1 and 5. Under all conditions studied but one (Experiment 7B of Table II), the reaction was found to follow apparently first order kinetics for at least two half-lives (Table II). Experiment 1 is that shown in Fig. 5. The anaerobic part of Experiment 2 of the table, the extraction was performed with all solutions first flushed with argon and all transfers made with a hypodermic syringe. Under this degree of anaerobic conditions, the rate of decomposition was decreased by a factor of almost 14. We must conclude that a supply of molecular oxygen is necessary for the decomposition. However, less strict anaerobic conditions, obtained by merely flushing the Thunberg cuvette with argon for 2 to 4 min, failed to decrease the decomposition rate from that observed in Experiment 1.

The addition of an excess of HCl did not slow the decomposition reaction (Experiment 3, Table II).

Organic peroxides were found to greatly increase the rate of sulfhemin decomposition. This effect was first suspected when it was observed that different commercial preparations of 2-pentanone produced different decomposition rates (Experiment 4) and that the rate was almost 10-fold lower in 2-butanone alone than in butanone-2-pentanone mixtures made with one preparation of 2-pentanone at the same temperature (Experiment 5). The presence of peroxides in the solvents was detected using the oxidation of 31$^{-}$ to 31$^{-}$ under neutral conditions as a criterion (20). Addition of a neutral KI solution to the first preparation of 2-pentanone was followed by an almost instantaneous development of the bright orange-brown color of iodine, which became progressively darker. In contrast, the 2-pentanone from a second commercial source and the 2-butanone initially developed only a very pale yellow on addition of KI, and the progressive darkening was much slower. We conclude that the first preparation of 2-pentanone contained a substantial concentration of peroxide, whereas the second preparation of 2-pentanone and the 2-butanone contained considerably less. However, the decomposition rate could not be further increased by addition of ferrous ammonium sulfate (Experiment 6), which was found in...
TABLE II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Temperature</th>
<th>Solvent system</th>
<th>Additive or other conditions</th>
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<th>min</th>
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<tr>
<td>1</td>
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<td>None</td>
<td>22</td>
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<td>None</td>
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<td></td>
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<tr>
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<td>5 μl/ml 4 N HCl</td>
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<td>4</td>
<td>4°</td>
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<td>5A</td>
<td>4°</td>
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<td>25°</td>
<td>Butanone alone</td>
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<td>40</td>
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<td>6A</td>
<td>4°</td>
<td>Butanone alone</td>
<td>10 μl/ml 0.1 M Fe(NH₄)₂(SO₄)₃</td>
<td>36</td>
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<td>25°</td>
<td>Butanone alone</td>
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<td>33</td>
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<td>7A</td>
<td>4°</td>
<td>Butanone alone</td>
<td>5 μl t-BuOOH</td>
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<td>8</td>
<td>4°</td>
<td>Butanone alone</td>
<td>0.9 μl t-BuOOH</td>
<td>7.0-5.3</td>
<td></td>
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</table>

* Half-life, determined as in Figs. 1 and 5.
* Lag period of approximately 0.1 of the half-life observed for the subsequent first order process.
* Biphasic kinetics observed (see Reference 14).

Another experiment to greatly decrease the ability of previously treated 2-pentanone to oxidize iodide. Comparison of Experiment 7 with Experiment 5 shows that addition of t-butyl hydroperoxide increased the rate by a factor of 60 relative to that in 2-pentanone alone at 4° when the t-BuOOH was approximately equimolar with the sulfhemin (Experiment 7A), and by a factor of 30 to 40 when the t-BuOOH was present at a 5-fold lower concentration (Experiment 7B). These results confirm the conclusion that the decomposition rate is very sensitively dependent on the concentration of organic peroxide in the system. It is also pertinent to note that even a stoichiometric amount of organic peroxide does not cause immediate decomposition of all of the sulfhemin. The reaction was not complete for at least 20 min.

Decomposition of Sulfhemin by the Action of Mercuric Ion—The sulfur of sulfhemin was also susceptible to attack by Hg²⁺ (Scheme 1). Mercuric acetate was found to convert sulfhemin immediately into a species with the optical spectrum of hematin (λₘₐₓ 640, 540, 510 nm). When the action of Hg(OAc)₂ on an orange solution of protohemin in 2-butanone was examined, the product was also found to have the spectrum of hematin. Shaking the product thus formed with aqueous HCl or with pH 2 glycine·HCl buffer converted the spectrum back to that of orange hemin (λₘₐₓ 640, 540, 510 nm). However, addition of chelating agents such as glycine or EDTA did not affect the spectrum of the hematin product. Addition of HCl alone, without extraction, was found to produce the spectral conversion of the hematin product to hemin. Acetic acid produced no spectral effect either to convert hemin to hematin without Hg²⁺ or to reverse the effect of Hg(OAc)₂. Finally, HgCl₂ was found to be ineffective in converting hemin to the species with the spectrum of hematin. It was concluded that the effect of Hg²⁺ on hemin, which is by definition the chloride salt, was to sequester chloride and so convert hemin to a species with hydroxide (or other oxygen-containing group) as ligand, the spectrum of which would be that of hematin. The ability of chloride to prevent or reverse this effect was specific and did not depend on providing acidity or on chelating the Hg²⁺. The effect of Hg(OAc)₂ on sulfhemin, then, was either to attack the sulfur directly as originally assumed or to remove chloride and convert sulfhemin to a hydroxide form which might then decompose rapidly to hematin. We have already seen above (Table II) that excess HCl does not seem to stabilize sulfhemin against oxidation. Since sulfhemin was decomposed by HgCl₂ in the presence of excess HCl, as well as by Hg(OAc)₂, we conclude that Hg²⁺ probably acts directly on the sulfur of sulfhemin to effect its removal.

DISCUSSION

The evidence presented here provides conclusive proof that only 1 atom of sulfur is incorporated when Mb is converted to sulf-Mb. This confirms the conclusion of Michel for sulf-Hb (5), which was based on small differences in the total content of sulfur between solutions of HbO₂ and those containing sulf-Hb as a minority species, with HbO₂ as the majority species.

Morel et al. (7) found 1.5 to 2 g atoms of radioactive sulfur incorporated per mole of sulf-Hb formed when they used over a 100-fold excess of radioactive sulfide in their preparation. About one-third of this radioactivity precipitated with the protein moiety when the prosthetic group was removed. In the present work, we have prepared sulf-Hb in high yield, using only a 1.67-fold excess of radioactive sulfide, and we found that precisely 1 atom of radioactive sulfur was incorporated. Moreover, on extraction of the prosthetic group of [³⁵S]sulf-Mb into 2-butanone, more than 85% of the labeled ³⁵S was co-extracted with the prosthetic group. This result is consistent with the structures proposed by Morel et al. (7) and by ourselves (9) for the prosthetic group of sulf-Mb in which a sulfur atom is added to one or both of a pair of β-carbon atoms of a pyrrole ring of the porphyrin, either as a thiol group plus hydrogen or as a three-membered ring episulfide, resulting in the saturation of the β-β double bond to produce a chlorin type structure.
In 1961, Nicholls (6) prepared sulf-Mb by titrating the myoglobin peroxide compound, MbIV, with H2S, and concluded that 1 mole of H2S could produce 1 mole of ferric sulf-Mb, although excess H2S was necessary for reduction of this compound to the ferrous form.

In contrast to the titration data of Nicholls (6), we have found that only 1 mole of sulfide reacts with 1 mole of MbIV (the higher oxidation state of Mb formed when ferric Mb reacts with H2O2) to produce ferrous sulf-Mb, rather than ferric sulf-Mb, as the initial product. To account for Nicholls' observations, we suggest that an oxidoreduction reaction between the excess MbIV and the ferrous sulf-Mb gave rise to ferric Mb and the ferric sulf-Mb which he observed. This stoichiometry is more consistent with the mechanism suggested by Nichol et al. (21) for the preparation of ferrous sulf-Hb from HbO2 via a cyclic process involving the reduction of HbO2 to HbIV by phenylhydroxylamine and not involving the formation of ferric sulf-Mb. This is also consistent with Nicholls' (6) observation that ferrous sulf-catalase is the initial product of the reaction between the oxidation state IV derivative of catalase (Compound II) and H2S. It also eliminates the question of the residual reducing equivalent which would be formed in Nicholls' mechanism (6).

These findings and the stoichiometry of radioactive sulfur incorporation suggest the following mechanism for the formation of sulf-Mb.

\[
\text{Globin-(Protoheme Fe-H2O)IV} + \text{H}_2\text{O} \rightarrow \text{ferric Mb}
\]

\[\text{Globin-(Protoheme Fe-H2O)IV} + \text{H}_2\text{O} \rightarrow \text{MbIV} + \text{1 oxidizing equivalent}\]

\[\text{Globin-(Protoheme FeOIV) + H2S} \rightarrow \text{MbIV} + \text{Sulfhemin FeII} + \text{HO}^-
\]

In the reaction of Equation 1, ferric Mb is oxidized by H2O2 to MbV (22), which subsequently decomposes spontaneously to form MbIV (21). In the reaction of Equation 2, the higher oxidation state derivative MbIV reacts with 1 mole of H2S, and an atom of sulfur is incorporated into the porphyrin moiety of the prosthetic group to form the chlorin of sulfhemin. The iron is left in the ferrous oxidation state, and is five coordinated like that of deoxy-Mb (12). At pH 8, most of the sulfide is in the form HS\(^{-}\), since the p\(K_a\) is 7.04 (23). Thus, the reaction may be viewed as the nucleophilic attack of HS\(^{-}\) on a \(\beta\) carbon atom of a pyrrole. Both reducing equivalents of the sulfide ultimately are transferred to the iron-ligand complex, to reduce (Heme FeO)IV to Heme Fe\(^+\) and \(\cdot\)OH. Thus, in analogy with the mechanism of Nichol et al. (21), one may speculate:

Here a charge on the \(\beta\) carbon of a pyrrole is stabilized by its allylic position. The oxidation-reduction stoichiometry of this mechanism is more consistent with the given epiporphyrin structure preferred by Morell et al. (7) than with a structure containing a thiol, as the thiol would retain one of the reducing equivalents of H2S. From a knowledge of the exact structure of sulfhemin, it will be possible to deduce with more certainty the subsequent steps in the molecular mechanism of the reaction.

All known cases of sulfhemate protein formation involve the intermediate higher oxidation state IV derivative of the molecule. Thus, Nichol et al. (21) have shown that the formation of sulf Hb in vivo in cases of intoxication by the analgesic \(p\)-aminophenol derivatives involves reduction in the red cell of HbO2 to HbIV, which then either reacts with sulfide to produce sulf-Hb or is reduced further to met-Hb. Furthermore, the in vitro synthesis of sulf-Hb from Hb and H2S was shown by Keilin (4) to require \(O_2\), so that probably here, too, HbO2 was first reduced by H2S to HbIV before reaction with sulfide to form sulf-Hb. Also, sulf-catalase has been shown to be formed from catalase Compound II, the oxidation state IV derivative, and H2S (6). The necessity of a higher oxidation state intermediate in the formation of sulfhemate derivatives of these heme proteins suggests that the decrease in electron density of the porphyrin system in the higher oxidation state renders the porphyrin ring more susceptible to nucleophilic attack by HS\(^{-}\).

In the present study we have also provided an explanation for the failure of part of the radioactivity in the experiments of Morell et al. (7) to follow the prosthetic group through a series of extractions. Sulfhemin, the prosthetic group of sulf-Mb, is found to be unstable once extracted from the protein. It decomposes to protohemin and what appears to be a mixture of sulfur-containing small molecules, including oxo-anions of sulfur. Significant quantities of HS, SO (or SO\(^{2-}\)) and elemental sulfur have been ruled out as products, whereas about 50% of the sulfur-containing product is SO\(^{2-}\). Under the conditions obtaining, with abundant \(O_2\) and peroxides present in the organic solvent, it is not unlikely that an initial sulfur-containing product of sulfhemin decomposition was further oxidized to sulfate (24).

The kinetics of decomposition of sulfhemin to hemin have been shown to be pseudo first order under all conditions studied. The rate is dependent on both the availability of \(O_2\) and the concentration of peroxides in the organic solvents used for extraction. These data suggest a free radical type autoxidation process in which organic hydroperoxides are required at least in the initiation process (25). Over-all first order kinetics in the reactant conditions where the rate was decreased by the absence of high initial peroxide concentrations but not where the rate was decreased by exclusion of \(O_2\) (Table II).

Sulfhemin can also be converted to hemin by the action of the thiophilic cation Hg\(^{2+}\) and probably by the action of the thiophilic amion CN\(^{-}\). In both cases the sulfur is removed from the molecule, presumably to form the stable complexes HgS and NCS\(^{-}\), respectively. The remaining porphyrin structure is probably left intact as judged by the optical spectra of hemin or hematin observed for the product.

Recently, we have demonstrated that in the intact protein, sulf-MbCO can be reconverted to MbCO in a photochemical process (26). This is the only mode of reversion known which
does not involve addition of an oxidation-reduction agent or a thiophilic compound. Nicholls (6) reported that \( \text{H}_2\text{O}_2 \) decomposed sulf-Mb to MbO\(_2\). Several other processes in which sulf-Hb has been found to revert to Hb (27) can be attributed (26) to the generation of peroxide in the reaction. These observations can now be readily understood in the light of the present results on the oxidative decomposition of sulfhemin in organic solvents. In fact, it becomes surprising that sulfhemin does not oxidize more readily in the intact protein sulf-Mb. That it is so stable in sulf-Mb bespeaks its presence in a sheltered environment in the protein.

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Jay A. Berzofsky, J. Peisach and B. L. Horecker


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