Denatured Hemoglobin in Sickle Erythrocytes

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ABSTRACT To study the nature of numerous inclusion bodies seen in red cells from patients with sickle cell disease (Hb SS), we have prepared red cell ghosts free of oxyhemoglobin and analyzed them by spectrophotometric and heme extraction methods. The absorption spectrum in the visible region of the ghost suspensions was typical of hemichromes. The spectrum was similar to that of denatured hemoglobin prepared by treatment of oxyhemoglobin S with mechanical shaking or heat. Similar treatment of cells containing only normal hemoglobin (Hb AA) showed a very small amount of denatured hemoglobin, approximately one-fifth of the amount in Hb SS cells. The amount of denatured hemoglobin determined after solution of membrane with 2.5% sodium dodecyl sulfate was 0.158±0.070% (1 SD) of the total cellular heme in Hb SS patients. In controls, the amount was 0.030±0.016%. Persons with Hb AA and reticulocytosis did not have an elevated amount of membrane-associated heme. In patients with hereditary spherocytosis and autoimmune hemolytic anemia, denatured stromal hemoglobin was normal or slightly elevated before and after splenectomy. The increased amount of denatured hemoglobin in Hb SS red cells may be related to the instability of sickle oxyhemoglobin.

INTRODUCTION

In hemolytic anemia due to unstable hemoglobins, inclusions of denatured hemoglobin are commonly found in circulating red cells, particularly after splenectomy. The shortened red cell survival in these disorders is due in part to increased rigidity of cells containing inclusions (1) and to membrane damage associated with intracellular precipitation of hemoglobin (2). Numerous small inclusions are also commonly seen in red cells from patients with sickle cell disease under special conditions, either by phase-contrast microscopy with high-intensity illumination or after acid elution of adult hemoglobin from cells (3). Sickle hemoglobin in the oxygenated configuration is unstable under special conditions of mechanical shaking or stirring, or with repeated cycles of oxygenation and deoxygenation (4, 5). The present study deals with our attempts to determine the nature and amount of denatured hemoglobin in red cell membrane fractions from patients with sickle cell disease and normal subjects after complete removal of oxyhemoglobin.

Received for publication 19 April 1976 and in revised form 14 December 1976.

METHODS

Patients. Patients with sickle cell disease ranged in age from 3 to 19 yr old. Blood was drawn at the time of a routine clinic visit and not during crisis or infection. Only one of these patients had a surgical splenectomy. The patients with hereditary spherocytosis and autoimmune hemolytic anemia were children 4–6 yr in age. The controls were mostly adult laboratory personnel, but also included several children with intact spleens.

Absorption spectra of red cell stromata prepared by various methods. Blood was drawn into heparin and red cells were washed three times with 0.85% saline solution and were centrifuged. 0.2-ml aliquots of washed red cells were hemolyzed with 40 ml of either (*a*) water, (*b*) 0.5 mM EDTA, 5 mM potassium phosphate buffer, pH 7.5, (*c*) 10 mM potassium phosphate buffer, pH 7.5, or (*d*) 20 mM potassium phosphate buffer, pH 7.5, to determine the best solution for removing native oxyhemoglobin from the stromal fraction. The hemolysate was centrifuged at 15,000 *g* for 20 min at 4°C. The stromata were washed four more times with the same buffer used for initial hemolysis. The washed stromata were resuspended in 4 ml buffer and the absorption spectra were recorded with an Aminco DW-2 UV-VIS spectrophotometer (American Instrument Co., Inc., Silver Springs, Md.). The conversion of oxyhemoglobin to hemichrome during the washing procedure was proven to be negligible by measuring the total oxyhemoglobin before and after the washing procedure.

Comparison of spectra of stromata after successive washes. The purpose of this series of experiments was to study the spectral changes of red cell ghosts as native oxyhemoglobin was eluted with successive washes. 1 ml of packed erythrocytes from normal controls or patients with sickle cell disease was hemolyzed by adding 19 ml of phosphate-EDTA buffer. After centrifugation at

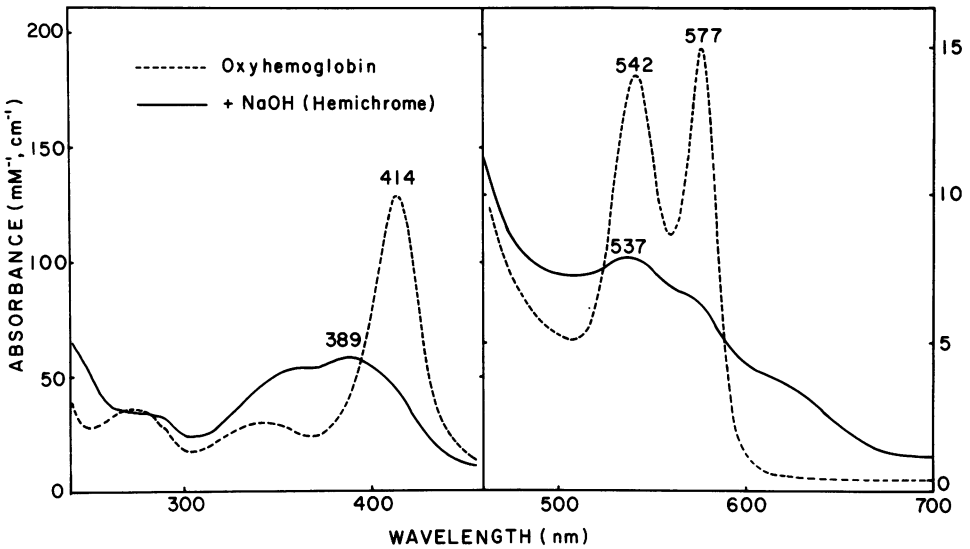


FIGURE 1 Absorption spectra of oxyhemoglobin and hemichrome. The hemichrome spectrum was obtained after treatment of 3 ml of oxyhemoglobin S with 10 μl of 4 N NaOH solution.

15,000 g for 20 min, 17 ml of the supernatant solution was carefully removed (supernate$_I$). The absorption spectra of this supernatant fraction were recorded between 250 and 700 nm with a Perkin-Elmer-Coleman 124 double beam spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) after appropriate dilution. The remaining 3 ml of hemoglobin and stroma mixture were gently homogenized manually with a Teflon Potter-Elvehjem homogenizer. A 0.1-ml aliquot of this mixture was resuspended in 2.4 ml of the phosphate-EDTA buffer with a Potter-Elvehjem homogenizer. Care was taken not to produce foam, which might lead to artificial precipitation of hemoglobin. The absorption spectra of this stromal suspension (stroma$_I$) were measured between 250 and 700 nm with an Aminco DW-2 UV-VIS spectrophotometer. The rest (2.9 ml) of the original stromal mixture was diluted with 17.1 ml of the phosphate-EDTA buffer and the procedure was repeated five more times to produce stromal fractions II–VI.

Heme concentrations in the supernatant solutions which contained mainly oxyhemoglobin were calculated by the use of the millimolar extinction coefficient of $mE_{577} = 15.0$ mM^{-1} cm^{-1}. In stromal fractions which contained both the oxyhemoglobin and hemichrome forms, the total heme concentrations were calculated from the optical density at the isosbestic points (the points of interaction) between the spectra of oxyhemoglobin and hemichrome (Fig. 1). The millimolar coefficients used were $mE_{525} = 7.8$ and $mE_{588} = 4.83$. The concentrations of heme were obtained by averaging the calculations from the two points. This method was used primarily to follow the elution of oxyhemoglobin from suspensions of stromata, and to confirm the presence of hemichrome and the absence of oxyhemoglobin in the final stromal preparation. Although the values obtained by this method with individual samples agreed within 10% experimental error on 10 duplicate or triplicate experiments (Table I), the heme concentrations determined by this method were always higher than those obtained by the extraction method described below because of a slight elevation of the base line due to turbidity. The extraction method was therefore used for all final quantitative determinations of heme content.

To determine if the hemichromes in the membrane fraction were reversible, dithionite was added and carbon monoxide was bubbled through the suspension. The spectrum was unchanged after this treatment, indicating that the hemichromes were of the irreversible type. The fractions of oxyhemoglobin in the mixture of oxyhemoglobin and hemichrome were calculated by the following equations:

$$\text{oxy-Hb (\%)} = \frac{x_1 - 0.795}{1.128} \times 100,$$

where $x_1 = E_{577}/E_{525}$.

The percentage of total proteins in the stromata was expressed as:

$$\text{stromal protein (\%)} = \text{stroma}_n E_{280}/\text{total } E_{280},$$

where n is the number of washings.

$$\text{stroma}_n E_{280} = E_{280} \times 3 \times \frac{2.4}{0.1} \times F_n, \text{ and}$$

$$\text{total } E_{280} = (\text{supernate}_I E_{280} \times 17) + \text{stroma}_I E_{280}.$$

Corrections were made for the loss of 0.1-ml suspensions out of 3-ml ghost suspensions used for spectrophotometer analysis, i.e., $F_n = (3.0/2.9)^{n-1}$, where F_n is the correction factor for losses due to sampling for analysis.

The absence of oxyhemoglobin in the stromata was confirmed by measuring the absorption spectra of the suspensions with an Aminco DW-2 UV-VIS spectrophotometer. When oxyhemoglobin remains in the stromata, the spectrum shows typical absorption peaks of oxyhemoglobin at 542 and 577 nm.

Heme content of red cell stromata. The following extraction method was used for quantitative determination of heme in the preparation of washed stromata. 0.5-1.0-ml aliquots of washed erythrocytes were hemolyzed by mixing with 40 ml of 5 mM of potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA. After standing for 5 min, the hemolysate was centrifuged at 15,000 g for 20 min.

The stromata were washed two more times with the same buffer. The pellets were washed once more by mixing first with 5 ml distilled water and then with 35 ml of the phosphate-EDTA buffer. The supernatant solutions were combined. After confirmation of the absence of oxyhemoglobin by analysis of spectra, the stromata were mixed with 2.3 ml of 2.5% sodium dodecyl sulfate (SDS)[1] solution, and left at room temperature overnight. Although foaming due to SDS can be controlled by gentle handling of the solution, the foaming can be effectively prevented by the addition of 1–2 ppm of SAG 10 (10% dimethylpolysiloxane emulsion, Union Carbide Corp., New York). The heme content of the stromata was determined by the absorbance at 404 nm of the resulting solution using 2.5% SDS as a blank. Millimolar extinction coefficients for denatured hemoglobin were derived from oxyhemoglobin S precipitated by mechanical shaking (4, 5). The precipitate is known to have a hemichrome spectrum (5). The precipitate was gently dissolved in 2.5% SDS solution. The millimolar extinction coefficients of heme in 2.5% SDS are $E_{404} = 90.8$, $E_{600} = 6.5$ cm^{-1}, mM^{-1}, and $E_{418} = 83.6$ and $E_{542} = 11.5$ cm^{-1}, mM^{-1} in the presence of KCN. The heme content in the supernate was measured as oxyhemoglobin.

The heme content in the stromata was expressed as a fraction of total heme in erythrocytes: stromal heme (%) = (stromal heme)/(supernatant heme and stromal heme) × 100.

RESULTS

Absorption spectra of red cell stromata. The amount of hemoglobin remaining in red cell ghosts depends on the concentration of the buffer with which cells are treated (6, 7). Dodge et al. (7) reported that retention of hemoglobin is increased by hemolyzing the cells with phosphate buffer on concentrations below 5 or above 20 mosmol. Our similar experiments with sickle erythrocytes confirm these results. A considerable amount of hemoglobin was retained when the cells were hemolyzed with water or with phosphate buffer at 10 mM or higher (Fig. 2A). The maximum hemolysis was obtained with 5 mM potassium phosphate buffer containing 0.5 mM EDTA. The hemoglobin retained in ghosts after repeated washings with water or with 20 mM phosphate buffer was found to be mainly in its oxyform as demonstrated by the prominent peaks at 542 and 577 nm in the absorption spectra of the ghost suspensions (Fig. 2A). In contrast, the red cell ghosts prepared with 5 mM phosphate buffer containing 0.5 mM EDTA showed mainly the spectrum of hemichrome. Hemichromes are derivatives of hemoglobin and other hemoproteins in which the sixth coordination position of heme-iron is bound to a nitrogen atom on the imidazole ring of histidine in position E7. Hemichromes are distinguishable by characteristic absorption spectra (8). Suspensions of precipitated hemoglobin prepared by mechanical shaking (4, 5) or by heating also show similar spectra (Fig. 2B).

There are distinct differences in the visual appearance of red cell ghosts prepared by the different methods of lysis. The pellets prepared with water exhibited a homogeneous pink color indicating the equal retention of oxyhemoglobin in each cell, whereas the pellets prepared with 10 or 20 mM phosphate buffer clearly showed two components: white ghosts and red erythrocytes. The pellets prepared by 5 mM phosphate containing 0.5 mM EDTA had two components of dark brown and white, the ratio depending on the amount of denatured hemoglobin in the stromata.

Comparison of spectra of stromata from normal and sickle erythrocytes. Absorption spectra of suspensions of stromata from normal and sickle erythrocytes at each washing step are compared in Fig. 3A and B. Initially the suspensions exhibit the spectrum of dissolved oxyhemoglobin. Upon repeated washings the fractions of hemichrome gradually increase as noted by the disappearance of the two sharp peaks of oxyhemoglobin at 577 and 542 nm and by the appearance of a hemichrome spectrum similar to that shown in Fig. 3A and B. The ratio of the absorbance at 280 nm to that at the Soret peak (414 nm) also increases as soluble oxyhemoglobin is washed out. After washing three to four times, these ratios become constant and the spectrum is mainly that of hemichrome.

The heme content, optical density at 280 nm, and fraction of hemichrome comparing total hemoglobin in $stroma_I$–$stroma_{VI}$ are illustrated in Fig. 4. The

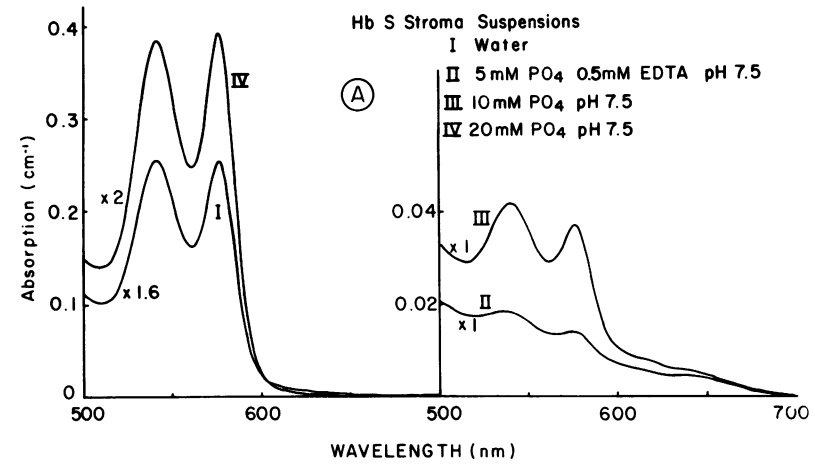
TABLE I

Reproducibility of Spectrophotometer Measurements of Membrane-Associated Denatured Hemoglobin *

Patient number	Patient type	Denatured Hb	Mean	Difference between high and low values
		%		
1	AA	0.10, 0.10, 0.11	0.10	0.01
2	AA	0.050, 0.053	0.058	0.003
3	AA	0.077, 0.080	0.079	0.003
4	AA	0.116, 0.116	0.116	0
5	SS	0.63, 0.67, 0.69	0.66	0.006
6	SS	0.65, 0.69	0.67	0.04
7	SS	0.42, 0.39	0.41	0.03
8	SS	0.54, 0.59	0.57	0.05
9	Spherocytosis	0.031, 0.031	0.031	0
10	Köln	1.28, 1.32	1.30	0.04

* The percentage of denatured hemoglobin was determined from spectra of washed membranes as described in Methods.

[1] *Abbreviations used in this paper*: ISC, irreversible sickle cells; SDS, sodium dodecyl sulfate.



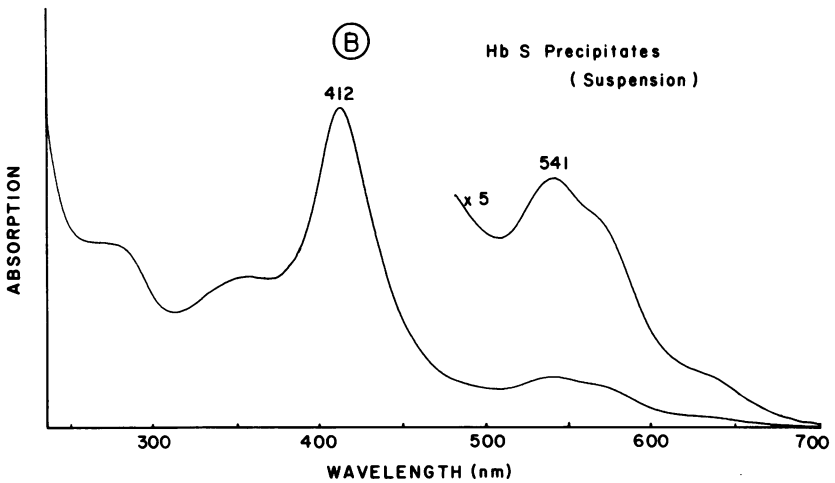
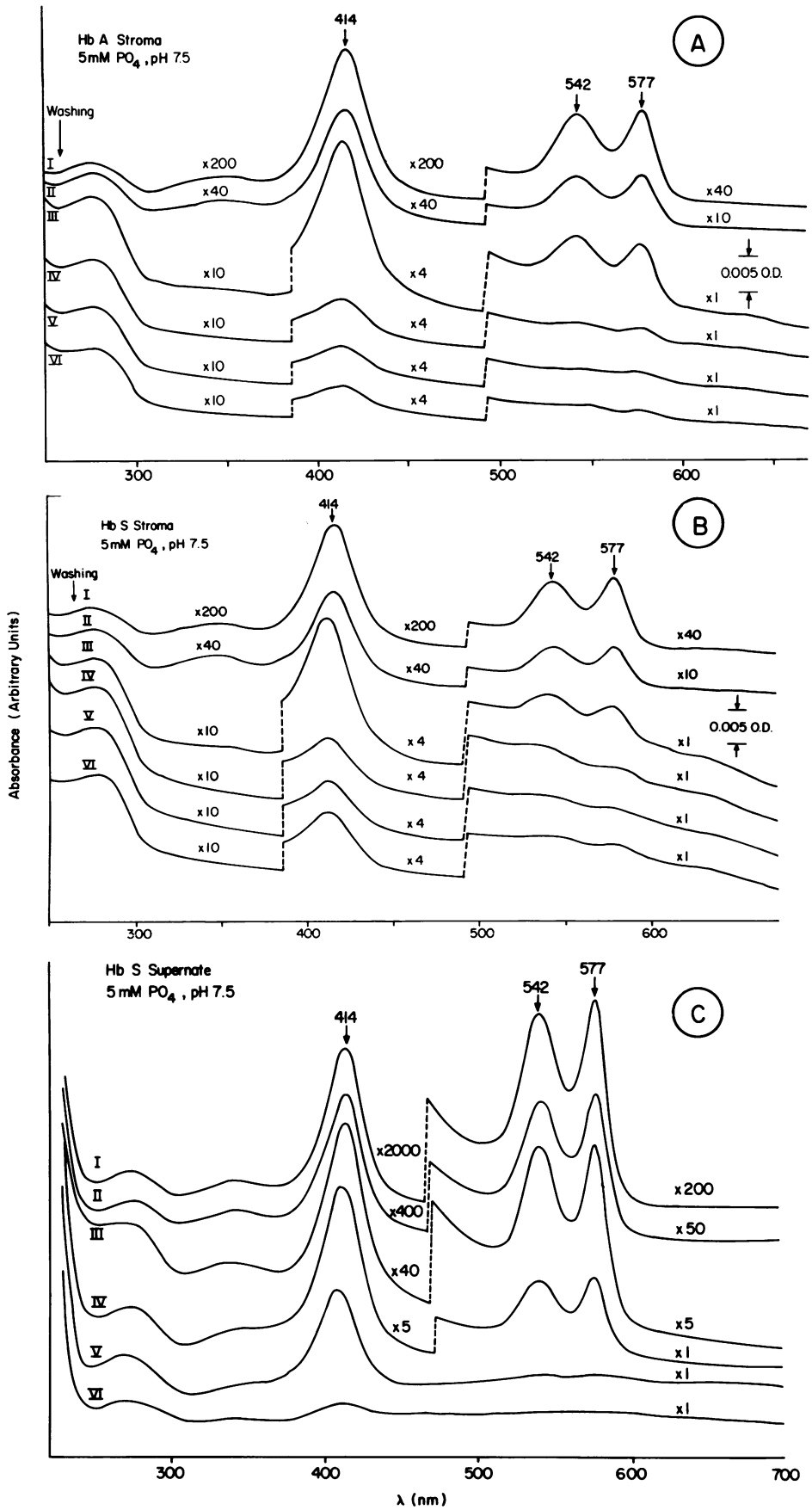


FIGURE 2 (A) Absorption spectra of stromal suspensions prepared by various methods. Note the oxyhemoglobin peaks at 542 and 577 nm in suspensions I, III, and IV. The spectrum of suspension II is mainly that of hemichrome. (B) Absorption spectrum of suspensions of hemoglobin S precipitate. A precipitate of oxyhemoglobin S by mechanical shaking was resuspended in 0.1 M potassium phosphate buffer, pH 7.5. The spectrum of the suspensions was measured with an Aminco split-beam spectrophotometer. The spectrum is characteristic of hemichrome (8).

data shown are from one example of 10 experiments in which the full calculations were done for at least three spectral measurements of stromata (samples after washes, e.g., I, III, VI) in experiments in which A and S cells were compared on the same day. In these studies, the mean A_{280}/total A_{280} (%) value for Hb AA cells in washes IV–VI was 1.7 ±0.2 (1 SD) (12 determinations), and the value for Hb SS cells was 3.4±0.55 (24 determinations). Comparison by a paired value t test gives a significant difference ($P < 0.001$), as does a comparison of the means by the t test ($P < 0.001$). In four sets of these studies done on the same blood sample, the mean value of A_{280}/total A_{280} for stromata IV–VI was 3.4±0.32. The relationship of A_{280}/total A_{280} to hemichrome is shown in Fig. 4, with values calculated from spectra (white bars in lower part of figure). As the red color disappears from the stromata

FIGURE 3 Absorption spectra of stromal suspensions and supernatant solutions at various washing steps. (A and B) Stromal suspension of Hb A and Hb S. (C) Supernatant washing solutions of Fig. 3A. The washing procedures are described in the text. Note that only of oxyhemoglobin is washed out.


Hb A Stroma
5mM PO4, pH 7.5
A
414
542
577
Washing
I
II
III
IV
V
VI
x200
x40
x10
x4
x1
0.005 O.D.
Hb S Stroma
5mM PO4, pH 7.5
B
Hb S Supernate
5mM PO4, pH 7.5
C
x2000
x400
x50
x5
300
400
500
600
700
Absorbance (Arbitrary Units)
λ (nm)

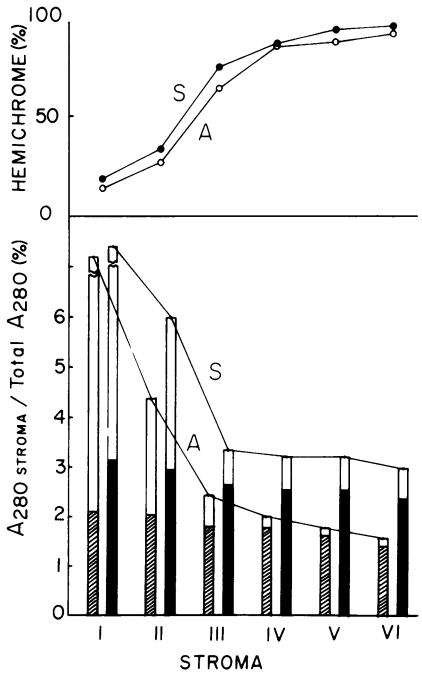


FIGURE 4 Optical properties and hemichrome content of suspensions of stromata at various washing steps. Calculations were made as shown in the text. The top panel, the percentage of hemichrome in total hemoglobin associated with stromata is shown for a patient with sickle cell disease (S) and a normal control (A). Note that the relative amount of hemichrome increases as oxyhemoglobin is washed out of stromata.

The bottom panel, the percentage of proteins in the stromata (A_{280} stroma) compared with the total protein in red cells (A_{280} Total), is shown for successive washing steps. The height of each column represents the percentage. The columns with a cross-hatched base show results from normal cells (A), whereas those with a solid base are from an experiment with cells from a patient with sickle cell disease (S). The clear bars were the expected E_{280} due to hemoglobin calculated on the basis of heme absorption at the Soret peak and represents the fraction of protein associated with heme (oxyhemoglobin and hemichrome). The cross-hatched or solid bars are the sum of membrane protein and hemefree globin.

by repeated washings, the fraction of hemichrome gradually increases. This is clearly due to the washing out of oxyhemoglobin as seen in the successive spectra of the supernatant solutions (Fig. 3C). Although it is not known if any soluble stromal protein is washed out during washing, there is no detectable loss of denatured hemoglobin in the supernate. After five to six washings, the stromata showed the dark brown color of hemichromes.

The absorption at 280 nm for sickle stromata is higher than that of normal stromata, indicating an increased protein content (Fig. 4). In $stroma_{VI}$ of normal cells, about 10% of the absorption at 280 nm is accounted for by denatured hemoglobin if we assume that there is no heme-free globin. Therefore, about 90% of this absorption may be attributed to membrane protein. In $stroma_{VI}$ of sickle cells, the percentage of denatured hemoglobin is at least three times the amount in normal cells. However, the increased stromal A_{280}/total A_{280} cannot be totally accounted for by the increased fraction of hemichrome. The remaining protein in sickle erythrocytes' stromata must therefore be either heme-free globin or increased membrane protein.

Denatured hemoglobin content in normal and sickle cells. The amounts of denatured hemoglobin were determined as total heme by dissolving the ghosts in 2.5% SDS solutions after removal of oxyhemoglobin. The results for red cells from patients with sickle cell disease, hereditary spherocytosis, autoimmune hemolytic anemia, and from controls are shown in Fig. 5.

In sickle erythrocytes, denatured hemoglobin was 0.158±0.070% (1 SD) of the total hemoglobin (range 0.080–0.300). The highest values were in the two oldest patients (19 yr old) and in an 11-yr-old girl splenectomized three yr before this study. A group of children with hereditary spherocytosis and autoimmune hemolytic anemia was also studied to evaluate the effect of reticulocytosis or splenectomy on

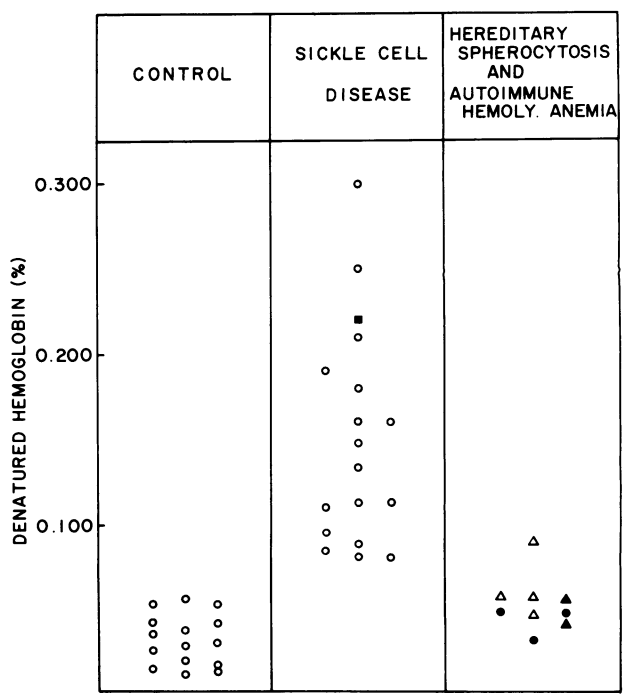


FIGURE 5 Comparison of the amounts of membrane-associated denatured hemoglobin in various patients and normal subjects. Sickle cell disease after splenectomy (■), spherocytosis before (●) and after (△) splenectomy, and autoimmune hemolytic anemia after splenectomy (▲).

denatured hemoglobin. Before splenectomy, the values were 0.030, 0.047, and 0.048, whereas in the splenectomized group the mean was 0.063±0.018 (range 0.041–0.090). The control group which included normal persons, several children with hematologic disorders other than hemoglobinopathies or hereditary spherocytosis, and one splenectomized child had a mean of 0.030±0.016 (range 0.010–0.055). To determine the amount of denatured hemoglobin in irreversible sickle cells (ISC), ISC were prepared artificially by incubating sickle erythrocytes under anaerobic conditions for 24 hr (9). Although more than 70% cells were ISC, the increase in the amount of denatured hemoglobin in these cells was negligible.

DISCUSSION

Although many previous investigations (6, 7, 10–13) have been concerned with the interaction of hemoglobin and the red cell membrane, little is known about the nature of hemoglobin in red cell ghosts. We have shown that there are at least two distinct types of hemoglobin associated with red cell ghosts. One is hemichrome-type denatured hemoglobin and the other is oxyhemoglobin. If the amount of hemoglobin in ghosts is determined as total heme, either by the pyridine hemochromogen method (7) or the cyanomethemoglobin method (1), the amount of hemichrome-type denatured hemoglobin cannot be distinguished from that of oxyhemoglobin remaining in the stromata. For this reason, the amount of hemoglobin on ghosts measured by these methods fluctuates with the variation in amount of oxyhemoglobin associated with the membranes due to differences in methods of lysis and washing. The amount of hemichrome (denatured hemoglobin) should be measured after confirmation of the absence of oxyhemoglobin by the direct measurement of suspensions of ghosts with a split-beam spectrophotometer. Since the retention of oxyhemoglobin varies largely with washing conditions, including ionic strength and pH, the binding of oxyhemoglobin to membranes appears to be nonspecific. On the other hand, the hemichrome-type denatured hemoglobin in red cell ghosts remains bound to membranes with washing, and varies in amount in different disorders. The spectrum of this hemoglobin is similar to that of precipitated hemoglobin formed by mechanical shaking or heat treatment (Fig. 2B), and represents a truly denatured, nonfunctional molecule. In the process of denaturation, hemoglobin forms two types of hemichromes, reversible and irreversible (8). The former can be converted to deoxyhemoglobin by reduction of hemichrome followed by anaerobic dialysis, whereas the latter cannot be renatured to oxyhemoglobin. The hemichromes in the stroma fraction in the present study were found to be irreversibly denatured.

Schneider et al. (3) observed Heinz bodies (intraerythrocytic aggregations of denatured hemoglobin) in erythrocytes obtained from patients with sickle cell disease. They measured the hemoglobin content of membranes after hemolyzing sickle cells with water and found approximately 10% of the total hemoglobin in the ghost fraction in sickle erythrocytes in contrast to 2.6% in normal cells. The present studies indicate that most of the remaining hemoglobin after lysis with water was in the oxyform (Fig. 2A) and did not represent a constant or specific form of membrane-associated hemoglobin. In the present study the amount of denatured hemoglobin in fresh erythrocytes from patients with sickle cell disease was only about 0.2% of the total, approximately five times higher than that in normal cells. It is more likely that the hemichrome-type denatured hemoglobin discussed in this paper is the type seen as red cell inclusions, because the spectrum of oxyhemoglobin changes to that of hemichrome with a variety of methods of precipitation, and also because hemichromes were found in the form of Heinz bodies due to the presence of excess hemoglobin subunits (14) or unstable hemoglobins (15). Although the amount of denatured hemoglobin in normal and sickle erythrocytes with respect to the total hemoglobin in erythrocytes is small, the amount with respect to structural protein is large. Approximately 5×10^5 hemoglobin molecules are denatured per single sickle erythrocyte. The amount of denatured hemoglobin in a sickle erythrocyte corresponds to its total structural protein. Since inclusion bodies cause rigidity in erythrocytes, the spleen possibly detects this change and retains these cells.

Increased intracellular denaturation of hemoglobin in sickle cell disease does not seem to be solely due to reticulocytosis or the absence of splenic function. Patients with reticulocytosis in this study did not have increased denatured hemoglobin, and those who were splenectomized had only a slight or no increase above the control group (Fig. 5). The validity of the methods used in this study in detecting intracellular precipitation of oxidized hemoglobin is indicated by 5–10-fold greater values than normal found in red cells from patients with Hb Köln, Hb H disease, or severe glucose-6-phosphate dehydrogenase deficiency.[2]

The mechanism of hemoglobin precipitation inside erythrocytes containing Hb S is not known. It cannot be due to the gas-liquid surface denatura-

[2] Asakura, T., K. Adachi, and E. Schwartz. Unpublished observations.

tion which occurs during mechanical shaking. Repeated passages of erythrocytes through very narrow capillaries might cause sufficient agitation of oxyhemoglobin to result in precipitation. Another possibility is the repetition of conformational changes between oxy- and deoxyhemoglobin S during circulation. Experiments in our laboratory have shown that hemoglobin S denatures at a faster rate than does hemoglobin A during repeated changes of the gas phase from air to nitrogen. The conformational changes between the two states may provide sufficient molecular agitation to cause precipitation.

The cause of most vaso-occlusive crises in sickle cell disease, and the differences in frequency of crises and severity of disease between patients, is not clearly understood. Increased precipitation of sickle hemoglobin inside the erythrocytes may be a contributing factor to the initiation of crises and chronic hemolysis. Intraerythrocytic precipitation of hemoglobin would cause rigidity of red cells and would contribute to stasis, sickling, and vaso-occlusion. The confirmation of this hypothesis requires extensive studies of many patients with sickle cell disease to determine the relationship between the amount of intraerythrocytic denatured hemoglobin and the clinical status of the patients.

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