Studies on Abnormal Hemoglobins

VIII. The Gelling Phenomenon of Sickle Cell Hemoglobin: Its Biologic and Diagnostic Significance

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HARRIS\(^{1}\) demonstrated that concentrated stroma-free hemolysates prepared from sickle cell anemia bloods are very viscous when the sickle cell (S)* hemoglobin is present in the reduced state. In such deoxygenated hemoglobin solutions tactoid formation can be visualized by phase microscopy.\(^{1, 6}\) The term “tactoid” refers to long, thin, spindle or boat shaped particles, whose molecules have an orderly arrangement.\(^{7}\) Only when the reduced S hemoglobin solution is sufficiently concentrated (above 10 Gm. per cent) do the intermolecular forces tend to become operative in producing such orientation and aggregation of the pigment molecules.\(^{1}\) The S hemoglobin tactoids have a striking resemblance to sickled erythrocytes.\(^{1}\) Since the hemoglobin concentration within the red cells is about 34 per cent, Harris concluded that a sickled erythrocyte represents essentially a “hemoglobin tactoid, thinly veiled and somewhat distorted by the cell membrane.”\(^{1}\)

When S hemoglobin solutions of sufficiently high concentrations are exposed to a continuous stream of CO\(_2\) gas to maintain the pigment in the reduced state, the hemolysates gel.\(^{1, 6}\) This gelling phenomenon is specific for sickle cell hemoglobin and cannot be obtained with any other type, either normal or abnormal, of human hemoglobin in the absence of S hemoglobin.\(^{6, 8}\) When such an S hemoglobin gel is reoxygenated, liquefaction takes place within a short period of time.\(^{6}\) The lowest S hemoglobin concentration of a hemolysate at which the gelling phenomenon can still be elicited may be designated as its lowest gelling point.

It was noticed in our laboratory that hemoglobin solutions prepared from sickle cell trait erythrocytes also exhibit gelling of the entire specimen under appropriate experimental conditions. This observation is of particular interest when it is realized that the S hemoglobin content of “trait” hemolysates amounts to only 20 to 50 per cent of the total pigment.\(^{9, 10}\) The remainder consisting of normal adult (A) hemoglobin. The S hemoglobin concentrations of sickle cell

\(^{*}\) At a recent symposium on abnormal hemoglobins,\(^{5}\) it was suggested that the following symbols be used: A for normal adult, F for fetal, S for sickle cell, and C for type III.\(^{1, 4}\) Hemoglobin, D was proposed for a rare type of abnormal hemoglobin which moves electrophoretically like S hemoglobin, but does not cause sickling when present in erythrocytes.\(^{5}\)
Several questions immediately present themselves in the light of these observations: (1) Does the lowest gelling point of a “trait” hemolysate differ from that of an “anemia” hemolysate? (2) Since the entire “trait” hemolysate gels—provided that the pigments are completely deoxygenated—is this indicative of an interaction (polymerization) between the S and A hemoglobin molecules? (3) Assuming that such an interaction between these two types of hemoglobin can readily be demonstrated, does the tendency of S hemoglobin to combine with other human hemoglobins (F, C, or D) differ characteristically for each distinct type of pigment? This paper reports experiments designed to deal with these questions.

Material and General Methods

All blood specimens used in this study were first examined with the routine hematologic procedures. The sickling phenomenon was demonstrated by means of the sodium metabisulfite method. Only patients with a marked normochromic normocytic anemia, an elevated reticulocyte count, and numerous sickled cells in the blood films were diagnosed as having typical sickle cell anemia.

From each blood specimen a stroma-free hemolysate was prepared and examined for the presence of fetal (F) hemoglobin with the alkali denaturation technic previously described. With this procedure, normal adult (A), sickle cell (S), and type C hemoglobin are completely destroyed within one minute. The concentration of the resistant F pigment is expressed in per cent of the total hemoglobin and is called the “one minute denaturation value.” This value represents the absolute amount of fetal hemoglobin minus the small quantity of this pigment which has also been denatured during the one minute exposure to the alkali test reagent. The total amount of F hemoglobin can be determined by means of fractional denaturation or, much simpler, by adding 5 per cent of the one minute denaturation value to the latter. In many instances it has been shown that this percentage of F hemoglobin is denatured during the test procedure regardless of the concentration of resistant pigment in the hemolysate. Fetal hemoglobin is almost always present in the blood of patients with sickle cell anemia in amounts up to 24 per cent but may also be irregularly encountered in the hemoglobin solutions prepared from erythrocytes of the “C variant” of the sickling disorders. In this variant, the main pigment consists of types C and S hemoglobin. In the hemolysates obtained from carriers of the sickle cell trait, types A and S hemoglobin are consistently demonstrable, but the F pigment is absent, provided the patient is older than 3 years.

The amounts of S hemoglobin in the hemolysates prepared from classical sickle cell anemia bloods were determined electrophoretically, or were computed from the formula: percentage of S hemoglobin = total hemoglobin (100 per cent) minus percentage of total F hemoglobin. It has been previously proven by comparative studies with electrophoresis and the alkali denaturation technic, that the non-S fraction of such hemolysates is, for practical purposes, entirely composed of the alkali resistant pigment. The percentage of S hemoglobin in the “trait” and “C variant” hemoglobin solutions was determined in the Tiselius apparatus, employing technics outlined in preceding contributions of this series.

Preparation of Highly Concentrated Hemoglobin Solutions

In order to elicit the gelling phenomenon in hemolysates prepared from the various kinds of sickling erythrocytes, a high concentration of total hemoglobin present in these solutions is required. To obtain 5 cc. of concentrates of 32 to 35 Gm. per cent total hemoglobin, the following technic was developed. 20 cc. of oxalated blood from nonanemic sickle cell trait carriers, or about twice this amount from patients with sickle cell anemia or the “C vari-
ant,
are taken by venepuncture. The red cells are separated from the plasma by centrifugation at 3000 rpm for 15 minutes and then the plasma is removed. The cells are transferred to a 30 cc. plastic centrifuge tube and washed three times for 10 minutes with isotonic saline, using a high speed refrigerated centrifuge at 18,000 rpm. After the third washing, the supernatant saline is aspirated completely. The tightly packed red cells are then transferred to a 65 cc. plastic tube, 0.5 cc. toluene and 25 glass beads (diameter 4 mm.) are added, the tube stoppered and very rigorously shaken for at least 10 minutes. The tube is then permitted to stand for about 5 minutes until all its fluid content has settled down. The entire preparation is again transferred to a small plastic centrifuge tube, draining off the viscous hemoglobin solution and trapping the glass beads with a spatula. The material is centrifuged at 18,000 rpm for 20 minutes and the nonhemoglobin layers aspirated. The hemolsate is then finally filtered through filter paper, yielding a clear, dark red solution. The hemoglobin concentration is read in a Beckman spectrophotometer in the usual manner.

DEMONSTRATION OF THE GELLING PHENOMENON OF S HEMOGLOBIN CONTAINING HEMOLYSATES UNDER STANDARDIZED CONDITIONS

A simple apparatus was developed for the demonstration and analysis of the gelling phenomenon under standardized conditions. In principle, a constant amount (1 cc.) of the hemoglobin concentrate is placed into a small Erlenmeyer flask and the pigment kept in the reduced state by exposure to a constant stream of CO2 gas, the flask being agitated in a constant speed shaker. To prevent water evaporation from the hemoglobin solution by the gas stream, the whole system is saturated with water vapor. Figure 1 is a schematic drawing of the set-up used.

The amount of gas (250 cc./minute) is regulated by a flowmeter attached to the CO2 tank and the gas passed through two bottles filled with water. Then the gas is permitted to enter the "hemoglobin flask," but not bubbled through the solution. To avoid evaporation, a loose water-soaked cotton pad is pushed about 1 inch into the outlet rubber tubing (fig. 1 6) and kept there in place by a needle or wire piercing both walls of the tubing. The portion of this rubber tubing distal to the cotton pad is thoroughly flushed out with water before it is connected to the outlet needle (fig. 1 5) of the "hemoglobin flask." The gas finally leaves the system through another wash bottle (fig. 1 7).

To insure proper rate of CO2 flow the whole system is tested before each gelling experiment in the following manner. The CO2 output from the tank is set at 250 cc./minute and the gas permitted to pass through the whole system as used later in the actual experiment, but with the "hemoglobin flask" being empty. Should the cotton pad be too large, the flow rate will drop. In such a case the cotton pad has to be replaced by a smaller one.

One cc. of the highly concentrated hemoglobin solution to be tested is placed in the "hemoglobin flask." The shaker is set at maximal speed and the content of the "hemoglobin flask" examined every 10 minutes for gelling. Gel formation can readily be observed by the complete absence of any movement of the hemolsate when the flask is rotated and shaken by hand and is easily distinguished from a mere increase in viscosity, thus providing a sharply defined endpoint for the test. Complete liquefaction of the gel is thereafter induced by connecting the apparatus to an oxygen tank and passing 250 cc. of O2 gas/minute through the system. After reoxygenation, the hemoglobin concentration of the sample is re-determined to check for any water evaporation. In most instances a close agreement of the pre- and postgelling hemoglobin values is obtained and only rarely does the difference exceed 3 per cent.

When several specimens had to be tested simultaneously, each "hemoglobin flask" was connected to a separate CO2 tank with its own flowmeter and its own set of three wash bottles. Since eight flasks can be attached to the shaker used in our laboratory, eight samples can be examined at the same time.

It may be mentioned that this apparatus can also be used to increase the pigment concentration of a hemolsate if this is desired. In such an experiment the flowmeter of the oxygen tank is set at 1500 cc./minute and several cc. of the solution to be concentrated are placed in a "hemoglobin flask" larger than that used for the test. The outlet needle (fig. 1 5) is replaced by a wider outlet glass tube which remains unconnected to any wash bottle.
The progress in pigment concentration can be checked from time to time by determination of the hemoglobin content of this specimen. This technic has been occasionally used in our studies when the stock hemoglobin solution turned out to be not sufficiently concentrated.

**Determination of the Lowest Gelling Points of S Hemoglobin Containing Hemolysates**

The lowest gelling point is defined as the minimal concentration of S hemoglobin, expressed as Gm. per cent, present in a hemoglobin solution which will still exhibit the gelling phenomenon under the standardized conditions described above. If electrophoretic determin-

![Diagram](image)

**Fig. 1.**—Setup for the demonstration of the gelling phenomenon of S hemoglobin solutions under standardized conditions.

1. CO₂ tank.
2. Flowmeter, graduated 0 to 1500 cc./minute.
3. Wash bottles: 2000 cc. Erlenmeyer flasks, filled with water 7 inches high; in- and outlet glass tubing: 6 mm. inner diameter; rubber stoppers.
4. Constant speed shaker: Burrell wrist action shaker, model BB with attachment for four flasks on each side; speed used: highest possible.
5. "Hemoglobin flask:" 25 cc. Erlenmeyer; inlet: glass tubing (2 mm. inner diameter); outlet: needle, gauge 20, one and one-half inches long; rubber stopper.
6. Loosely packed cotton pad, about one-fourth inch in diameter, held in place by needle (gauge 27) both of whose ends are bent for fixation.
7. Outlet: 200 cc. wash bottle, filled with 150 cc. water.

nation of the percentage of S hemoglobin in the concentrate is not available, the lowest gelling point may be expressed as the minimal total hemoglobin concentration (Gm. per cent) at which the gelling phenomenon can still be elicited. Thus, the lowest gelling point can be expressed either as Gm. per cent S or as Gm. per cent total hemoglobin.

All experiments were performed on 1 cc. samples at room temperature and no test was run for longer than one hour, thus arbitrarily setting this time limit for gel formation. After this period an increase of the hemoglobin concentration of the sample is apt to occur which may invalidate the experiments.

To find the lowest gelling points, the gelling phenomenon is first demonstrated in the original hemoglobin concentrates obtained from the various types of sickling erythrocytes. Such a stock concentrate, which should have a total hemoglobin concentration of 32 to 35 Gm. per cent, is then diluted to a series of lower concentrations in 1 cc. portions, and each of these samples is tested for gelling. The difference in the hemoglobin content of two successive dilutions should be approximately 2 Gm. per cent, and dilution is continued until a concentration is reached at which no gelling occurs. Then the intermediate concentration between the "still gelling" and the "nongelling" specimens is prepared and examined.
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Thus, for example, if gelling still occurs at a total hemoglobin concentration of 24 Gm. per cent, but is not observed at 22 Gm. per cent, a sample of 23 Gm. per cent is tested. Each 1 cc. sample which has once undergone gelling is discarded, since it was noted that gelling may cause irreversible changes in the pigment preparations (see Results).

It will be described that the lowest gelling point of an S hemoglobin concentrate varies distinctly with the use of different types of diluents. The diluents employed in this study belonged to two categories; (1) those of the nonhemoglobin type: (a) distilled water, (b) veronal or phosphate buffer of pH 7.4, which is also the pH of the hemolysate, and (c) 30 per cent salt-poor bovine albumin; (2) hemoglobin solutions prepared from (a) bloods from normal adults (containing only A hemoglobin) and (b) from cord bloods (containing 70 to 85 per cent F hemoglobin in addition to A hemoglobin). When hemoglobin solutions were used as diluents, their concentrations were closely matched to that of the stock hemolysates. Therefore, the total hemoglobin concentration in such diluted mixtures is kept constant, although the S pigment content diminishes. For example, if a 32 Gm. per cent sickle cell anemia hemolysate is diluted with a 32 Gm. per cent A hemoglobin solution in relation 1:1, the absolute hemoglobin concentration of this mixture remains 32 Gm. per cent, although the concentration of the "S hemolysate" decreases to 16 Gm. per cent.

Stock concentrates of 32 to 35 Gm. per cent total hemoglobin were obtained from forty sickle cell trait carriers, twenty-eight sickle cell anemia patients, and from six individuals showing the clinical and electrophoretic features of the "C variant" of sickle cell disease. The S hemoglobin content was determined in fifteen of the forty typical "trait" hemolysates by means of electrophoresis and varied from 33.5 to 50 per cent. Only these fifteen hemolysates will be discussed in greater detail. In the remaining twenty-five "trait" hemolysates the gelling experiments gave results identical with those of known S hemoglobin content. Since it was not feasible to take sufficient quantities of blood from any individual patient with severe sickle cell anemia to perform titrations for obtaining all the lowest gelling points, the twenty-eight patients with this disorder were arranged into two equal groups of fourteen. The blood specimens of the members of each group showed a quite similar range of values for S hemoglobin, which varied from 85 to 98 per cent. Thus, comparable results about the influence of different diluents on the gelling phenomenon were achieved. In the six persons with the "C variant" of sickle cell disease, the major component on electrophoretic analysis was C hemoglobin, amounting to 53 to 66.5 per cent, and the predominant minor component was S hemoglobin (33.5 to 47 per cent). However, small amounts of F hemoglobin (up to 3 per cent) were also detected in four out of the six C + S hemolysates. Essentially, the S pigment values of the "C variant" bloods are within the limits established for the sickle cell trait, the main difference being that the abnormal C hemoglobin takes the place of the normal adult hemoglobin present in the "trait."

RESULTS

In general, the lowest gelling points of S hemoglobin containing hemolysates were found to be related to three variables: (a) the percentage of S pigment in the concentrate, (b) the type and quantity of the companion pigment in the solution, and (c) the kind of diluent used in the determination.

Determination of the Lowest Gelling Points Using Distilled Water or Buffer as Diluents

Identical findings were obtained when distilled water or buffer (pH 7.4) were employed as diluents. Dialysis of the hemolysates against water for 24 hours exerted no influence on the results.

Figure 2a shows a scattergram of the lowest gelling points found for the "anemia," "trait," and "C variant" hemolysates tested. The values are expressed as the lowest total hemoglobin concentration (Gm. per cent) at which gelling could still be elicited. As can be noticed, the lowest gelling points of these three
types of hemolysates differ distinctly. "Anemia" (S + F) hemolysates still gel when the total hemoglobin concentrations are as low as 20 to 25.5 Gm. per cent. "Trait" (A + S) hemolysates require total pigment concentrations of 29.7 to 33.2 Gm. per cent for gelling. The values for the "C variant" (C + S) hemolysates lie between these two ranges and amount to 26 to 28 Gm. per cent.

When the lowest gelling points are expressed in Gm. per cent S hemoglobin (based on electrophoretic analysis of the original samples) the scattergram (fig. 2b) reveals that the minimal amounts of S hemoglobin required to elicit gelling
are much greater for the S + F than for the A + S and the C + S hemolysates. The last two, however, cannot be readily distinguished by this particular way of presentation of the lowest gelling points. Some of the values for the C + S hemolysates are found to be irregularly distributed between those for the A + S hemoglobin solutions. This is due to the inequality of the S pigment content of the various specimens examined and to the considerable, but different, influence exerted by the A and C companion pigments on the gelling phenomenon of S hemoglobin. The existence of such influences becomes manifest when the lowest gelling points obtained from A + S and C + S concentrates with almost identical percentages of S hemoglobin are compared (table 1). These results suggest that both A and C hemoglobin interact with the S pigment during the gelling process. However, a certain quantity of C hemoglobin decreases the amount of S pigment necessary for gelling to a greater extent than does an equal quantity of the A compound. Since, in “anemia” hemolysates, neither A nor C hemoglobin are present, and F hemoglobin does not interact significantly with S hemoglobin—as will be shown later—gel formation, in these “anemia” hemoglobin solutions, probably depends exclusively upon the available quantity and the mutual interactions of these S hemoglobin molecules.

To determine what relationship exists between the lowest gelling point, expressed as Gm. per cent S hemoglobin, and the amount of non-S pigment, expressed as the percentage of the total hemoglobin, these values were plotted in a diagram for the A + S and C + S hemoglobin solutions. A straight line relationship was revealed (fig. 3a). No such interdependence could be established for the anemia (S + F) hemolysates (fig. 3b). Why this is so represents an as yet unsolved problem.
Comment. These experiments demonstrate that the lowest gelling points of the "anemia", "trait", and "C variant" hemolysates are distinctly different, when expressed as total (Gm. per cent) hemoglobin values. Further analysis of the data indicates that the presence of A hemoglobin in the "trait" hemolysates decreases, and that type C hemoglobin reduces even further the minimal amount of S pigment required for gelation.

![Graph](image_url)

**Fig. 3.**—Relationship between the percentages of non-S hemoglobin of the stock concentrates and the lowest gelling points, expressed in Gm. per cent S hemoglobin of A + S, C + S, and S + F hemolysates.

![Graph](image_url)

**Fig. 4.**—Lowest gelling points of hemoglobin solutions prepared from sickle cell anemia and sickle cell trait erythrocytes. Diluent: A hemoglobin solution. The ranges of the lowest gelling points with water as a diluent are given as reference values.

**Determination of the Lowest Gelling Points Using A Hemoglobin Solutions as Diluent**

In order to study further the influence of A hemoglobin on the gelling phenomenon of the S pigment, the lowest gelling points of "anemia" and of "trait" hemolysates were determined by using A hemoglobin solutions as a diluent instead of water or buffer. As has been previously mentioned, the A hemoglobin diluent had the same total hemoglobin concentration (32 Gm. per cent) as the
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stock pigment concentrate prepared from the sickling red cells. In the dilution mixtures the absolute hemoglobin concentration remains, therefore, constant, although the S pigment concentration decreases progressively.

Figure 4 represents a scattergram of the lowest gelling points obtained with “trait” and “anemia” hemolysates. As can be observed, the lowest gelling points of the anemia hemoglobin solutions change considerably by dilution with normal hemoglobin. Expressed in Gm. per cent S hemoglobin, the lowest gelling points of the anemia hemolysates with water as diluent vary from 19.1 to 24.0 Gm. per cent, whereas with A hemoglobin as diluent they were transferred in almost all instances into the “lowest gelling range” of the “trait” hemolysates (10.6 to 16.3 Gm. per cent). The lowest gelling points of some “trait” hemolysates could also still be somewhat decreased by dilution with A hemoglobin.

Comment. These experiments provide additional evidence that A hemoglobin participates in the gelling phenomenon of S hemoglobin by replacing the latter in part and interacting with the remaining sickle cell pigment.

![Diagram](image)

**Fig. 5.**—Lowest gelling points of hemoglobin solutions prepared from sickle cell anemia and sickle cell trait erythrocytes. Diluent: cord blood hemoglobin solution. The ranges of the lowest gelling points with water as a diluent are given as reference values.

**Determination of the Lowest Gelling Points Using Cord Blood Hemolysates (F Hgb. 70 to 85 per cent, and A Hgb. 15 to 30 Per Cent) as Diluent**

Although it would have been desirable to use pure F hemoglobin solutions as a diluent, the technical difficulties in obtaining such pigment solutions in large quantities and high concentrations made this approach impractical. However, our experimental results suggest that evaluation of the effect of F hemoglobin on the gelling phenomenon can also be achieved by using cord blood concentrates containing 70 to 85 per cent F hemoglobin. “Trait” and “anemia” hemolysates were diluted with such cord blood concentrates in an identical manner as described with the A hemoglobin solutions. The findings are collected in figure 5 which reveals that by dilution with matched cord blood hemolysates the lowest gelling points of the “anemia” and “trait” hemoglobin solutions are only slightly influenced; it remains doubtful whether the slight alterations seen are not due to the small amount of A hemoglobin present in this diluent. At any rate, F hemoglobin behaves quite differently in influencing the gelling phenomenon when
compared to normal adult or to C hemoglobin. This behavior is of particular interest since F hemoglobin is the established companion of S pigment in the anemia hemolysates.

Comment. F hemoglobin seems to exert no significant influence on the gelling phenomenon of S hemoglobin.

Determination of the Lowest Gelling Points Using 30 Per Cent Salt-Poor Bovine Albumin as Diluent

It seemed of interest to determine whether a nonhemoglobin protein used as a diluent could also alter the lowest gelling points of S hemoglobin solutions. The commercially available (Armour & Co.) 30 per cent bovine serum albumin was selected because of its relative similarity to the protein moiety of hemoglobin. As can be seen from the scattergram (fig. 6) the albumin solutions also decrease the lowest gelling points of “anemia” and “trait” hemolysates, but to a somewhat lesser extent that do the A hemoglobin solutions. In figure 6a the lowest gelling points are expressed in Gm. per cent total hemoglobin, and in figure 6b as Gm. per cent S hemoglobin, based on the electrophoretic analyses of the original samples. The lowest gelling points of the sickle cell anemia hemolysates, amounting to 20 to 25.5 Gm. per cent total hemoglobin with water as diluent, shifted to 13.2 to 22.5 Gm. per cent when diluted with albumin, and a similar trend was found for the A + S hemolysates. Thus, serum albumin, which is not a normal constituent of the red cells, is also capable of influencing the gelling phenomenon of S hemoglobin. However, with albumin, contrary to the observation with water as a diluent, no straight line relationship between the amounts of A pigment and the lowest gelling points, expressed as per cent S hemoglobin, could be established for the A + S hemolysate.

Comment. Addition of 30 per cent bovine serum albumin also decreases the amount of S hemoglobin necessary to elicit gelling, but to a lesser extent than does normal adult hemoglobin.

Further General Observations on the Gelling Phenomenon of S Hemoglobin

In the experiments so far described the lowest gelling points were determined in separate 1 cc. portions, obtained by diluting the stock concentrates, and agitated for not longer than 1 hour. In the beginning of our studies we had attempted to determine the lowest gelling points by using one single 1 cc. sample for a whole series of dilutions; after reoxygenation of a gelled sample, its concentration was brought down to the next dilution, and the test run again on this same specimen. This procedure was continued until the lowest gelling point was reached. However, it was noticed that with this technic the lowest gelling points were considerably lower after several hours of shaking than with single samples of corresponding concentrations, agitated for 1 hour only. This observation seems to suggest that some partly irreversible changes may occur in the gelled specimen. It was also noticed that when gelling was continued for several hours, complete liquefaction of the gel could not be accomplished by reoxygenation. It is for these reasons that the technic of using separate 1 cc. portions in the titrations of the lowest gelling points was adopted and that all samples, once gelled, were discarded. Whether some denaturation of the protein occurs during prolonged
agitation and exposure to CO₂ remains to be elucidated. Shen et al. also noted that the sickling phenomenon of erythrocytes may become irreversible when the cells are incubated for 24 hours.²

![Graph showing lowest gelling points of hemoglobin solutions prepared from sickle cell anemia and sickle cell trait erythrocytes. Diluent: 30 per cent bovine albumin. The ranges of the lowest gelling points with water as a diluent are given as reference values.]

**The Gelling Phenomenon of an Exceptional A + S Hemolysate**

One of our "trait" hemolysates, not included in the data given above, contained only 20.3 per cent S hemoglobin, as determined by electrophoresis. The red cells of this individual had a mean corpuscular hemoglobin concentration of 35 Gm. per cent. Almost all cells sickled with exposure to sodium metabisulfite. Therefore, a mean corpuscular S hemoglobin concentration of about 7 Gm. per cent was sufficient to produce the sickling phenomenon. The lowest gelling point of this hemolysate, expressed as total hemoglobin, was also 35 Gm. per cent, and, expressed as S hemoglobin, amounted to 7 Gm. per cent (see fig. 3a). The close correlation of the values for the mean corpuscular hemoglobin concentration
and the lowest gelling point in this particular hemolysate, may perhaps indicate that about 20 per cent of S hemoglobin in a normochromic normocytic cell represents the minimal necessary amount required for obtaining a positive sickling test in a "trait" erythrocyte. When the S content falls below this level, the sickling phenomenon cannot be elicited any more, although the individual is still a carrier of a latent sickle cell trait. One observation of this kind has been previously reported in this series.\textsuperscript{13}

**Discussion**

It is now well established that there exist two normal variants of hemoglobin, the adult A and the fetal F, and three pathologic types, S (sickle cell) hemoglobin, and the pigment compounds named C and D.\textsuperscript{2} Electrophoretically, A and F hemoglobin move identically but slower than S hemoglobin under the conditions most suitable for the separation of the latter component (pH 6.5\textsuperscript{10, 18}). C hemoglobin moves faster than S.\textsuperscript{3} D hemoglobin has the same mobility as S hemoglobin\textsuperscript{4} but does not cause sickling of red cells. Only F hemoglobin has an increased resistance to alkali denaturation.

S hemoglobin is only rarely found in a hemolysate without the company of another type of hemoglobin.\textsuperscript{10, 18} It is associated with A pigment in the sickle cell trait, with C hemoglobin in the "C variant," and with the rare D component in the "D variant" of the sickling disorders. Fetal hemoglobin is the companion pigment in classical sickle cell anemia. In the absence of S pigment, A hemoglobin may be associated with either the C or the D compound, thus giving rise to the A + C or A + D traits.\textsuperscript{3, 5}

The "differential" hemoglobin analysis\textsuperscript{19} of a hemolysate prepared from sickling erythrocytes requires at present the combined use of electrophoretic methods, of the alkali denaturation technic, and of solubility studies.\textsuperscript{18} There is an urgent need to develop simple, less expensive, and less time consuming procedures for obtaining information about the hemoglobin patterns in the various types of sickling disorders. The study of the gelling phenomenon of S hemoglobin containing hemolysates was undertaken with this possible goal in mind.

The formation of tactoids and the tendency to gelation appears to be a specific characteristic of reduced sickle cell hemoglobin and is not encountered with either the A, F, or C pigments in the absence of S hemoglobin. We could not demonstrate tactoids or gelling when hemolysates prepared from normal (A), cord blood (F + A),\textsuperscript{6} or A + C\textsuperscript{8} erythrocytes were examined. Specimens containing D hemoglobin were not available for this study.

The appearance of tactoids and, at higher concentrations, of gels indicate an orderly orientation of the S hemoglobin molecules in the hemolysates.\textsuperscript{1, 7} According to Flory\textsuperscript{19, 20} and to Ferry\textsuperscript{21} gel formation, in certain systems, may be visualized as being due to polymerization of interacting molecules which form "tri-functional" \( \text{S Hgb.} \) or "polyfunctional" units. Such units will combine to form a branched network which will suddenly appear as a gel after condensation has proceeded to a certain point.\textsuperscript{21} This gelling point thus
represents the complete synthesis of a tridimensional network\textsuperscript{21} which pervades the entire sample.

By means of determinations of the lowest gelling points of hemoglobin concentrates prepared from sickling “trait”, “C variant”, and “anemia” erythrocytes, it could be shown that the minimal amounts of S hemoglobin necessary for gelling vary distinctly with the kind and quantity of the companion pigment present in the solution. A and C hemoglobins definitely decrease the quantity of the S compound necessary for gel formation. If gelling is visualized as the polymerization of polyfunctional units of S hemoglobin, then the A or C hemoglobin molecules can replace, in a specific manner, a certain number of S pigment molecules and, by interaction with the remaining S hemoglobin units, participate in the formation of the network which constitutes the gel. Fetal hemoglobin seems to be almost incapable of such an interaction. This interpretation of the S hemoglobin “sparing” effects of A and C pigment in gel formation is also supported by the findings that when the percentages of the non-S pigment present in the A + S and C + S hemolysates are plotted against their corresponding lowest gelling points expressed in Gm. per cent S hemoglobin, straight line relationships are revealed (fig. 3a). C hemoglobin surpasses the normal adult pigment in its ability to replace sickle cell hemoglobin during the process leading to gelation (fig. 3a and table 1). The gel network of the “anemia” hemolysates is apparently, in its entirety, composed of polyfunctional S hemoglobin units. By using A hemoglobin solutions as a diluent, a certain quantity of S hemoglobin can be replaced by A pigment, and the lowest gelling points of the “anemia” hemolysates can thus be transferred into the range established for the A + S hemoglobin solutions.

S hemoglobin tactoids most likely represent the building stones of the gelled network which will develop at a critical hemoglobin concentration. The tactoids found in “trait” or “C variant” hemolysates probably also contain the companion pigments A or C, respectively. In vitro, tactoids are only demonstrable when the S hemoglobin concentration is at least 10 Gm. per cent.\textsuperscript{1} However, some A + S erythrocytes contain only about 20 per cent S pigment. Assuming that the mean corpuscular hemoglobin concentration is 34 ± 2 Gm. per cent, the mean corpuscular S hemoglobin concentration is then theoretically only about 7 Gm. per cent. Actually, the lowest gelling point of a blood specimen containing 20 per cent S pigment was found to be about 7 Gm. per cent S hemoglobin. Thus, sickling could still be elicited due to extensive interaction of A and S hemoglobin. Whether other intraerythrocytic, nonhemoglobin proteins may also participate in a similar manner in tactoid or gel formation during the sickling process is not yet known. The demonstration that serum albumin can also interact with S hemoglobin is of interest in regard to this problem.

Harris\textsuperscript{1} has described the sickled red cell as “essentially a large hemoglobin tactoid, thinly veiled and distorted by the cell membrane.” In the light of our experimental results, a sickled erythrocyte represents an S hemoglobin tactoid or gel, specifically influenced by the companion pigment which interreacts with the S compound. In the sickled “trait” erythrocyte the assumption of the altered shape is due to the interaction of sickle cell hemoglobin with normal adult hemoglobin and in the “C variant” with pathologic C hemoglobin. Theoretically, the limitations set to the sickling process in a cell with a normal mean corpuscular
hemoglobin concentration will be determined by the amount of reduced S hemoglobin available, as well as by the "S pigment sparing action" of the companion pigment and possibly other intraerythrocytic constituents as well. Only in the sickle cell anemia erythrocytes does the formation of tactoids and gels appear to be almost solely dependent on polyfunctional units of reduced S hemoglobin, since the accompanying fetal pigment does not significantly interact with sickle cell hemoglobin.

The present investigation has thrown some light on certain problems of the gelling phenomenon of S hemoglobin, but many aspects remain to be clarified. Although C hemoglobin decreases the amount of S hemoglobin necessary for gelling more extensively than does a corresponding quantity of A pigment (fig. 3a and table 1), the differences in the lowest gelling points of the C + S and A + S hemolysates, when expressed as Gm. per cent total hemoglobin (fig. 2), are greater than when expressed in Gm. per cent S hemoglobin. Therefore, we have not been able to correlate the lowest gelling point of a sample, when expressed as Gm. per cent total hemoglobin, to its S pigment content. Apparently the companion pigments A or C participate in the gel formation in a more complicated manner than by merely replacing a certain quantity of sickle cell hemoglobin. We are also unable to explain why different sickle cell trait hemolysates, when diluted with an A hemoglobin solution, do not show identical lowest gelling points. Another unsolved problem is presented by the fact that sickle cell anemia hemolysates with the same S hemoglobin content show different lowest gelling points (fig. 3b). All these findings require elucidation by expert physicochemical studies.

It may be worth while to emphasize that the in vitro studies reported in this communication are not directly applicable to the behavior of the various kinds of sickling erythrocytes within the blood stream. Complete reduction of the whole intraerythrocytic hemoglobin is almost never found in vivo and, therefore, apparently only the anemia cells with their high S hemoglobin content assume the sickled shape within the venous compartment of the circulation.

The fact that the lowest gelling points of the "trait," "C variant," and sickle cell anemia hemolysates differ distinctly has led to the development of the diagnostic gelling test which is now on trial in our laboratory. In this procedure a stock concentrate of 32 to 35 Gm. per cent, as well as two aqueous dilutions, of 27 and 24 Gm. per cent total hemoglobin, are prepared from sickling red cells and examined for gel formation. If gelling occurs in the 24 Gm. per cent hemolysate, the diagnosis of sickle cell anemia is established (fig. 2a). Should the stock concentrate gel, but gelling not occur in the two dilutions, the hemolysate has been obtained from A + S ("trait") erythrocytes, even if the patient has a severe anemia. In such instances the anemia is unrelated to the sickling process. The presence of the "C variant" may be suspected if gelling is only observed with the 32 and 27 Gm. per cent concentrations, but not with 24 Gm. per cent. It may be mentioned that some of our patients with the "C variant" were first recognized by means of this procedure, and their "differential" hemoglobin pattern then confirmed by electrophoretic and alkali denaturation studies. However, we have also encountered three hemolysates obtained from other atypical cases of sickle cell disease which gave results in the gelling test similar
to the hemolysates of the “C variant.” These atypical specimens, prepared from the sickling erythrocytes of nonanemic individuals, contain, on electrophoresis, 70 to 80 per cent of a component having the mobility of S hemoglobin, and, as companion pigment, normal adult hemoglobin, and, in two out of three instances, also a small amount (up to 3.4 per cent) of F hemoglobin. Whether these atypical hemolysates contain D hemoglobin is not yet known and requires further genetic and solubility studies. At present, electrophoretic analysis of the hemolysate is still necessary if gelling is found to occur in only two out of the three standard solutions used in the diagnostic test.

Although the diagnostic gelling test is a nonspecific procedure, it has had great value in our hands as a screening method, thus diminishing considerably the necessity for using the more cumbersome electrophoretic techniques in the analysis of the sickling disorders. However, evaluation of the limitations of the diagnostic gelling test still needs investigations on an extensive scale. Such studies are now in progress in our laboratory.

**Summary**

1. When sufficiently concentrated sickle cell hemoglobin containing solutions are exposed to a constant stream of CO₂ gas, the hemolysates gel. This gelling phenomenon is indicative of the presence of S hemoglobin and cannot be obtained with any other type of human hemoglobin in the absence of S pigment. The lowest S hemoglobin concentration (Gm. per cent) of a hemolysate at which the gelling phenomenon can still be elicited is designated as its lowest gelling point.

2. A simple apparatus was developed to analyze the gelling phenomenon under standardized conditions. It could be shown that the lowest gelling points of hemolysates prepared from erythrocytes of the sickle cell trait (containing A + S hemoglobins), of the “C variant” (containing C + S hemoglobins), and from sickle cell anemia cells (containing S + F hemoglobins) differ distinctly. Further experiments suggest that the presence of A hemoglobin decreases the minimal amount of S pigment required for gel formation, and that type C hemoglobin reduces this amount even further. F hemoglobin seems to exert no significant influence on the gelling phenomenon. Serum albumin is also capable of decreasing the amount of S hemoglobin required for gelation.

3. A sickled erythrocyte is visualized as an S hemoglobin tactoid or gel, specifically influenced by the companion pigment which interacts with the S compound. Thus, in the sickle cell trait, a positive sickling test is not only caused by the presence of S hemoglobin, but also by its interaction with A hemoglobin. Only in the sickle cell anemia cells does sickling seem to depend solely upon the interaction of the S hemoglobin molecules.

4. The readily demonstrable differences of the lowest gelling points of hemolysates prepared from the various types of sickling red cells form the basis of the diagnostic gelling test which distinguishes sharply between sickle cell anemia and sickle cell trait erythrocytes. By this procedure atypical cases of sickle cell disease, for example, those whose erythrocytes contain C hemoglobin, may also be detected.
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Studies on Abnormal Hemoglobins: VIII. The Gelling Phenomenon of Sickle Cell Hemoglobin: Its Biologic and Diagnostic Significance

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