GELS OF NORMAL AND SICKLED HEMOGLOBIN

Comparative Study

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(Received for publication 8 December 1969)

The specific defect in molecules of sickle hemoglobin (HbS) has been known for many years (1), but the nature of the assembled polymers of HbS which distort susceptible erythrocytes remains unclear. Several structural forms of sickled hemoglobin have been observed in electron microscopic studies, including a mat-like arrangement of fibers (2), empty hexagonal crystals (3), microtubules (4), and dense rods (5, 6). It is unlikely that the process of sickling results in formation of all of the different structural arrangements described by various workers.

Previous studies reported from this laboratory have examined the fine structure of sickled hemoglobin in thin sections and whole mounts of intact cells, thin sections and whole mounts of sickled cell-free solutions of HbS, and thin sections and whole mounts of crystallized samples of cell-free sickled hemoglobin (5–10). In all the investigations sickling resulted in the assembly of reduced HbS molecules into long 170–190 A polymers resembling dense rods. Tubular polymers similar to the microtubules described by Murayama (4) and the honeycomb crystals observed by Stetson (3) were not found in our material. Therefore, it was concluded that the process of sickling was due to a sol-gel transformation in which molecules of HbS assembled into solid rods.

The findings, however, did not explain the origin of the hollow polymers of hemoglobin found by other workers. For purposes of the present investigation it was assumed that microtubules of hemoglobin were aberrant polymers unrelated to erythrocyte sickling. To support this assumption, it would be necessary to demonstrate that sickle hemoglobin can form tubular polymers, but under conditions which do not promote sickling. Also, it would be helpful to show that other hemoglobins can form tubular polymers, but not the rods characteristic of reduced sickled hemoglobin.

The results of the present investigation indicate that normal human oxyhemoglobin and reduced hemoglobin, and sickle oxyhemoglobin can form tubular polymers resembling microtubules when cell-free solutions are gelled by potassium phosphate buffer.

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Materials and Methods

1. Preparation of Hemolysates.—A detailed description of the method used to prepare hemolysates, induce gel formation, and fix and embed the hemoglobin samples for study in the electron microscope was reported previously (7 and footnote 1). Blood collected from normal donors and individuals with homozygous sickle cell anemia was mixed immediately with citrate citric acid, pH 6.8, in a ratio of nine parts blood to one part anticoagulant. Red blood cells were separated by centrifugation and washed three times with phosphate buffered saline, pH 7.1. The washed cells were packed by centrifugation and lysed by combining with an equal volume of distilled water and 0.4 volume of toluene (11). After 24 to 72 hr the toluene and membrane layers were removed from the hemoglobulin by pipette. Subsequently, the hemolysates were filtered by gravity and centrifuged in several steps to remove residual stromal elements. The final centrifugation was carried out for 1 hr in a Beckman L-250 preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 100,000 *g*. Hemoglobin concentrations of the hemolysates varied between 8.7 and 16.8 g/100 ml. Cellulose acetate electrophoresis and ultraviolet spectrophotometry have shown that these samplies are essentially pure oxyhemoglobin with only a trace of stromal protein.[1] The hemolysates were used immediately or stored at 4°C. Blood was obtained from 2 patients with homozygous sickle cell anemia and 11 normal individuals for this investigation, and 50 ml of blood was used to prepare each hemolysate.

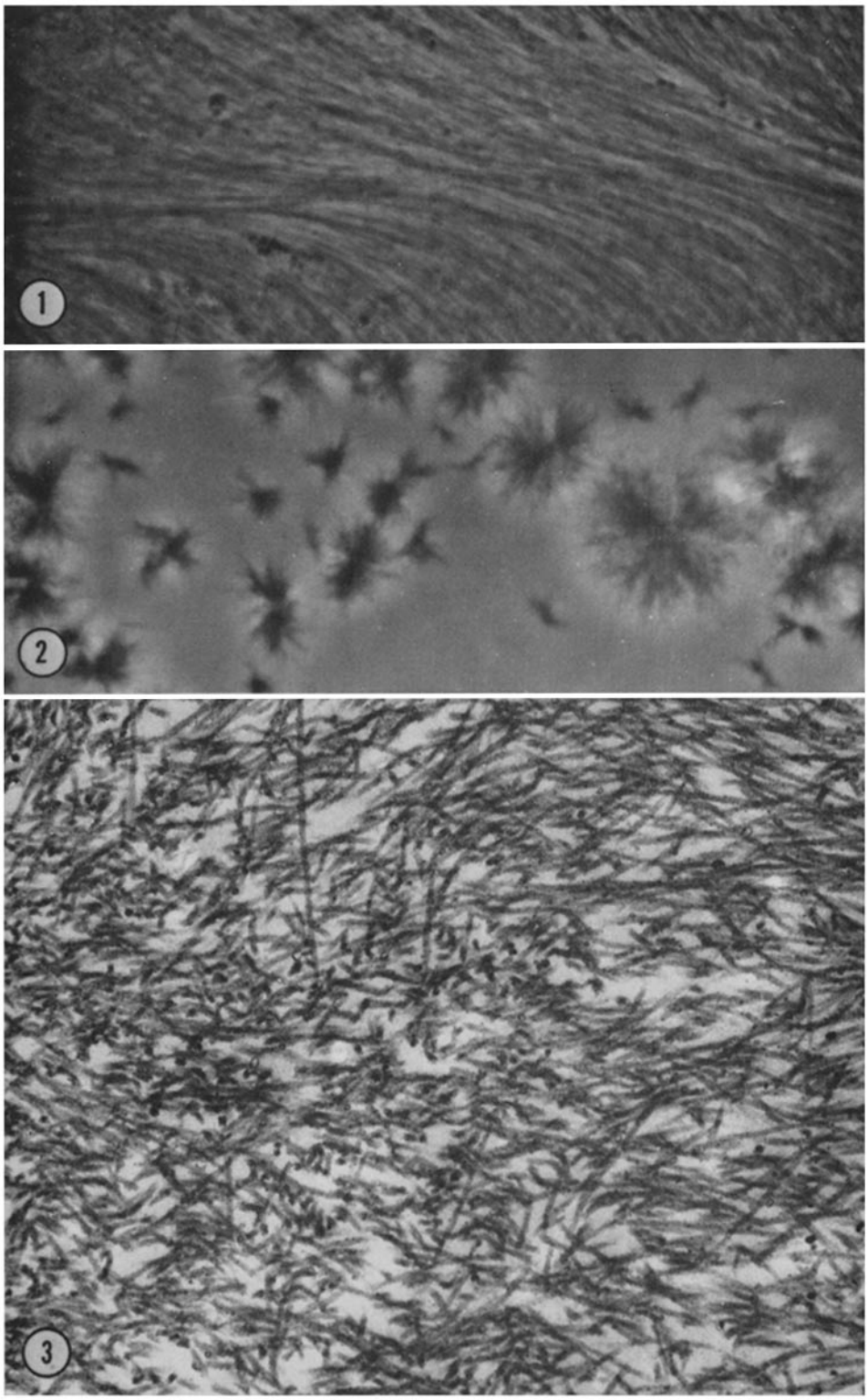
2. Preparation of Hemoglobin Gels.—Gels of normal and sickle oxyhemoglobin were developed by combining 0.5 ml of cell-free hemoglobin solution with 2.5 ml of 2.8 M potassium phosphate, pH 6.8–7.2. To prepare gels of reduced hemoglobin, solutions of normal and sickle hemoglobin and the phosphate buffer were made up to 1% with sodium metabisulfite, then combined in the ratios mentioned above. Drops of the oxyhemoglobin and reduced hemoglobin gels were observed under cover slips by phase optics before addition of buffer and at intervals during gelation. The gels of reduced sickle hemoglobin formed in a few minutes to 1 hr. Gels of normal and sickle oxyhemoglobin and reduced normal hemoglobin required 1 to 7 days to solidify. This time could be shortened to 1 hr by combining the hemoglobin solution with a one-tenth volume of preformed gel before addition of buffer.

3. Preparation of Gels for Electron Microscopy.—The methods used in this laboratory to fix, dehydrate, and embed hemoglobin gels were described in other reports (6, 7, 9, and footnote 1). Small portions of gel were dropped into approximately five volumes of fixative. The fixation schedules included glutaraldehyde followed by osmic acid, glutaraldehyde combined with osmic acid, and osmic acid alone. All produced adequate fixation, although osmic acid alone produced the best preservation of normal hemoglobin gels. All samples were dehydrated through a graded series of alcohols and embedded in Epon 812. Thin sections of the plastic-embedded hemoglobin gels were usually stained with uranyl acetate and lead citrate before examination in a Philips 200 electron microscope.

RESULTS

1. Gels of Reduced Sickle Hemoglobin.—The gels of sickled hemoglobin contained masses of thin fibers when examined in the phase contrast microscope (Figs. 1, 2). At initial concentrations of hemoglobin above 12 g/100 ml the fibers were in parallel masses (Fig. 1), but at lower concentrations the thin fibers were aggregated in radial arrangements of various sizes (Fig. 2). The smallest aggregates visible consisted of a few filaments arranged in the form of the letter "x".

[1] White, J. G. and B. Heagan. Tubular polymers of normal human hemoglobulin. *Amer. J. Pathol.* In press.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Larger clumps resembled sheaves of wheat or complete radial patterns, suggesting spokes radiating from a central hub in all directions.

Stellate patterns were preserved in the plastic embedded gels (Fig. 3). Thin sections revealed long fibers extending in all directions from a central hub. At high magnification the fibers were solid rods identical in appearance with the polymers of HbS observed inside intact sickled erythrocytes (Figs. 4, 5).

2. Gels of Sickle and Normal Oxyhemoglobin and Reduced Normal Hemoglobin.—Gels which developed in solutions of sickle and normal oxyhemoglobin, and reduced normal hemoglobin were similar to each other, but differed markedly from gels of reduced sickle hemoglobin (Figs. 6, 7). The gels contained masses of thick and thin fibers. Thin filaments were occasionally gathered into irregular meshworks surrounded by thicker fibers (Fig. 6), but for the most part the arrangement of fibers was random (Fig. 7).

Thin sections of the gels revealed that the thin fibers were tubular polymers

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FIG. 1. A gel of reduced, cell-free sickled hemoglobin viewed in the phase contrast microscope. The initial concentration of the hemoglobin solution was 16.8 g/100 ml. Polymers of sickled hemoglobin form parallel masses at this concentration. × 1480.

FIG. 2. The appearance of cell-free sickled hemoglobin when the initial concentration is below 12 g/100 ml. Fibers of sickled hemoglobin are gathered in radial configurations of various sizes. The smallest aggregates resemble the letter "x." Larger masses are similar to sheaves of wheat or complete radial patterns with spokes radiating from a central hub in all directions. × 1880.

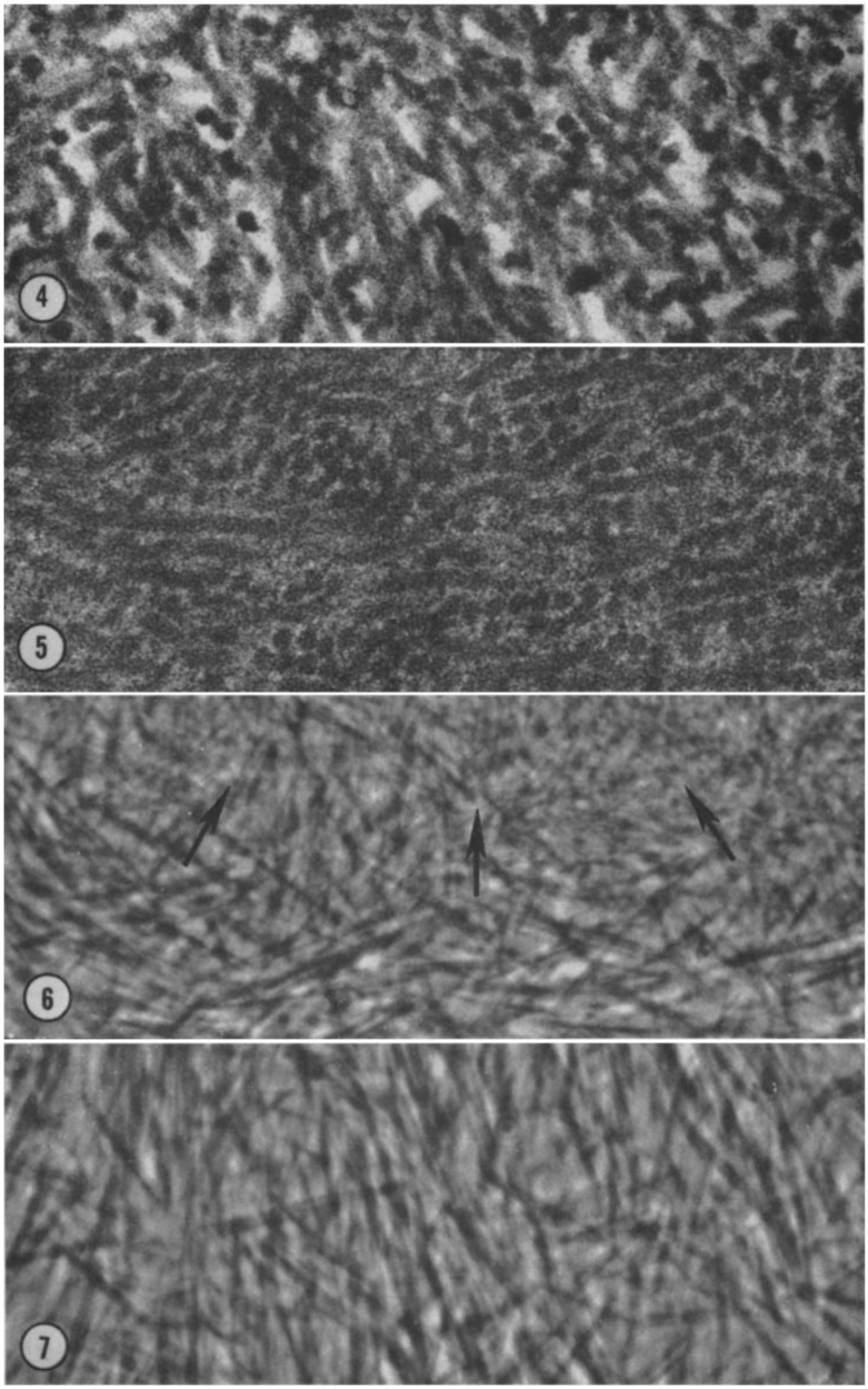
FIG. 3. The fine structure of cell-free sickled hemoglobin. A radial pattern has been preserved in this example. Polymers spread from the central area in all directions. The central axis is beyond the left side of the picture where many of the fibers are cut in cross-section. More of the polymers to the right appear in the plane of section as they spread from the central axis. × 49,800.

FIG. 4. The central area of a gel of cell-free sickled hemoglobin. Polymers are sectioned in various planes. In cross-section the fibers appear to have solid cores and are approximately 170–190 A in diameter. × 85,600.

FIG. 5. The appearance of sickled hemoglobin in an intact sickled cell. Cytoplasmic matrix surrounds each polymer. The polymers of HbS in intact erythrocytes appear identical with those present in gels prepared from cell-free solutions of reduced sickle hemoglobin. × 79,200.

FIG. 6. Phase microscopic view of a gel prepared by combining normal cell-free oxyhemoglobin with potassium phosphate buffer. The organization of the fibrous elements is strikingly different from that noted in gels of reduced sickle hemoglobin. Polymers are randomly dispersed in gels of normal reduced hemoglobin, except for the occasional tendency of thin filaments (arrows) to be segregated from larger fibers. × 1840.

FIG. 7. The gel in this example was prepared by combining sickle oxyhemoglobin with phosphate buffer. Polymers in this gel and their distribution are identical with the composition of gels formed from cell-free solutions of oxy- and reduced normal hemoglobin. × 2400.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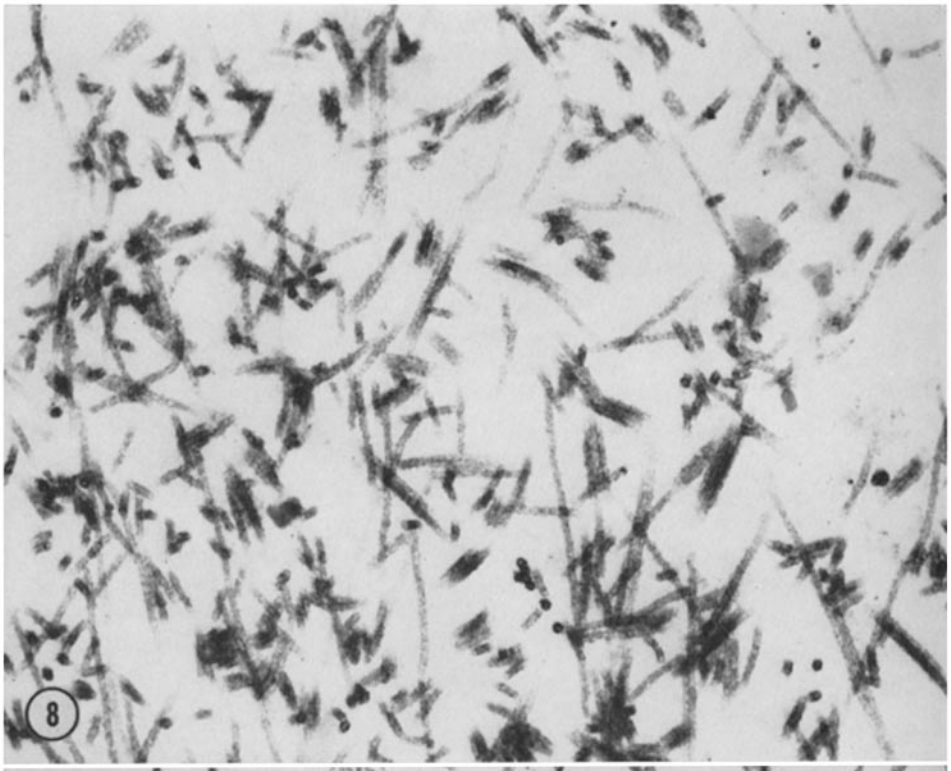
resembling microtubules (Fig. 8). The thick fibers consisted of a variable number of closely packed tubular polymers (Fig. 10). Cross-sections of the polymers revealed hollow circular profiles approximately 240 A in diameter with walls about 60 A thick (Figs. 8, 11, 13). The individual polymers resembled the microtubules of sickled hemoglobin observed by Murayama Fig. 9 (4) and sections of the parallel bundles of tubules in various planes were similar to the empty hexagonal crystals described by Stetson Figs. 12, 13 (3) in intact sickled cells.

DISCUSSION

The present investigation has shown that the structure and arrangement of polymers in gels of reduced sickle hemoglobin (RHbS) differed strikingly from the composition of polymers in gels prepared from normal oxyhemoglobin (OHbA), normal reduced hemoglobin (RHbA), and sickle oxyhemoglobin (OHbS). The thick and thin fibers comprising gels of OHbA, RHbA, and OHbS appeared randomly dispersed in the phase contrast microscope, except for a tendency of thin elements to form islands in the midst of thicker fibers. At high hemoglobin concentrations the fibers present in gels of RHbs were arranged in parallel masses similar to those described by Harris (12), but below 12 mg/100 ml the polymers were always gathered into radial configurations. The tendency toward parallel or radial organization readily distinguished sickle gels from unsickled gels in which thick and thin fibers appeared randomly associated when viewed in the phase contrast microscope.

Electron microscopy revealed differences between sickled and unsickled gels which had not been observed by phase optics. The gross patterns of polymer organization were preserved in thin section, but a degree of association became evident in unsickled gels which had not been seen at low magnification. The thick fibers were composed of variable numbers of thin filaments compacted together in tight parallel bundles. Stetson's study (3) of intact sickled erythrocytes revealed a similar packing of hemoglobin subunits into a paracrystalline lattice, and illustrations accompanying Murayama's report (4) show that microtubules of HbS form parallel bundles in which the walls of adjacent polymers appear pressed tightly together. However, the appearance of cell-free solutions of sickled hemoglobin led Harris (12) to the conclusion that polymers of HbS tend to align parallel and *equidistant* to each other. Study of intact sickled cells (5–9) and concentrated cell-free solutions of sickled hemoglobin (7) in this laboratory have confirmed the tendency of HbS polymers in parallel bundles to remain separated. Bertle's investigations of intact sicled erythrocytes revealed an identical organization of separated polymers in parallel bundles (13, 14). The alignment of hemoglobin polymers in wall to wall contact in gels of OHbA, RHbA and OHbS was a major feature which distinguished them from gels of reduced sickled hemoglobin.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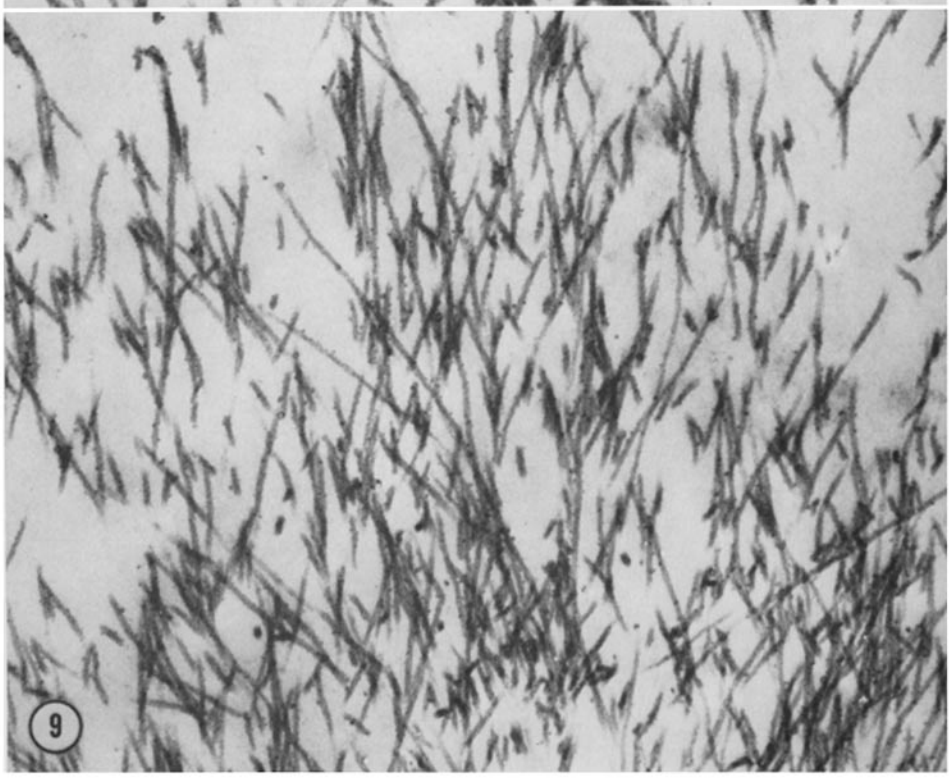
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The radial organization of polymers in RHbS gels formed in solutions of less than 12 g/100 ml also reflected the tendency of HbS polymers to spread apart. Harris (12) suggested that the polymers naturally tend to align in parallel, equidistant associations, but this relationship is evident only in concentrated cell-free solutions (7) and in the intact sickled cell (6). Investigations in this laboratory have indicated that forces causing polymers to twist into radial configurations are stronger than the bonds holding the fibers parallel and equidistant to each other. When sickled cells are crushed (8) or exposed to hypertonic stress (9) the parallel bundles shift into branching or radial configurations identical with those observed in the cell-free solutions of sickled HbS. Sickled hemoglobin polymers, in contrast to other hemoglobin polymers, appear to have a unique character which prevents them from becoming aligned in wall to wall contact, separates them when they are associated in parallel bundles, and promotes radial relationships when the hemoglobin solution is not concentrated. This unknown force generated by HbS polymer interaction may be a critical factor in supplying the stress which deforms susceptible erythrocytes (9).

The origin of forces which result from polymer interaction has not been defined but may result from the molecular structure of the polymer itself. Efforts to determine the structure of HbS polymers have been complicated by reports from different laboratories suggesting a variety of molecular arrangements considered characteristic of sickled hemoglobin (2–6). The present investiga-

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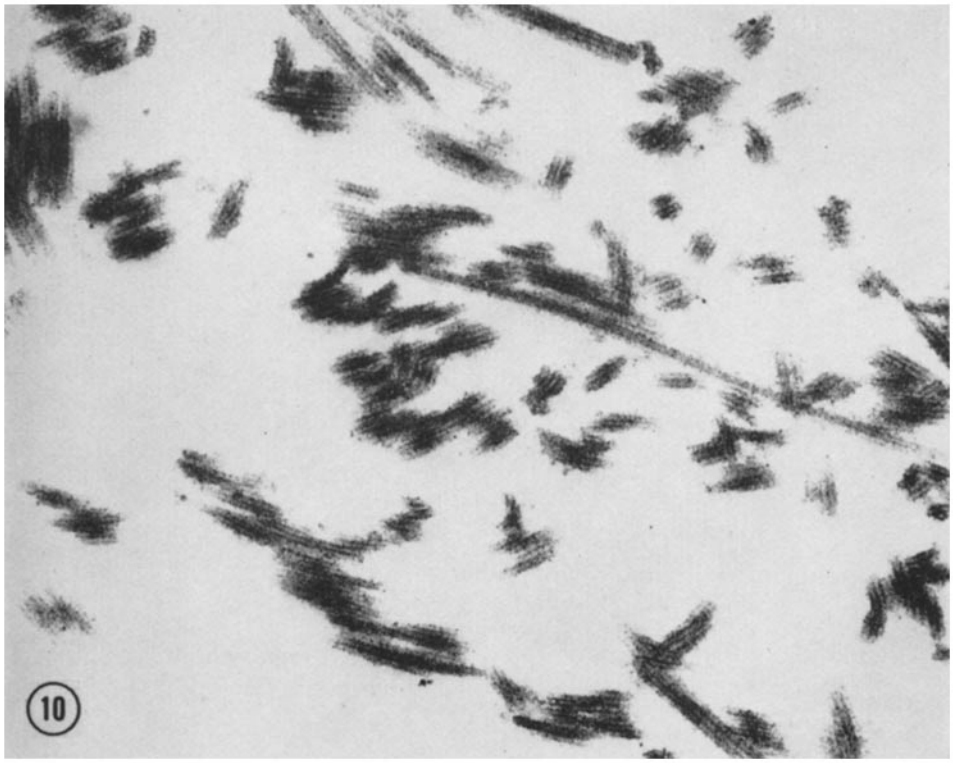
FIG. 8. Thin section of a normal hemoglobin gel. Polymers making up the gel are randomly arranged. They occur singly or in small bundles. On cross-section the polymers reveal hollow circular profiles. The tubular polymers of hemoglobin are essentially identical in appearance with microtubules found in most plant and animal cells. × 36,500.

FIG. 9. A gel of cell-free reduced sickle hemoglobin for comparison with the gel of normal hemoglobin shown in the previous illustration. The area is near the periphery of a radiating mass of sickled hemoglobin polymers. At this magnification the polymers of sickled hemoglobin are quite similar to those in gels of normal hemoglobin, except for the absence of hollow cores. × 37,300.

FIG. 10. Both the normal hemoglobin and phosphate buffer for this example were made up to 1% with metabisulfite before combining to make the gel. The organization is identical with that in gels of sickle and normal oxyhemoglobin. This area was chosen to show the bundles of tubular polymers cut in various planes which apparently constitute the thick fibers seen in the phase microscope. × 38,100.

FIG. 11. A gel of sickle oxyhemoglobin in which bundles of tubular polymers are cut in cross section. Each large bundle consists of single tubules pressed tightly together. The number of tubules in each bundle is quite variable, as can be noted in this example. × 52,200.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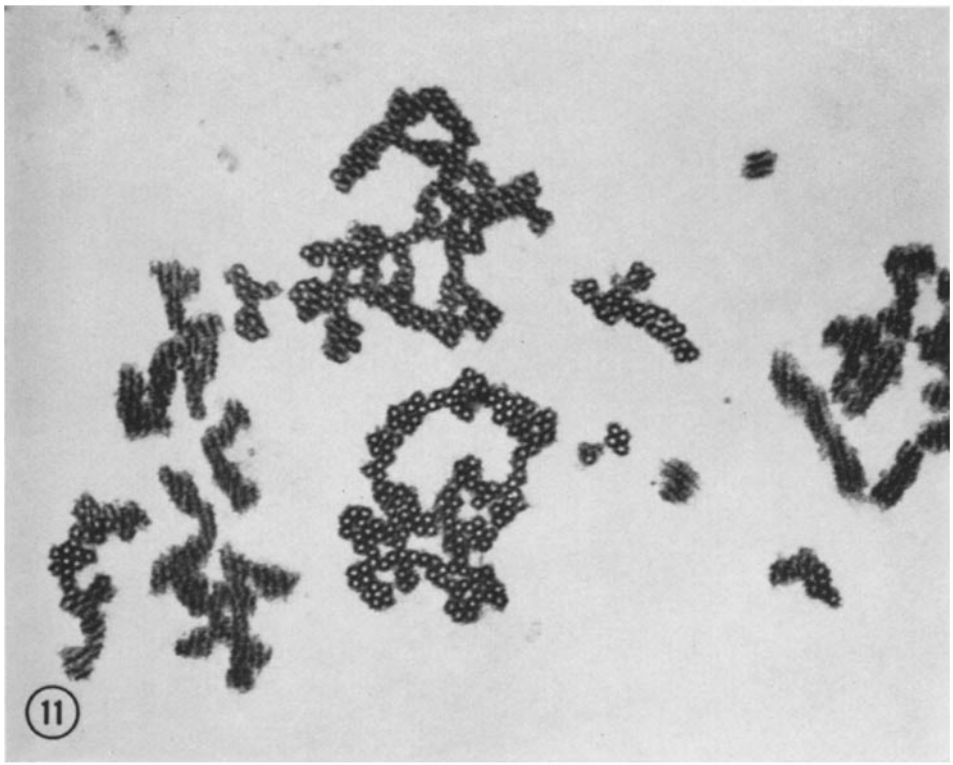
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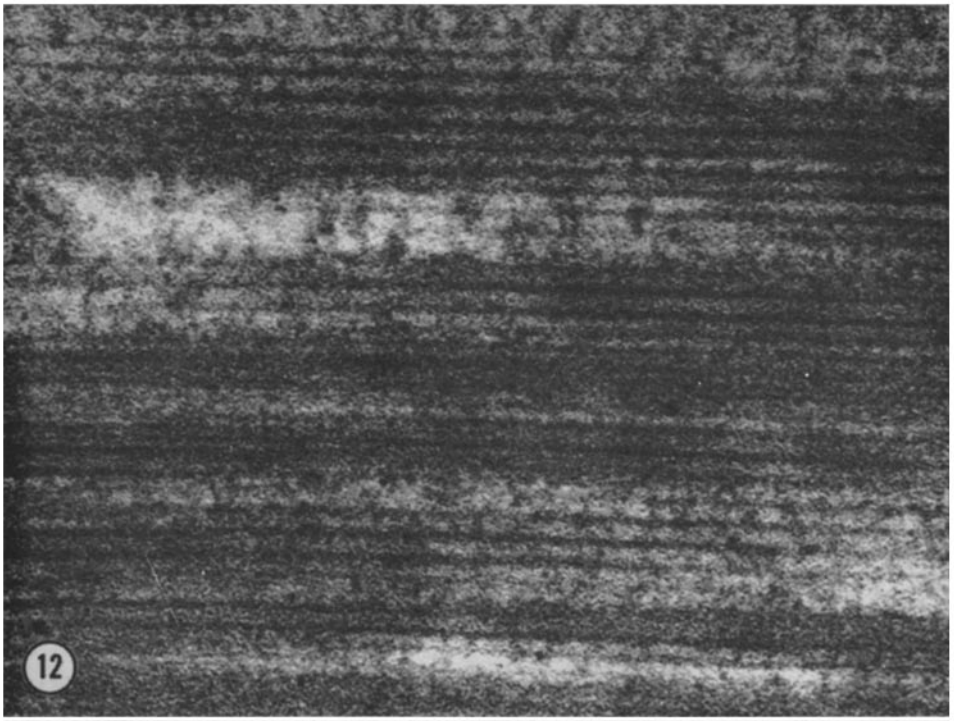

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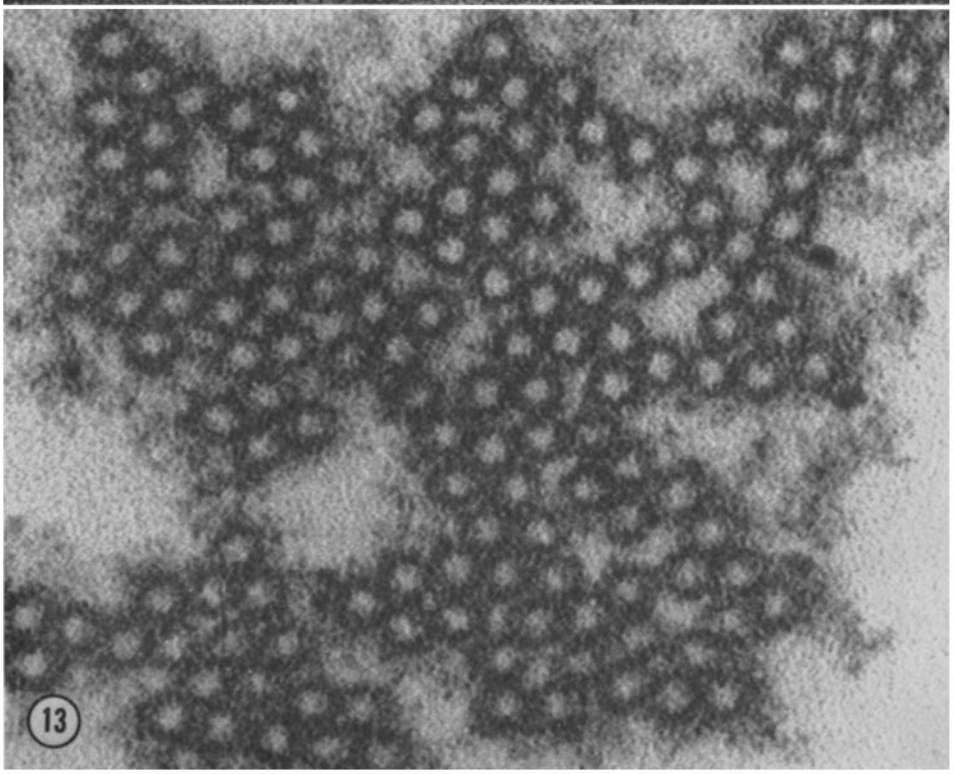

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tion offers a possible solution to this problem. Gels formed from cell-free samples of OHbA, RHbA, and OHbS consisted entirely of hollow-cored polymers arranged singly or in tightly packed parallel bundles. The isolated tubular polymers in these preparations were essentially identical with the microtubules found in nearly all plant and animal cells. Murayama (4) found that sickled hemoglobin assumed the form of microtubules when he examined negatively and positively stained whole mounts of sickled hemolysates that were freeze-dried on the surfaces of carbon-coated grids. The tubular polymers he observed averaged approximately 170 A in diameter. Tubules in our unsickled hemoglobin gels were slightly larger, approximately 240 A in width, but differences in methods may account for this variation.

Stetson's study (3) of intact sickled erythrocytes revealed a series of parallel lines which in cross-section resembled an empty honeycomb. Dark lines surrounding the light cores were roughly hexagonal in shape and so closely packed that subunits of the honeycomb appeared to share the same walls. The diameter of the subunits was 150 A, considerably less than the width of the tubular polymers observed in the present study. Yet longitudinal sections and cross-sections of the bundles of tubules in gels of OHbA, RHbA, and OHbS revealed an appearance remarkably similar to the paracrystalline arrangement described by Stetson.

The gels of reduced sickled hemoglobin did not contain tubular elements resembling microtubules. All of the polymers present in sickled gels were uniform dense rods without hollow cores. These structures were identical with the polymers of HbS observed in sections and whole mounts of intact sickled cells, and in negatively stained whole mounts of the sickled gels (5–10). On the basis of the evidence presented here and results of previous investigations, it seems reasonable to suggest that solid rods represent the product of the sickling phenomenon in vivo and in vitro.

The present study has shown that sickle hemoglobin in the oxy state can

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FIG. 12. A parallel bundle of tubules in a gel of normal hemoglobin sectioned in the longitudinal plane. The tubular polymers of hemoglobin are pressed tightly together. Sickled hemoglobin polymers in cell-free gels and intact sickled erythroyctes do not appear to develop this close relationship. However, the arrangement shown in this illustration resembles closely the longitudinal sections of sickled hemoglobin observed by Stetson in intact cells (3). × 198,200.

FIG. 13. Cross-section of a bundle of tubular polymers present in a gel of normal hemoglobin. At high magnification the closely packed polymers suggest the empty honeycomb arrangement observed by Stetson, but are larger than the structures he identified inside sickled erythrocytes. Each tubule is approximately 240 A in diameter with walls about 60 A thick. The walls may consist of subunits as this illustration suggests, but this has not been determined with certainty. × 324,400.

elaborate into hollow polymers similar to those which others have considered characteristic of sickled HbS (3, 4). However, normal oxyhemoglobin and reduced hemoglobin assembled into identical hollow polymers under the same conditions, whereas reduced sickle hemoglobin formed only dense rods. We are forced to conclude from these observations that tubular polymers are not the result of the sickling phenomenon and have nothing to do with erythrocyte sickling.

The method used in this study to prepare gels of hemoglobin is an adaptation of the procedure Drabkin developed to prepare oxyhemoglobin crystals (11). Murayama (4) employed this method to crystallize sickle hemoglobin which he then dissolved to prepare sickled hemoglobin solutions and the whole mounts containing HbS microtubules. Stetson (3) also modified Drabkin's procedure to prepare crystals of reduced sickled hemoglobin which served to confirm his concept that sickling was due to intracellular crystallization. We carefully evaluated the evolution of reduced hemoglobin crystals prepared by Stetson's technique, and have shown that gel formation is a separate sate which precedes crystallization of HbS (7). Furthermore, the transformation of gels to crystals involves fusion of rods of sickled hemoglobin and complete molecular reorganization into a crystalline lattice (7, 10). These findings separated sickling from crystallization and established the fact that potassium phosphate buffer with metabisulfite could produce gels containing polymers identical with those present in intact sickled cells.

Since a method originally designed to cause crystallization could induce formation of gels of reduced HbS, it was reasonable to apply the same procedure to normal hemoglobin. The gels which formed in cell-free samples of OHbA and RHbA took considerably longer to form than gels of reduced sickle hemoglobin, suggesting that polymerization might be related to denaturation of the hemoglobin. However, the procedure used was identical with that of Drabkin, Stetson, and Murayama in its essentials, and it is doubtful that our slight modification could have resulted in protein damage not evident in their preparations. Furthermore, we have carried out physical and chemical analyses of the hemoglobin solutions, intact gels, and dissolved gels (1). Except for the development of a small amount of methemoglobin in older samples (which can be prevented) the gels are essentially pure hemoglobin protein comparable with hemoglobin standards studied simultaneously. Investigations into the effects of cold, heat, EDTA, heparin, different salt buffers, colchicine, vincristine, urea, and efforts to rule out a role of structural proteins, stromal proteins, and phospholipids in gel and polymer formation have been completed, and are the subject of another report.[2] The results indicate that the gels are composed of undenatured hemo-

[2] White, J. G. and B. Heagan. Chemical and physical properties of normal human hemoglobin gels. In preparation.

globin polymers and, at least in the case of sickled gels, represent a close approximation of the intracellular state of sickled HbS.

How the tubular polymers present in gels of OHbA, RHbA, and OHbS relate to the intracellular state of hemoglobin is not clear. Yet, the polymers provide an entirely new state of hemoglobin for in vitro studies which may be more representative of intracellular hemoglobin than dilute solutions. The hemoglobin gels have been used in this investigation to clarify the nature of the sickled hemoglobin polymer. They may prove even more valuable in defining other hemoglobinopathies causing increased erythrocyte fragility and for understanding the role of hemoglobin molecular interactions in establishing the discoid shape of red cells and removal of senescent erythrocytes from the circulation.

SUMMARY

Electron microscopic studies of the sickling phenomenon have described at least three different structural arrangements of sickled hemoglobin, including empty hexagonal crystals, microtubules, and solid rods. It is unlikely that sickling results in several different polymers, and it is essential to determine the true structure of sickled hemoglobin in order to define the mechanism of molecular assembly. The present study has explored the fine structure of gels formed in cell-free solutions of normal and sickle reduced and oxyhemoglobin. Gels of reduced sickled hemoglobin consisted entirely of solid rods. The gels formed from sickle oxyhemoglobin, normal oxyhemoglobin, and normal reduced hemoglobin contained masses of hollow polymers essentially identical in appearance with microtubules. These findings indicate that solid rods are the characteristic polymers of sickled hemoglobin and tubular polymers represent aberrant structures which are not related to erythrocyte sickling.

Supported by grants from the U.S. Public Health Service No. HE-11880, AI-05153, and CA-08832 and the Cardiovascular Clinical Research Program Project.

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