The American Journal of PATHOLOGY

JANUARY 1970 • Volume 58, Number 1

The Fine Structure of Cell-Free Sickled Hemoglobin

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THE CONVERSION OF SOLUBLE SICKLE HEMOGLOBIN (HbS) into rod-like structures is regarded as the probable basis for the sickling of susceptible erythrocytes.[1,2] Yet, many questions concerning the nature of HbS molecular assembly and the associations between polymers of HbS which lead to deformation of red cells from patients with sickle cell disease remain to be answered. One unresolved problem concerns the form assumed by the polymers of HbS after elaboration. Various workers have described the polymers as rods,[3-5] crystals[6,7] or microtubules.[8]

The existence of several different opinions regarding the fine structure of sickled hemoglobin is puzzling. One explanation may be that different methods used to prepare material for study in the electron microscope could cause structural alterations in hemoglobin polymers. Another possibility is that polymerization of HbS in cell-free solutions leads to formation of structures which differ from those observed in intact sickled cells.

The present study has attempted resolution of the problem by comparing the fine structure of polymers and crystals formed in cell-free solutions of sickled hemoglobin with the rods observed in intact erythrocytes. Results indicate that rods are the characteristic form assumed by polymers of HbS outside as well as inside the sickled cells.

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Supported by Grants HE-11880, AI-05153, and CA-08832, US Public Health Service, Cardiovascular Clinical Research Program Project, Minnesota Heart Association, and the Minnesota Division of American Cancer Society.

Accepted for publication September 20, 1969.

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Published by Medical Department
HARPER & ROW, PUBLISHERS

Materials and Methods

General

The methods used in this laboratory to secure blood, separate cell fractions, and prepare samples for study in the electron microscope were described in detail in previous reports.[4,9,10] Blood was obtained from 2 adults and 1 child with documented homozygous sickle hemoglobin disease for the present study. Erythrocyte rich samples obtained by differential centrifugation were washed 3 times and resuspended in buffered saline. Small samples were sickled by reducing oxygen tension, then bubbling with carbon dioxide, or combining with an equal volume of 2% sodium metabisulfite. The sickled erythrocytes were fixed for electron microscopy as previously described.[4,9,10]

Hemoglobin Solutions

The method described by Drabkin[11] was used in a modified form for preparation of cell-free solutions of sickle hemoglobin. Triply washed erythrocytes separated from 50–100 cc of blood from patients with sickle cell disease were packed by centrifugation at 10,000 *g* for 10 min. Supernatent salt solution was removed and replaced by an equal volume of distilled water and 0.4 %by volume of toluene to secure hemolysis. The samples were refrigerated for 1–72 hr, and the tolune and membrane layer removed. Hemoglobin concentrations in the several samples varied between 6.4 and 12.8 g%.

Small portions of the cell-free HbS were sickled by the techniques used to induce sickling in intact cells.[4,10] Other samples were made to 1% with sodium metabisulfite, and 2.8 M sodium phosphate buffer in 1% sodium metabisulfite (pH 6.8) was added drop by drop according to the method suggested by Stetson.[7] Turbidity developed in these solutions after approximately 2 ml of buffer had been combined with 1 cc of HbS solution. The precipitate was removed by centrifugation. One or two more drops of buffer were added to the supernate and the tube shaken. Within a few minutes the solution became turbid again. Drops of this material or the initial precipitate were placed on glass slides under coverslips, or fixed after centrifugation for study in the electron microscope.[4,10]

Crystals of sickled hemoglobin were obtained by adding a slight excess of buffer to the HbS solution beyond that required to produce a sol-gel transformation.[7] The material was prepared 2–3 hr later for study in the electron microscope or drops were placed on glass slides for study by phase optics.

Results

Gels of HbS manifested a variety of associated filamentous structures under coverslips in the phase contrast microscope (Fig 1–4). A few fine filaments arranged in the form of an "X" and tufts of fibers resembling bow ties were the first structures to appear (Fig 1). Larger concentrations of fibers were grouped in clusters, sheaves, or in radial arrangements forming complete circles (Fig 2 and 4). The small bundles of fibers and pinwheel arrangements were birefringent when examined with polarizing optics. Bundles of parallel fibers were rarely observed unless the whole solution converted to a gel. This occurred only when the initial hemoglobin concentration exceeded 10 g% (Fig 3).

The structural arrangements of sickled material from cell-free HbS prepared for electron microscopy resembled the sheaves or pinwheel patterns observed with phase optics (Fig 5). Thin fibers radiated in all directions from a central hub or axis (Fig 6 and 7). At higher magnification the uniform structure of HbS was apparent (Fig 8). Each fiber was 170–200 Å in diameter. Cross sections revealed a uniformly dense substructure. Only rare examples of filaments 65 Å in diameter were observed. Hollow structures resembling microtubules were not apparent.

Crystals formed from sickled hemoglobin were studied in the phase and electron microscopes. Samples of sickled cell-free HbS converted from gels to crystals as the samples dried under coverslips (Fig 4C and D). Pinwheel arrangements of HbS retained their form but appeared more rigid. In the electron microscope the starburst pattern of fibers radiating from the central hub was also preserved (Fig 9). The fibers of crystalized sickled hemoglobin differed significantly from the uniform rods in the sickle-gel (Fig 10). In cross sections the fibers were square, rectangular, or rhomboid in shape. At the periphery of the radiating spokewheel patterns the crystals were rigid compared to the undulating rods observed in gels. Parallel lines with a periodicity of 65–70 Å were evident in the substructure of the crystals, and in many examples the lines formed intersecting patterns characteristic of a crystalline lattice. Large block-like crystals were also present which were identical to crystals of normal hemoglobin.[9]

Discussion

A principle aim of this investigation was to resolve the question as to whether the polymers formed by molecules of reduced HbS were rods, crystals, or microtubules. Pauling *et al*[1,12] were first to suggest that sickling might be due to formation of HbS rods. This theory was substantiated by Harris[2,13] who observed spindle shaped bundles of rod-like particles lying parallel and equidistant to each other in stroma-free solutions of sickled hemoglobin. Stetson[7] also studied cell-free solutions of sickled hemoglobin. He observed that sheaves of needles resembling crystals formed in the presence of salt and a reducing agent. Similar structures were identified in thin sections of intact sickled erythrocytes, leading to the conclusion that sickling was due to crystalization. Murayama[8] examined the fine structure of polymers formed in cell-free solutions, and identified a different structural arrangement of HbS. He found hollow-cored polymers in freeze-dried whole mounts which appeared identical to microtubules. As a result, he suggested that sickling was due to aggregation of HbS molecules into microtubules which de-

formed the shape of susceptible erythrocytes. More recent studies of the fine structure of HbS in sections of intact sickled cells have failed to demonstrate microtubules or structures with the typical triaxial lattice of crystals.[3-5,9,14] The apparently contradictory observations on the fine structure of sickled hemoglobin need to be clarified if the mystery of molecular assembly underlying erythrocyte sickling is to be solved.

Results of the present investigation support the concept that polymers of HbS are rods. Polymers which developed in solutions of HbS exposed to low oxygen tension or reducing agents were identical to the rods observed previously in intact sickled erythrocytes.[3,4,10] Structures resembling microtubules were not apparent in the gels. The absence of empty circular profiles in sections of stroma-free sickled hemoglobin and intact erythrocytes argues against the microtubule as the basic structure of sickled HbS.

The method described by Stetson[7] was employed with slight modifications in this study to define the role of crystalization in the sickling phenomenon.[6] It rapidly became apparent that the technique produced gels indistinguishable from those induced by low oxygen tension and reducing agents, as well as crystals. When examined in the phase microscope the gels and crystals were practically indistinguishable. In fact, HbS gels underwent transformation to crystals if allowed to dehydrate under coverslips overnight. The basic structural difference was apparent in the electron microscope. Arrangements of fibers were similar in the gels and crystallized samples, but the fine structure of radiating elements differed completely. Whereas gels contained uniform rods without internal structure, the long fibers of the crystals revealed the characteristic triaxial lamellar substructure of crystalline lattices.[9] These findings argue against the concept that HbS polymers are crystals.

The findings of this study support the concept that sickling involves a sol-to-gel transformation of soluble sickle hemoglobin into polymers which are best described as rods. In addition, the observations on gels formed in stroma-free solutions of HbS offer insight into the association of polymers which may be a fundamental factor in the sickling phenomenon. Harris'[2] finding that the cell-free solutions of sickled hemoglobin contained spindle-shaped bundles of rod-like particles gave substance to the sol-gel theory of erythrocyte sickling. A major factor in his conclusions was the observation that rods making up the spindle-shaped bundles were aligned parallel and equidistant to each other. Similar bundles of rods arranged in parallel association have been observed in intact sickled cells.[4,5] These findings suggest that interaction of polymers of HbS inside intact cells and in stroma-free solutions preferentially causes

formation of parallel bundles. Identification of the physical or chemical forces favoring alignment of polymers parallel and equidistant to each other has not been made.

The HbS hemolysates examined in this study did not approach the concentrations evaluated by Harris.[2] Samples containing greater than 10 g% of HbS did develop masses of fibers arranged parallel to each other. At lower concentrations in which polymers could interact freely, however, parallel arrangements were not evident. The clusters of HbS polymers consistently assumed radial patterns of various sizes. When few fibers were present a criss-cross pattern resembling the letter "X" was produced. Larger numbers of fibers formed patterns resembling bow ties, sheaves of wheat or complete circles of radiating elements. It was clear from electron microscopic evaluation that the radial patterns were three dimensional in nature with rods extending from a central axis in all directions.

These findings suggest that HbS polymers do not necessarily assume parallel associations. The concentration of hemoglobin may be the major factor in formation of parallel bundles of rods, rather than unknown forces between polymers favoring that alignment. At the concentrations used in this study interaction between polymers was clearly apparent, but rotational forces resulting in radial arrangements dominated the picture. The tendency of rods to spread rather than align parallel to each other may be very important. Sickling results from stress imposed on the cell wall by rods of HbS which overcome the resistence maintaining biconcave shape. If parallel orienting forces are dominant, all polymers should form a single massive bundle elongating erythrocytes into the spinde-shaped configurations observed by Harris[2] in cell-free solutions. The crescent shaped cells from which the disease derives its name support that possibility. Yet many sickled cells are distorted in multiple planes, and organization of polymers into parallel bundles is not always apparent.[3–5,10,14] Rotational forces favoring radial rather than parallel association could provide the stress necessary to overcome membrane resistance at multiple sites. The findings described here support this possibility. Additional evidence favoring this concept would be provided if removal of the membrane of sickled erythrocytes was followed by preferential rearrangement of parallel bundles of polymers into radial patterns. That rotational forces do indeed overcome the parallel orienting factors in bundles of HbS polymers will be demonstrated in a subsequent report.

Summary

The fine structure of stroma-free sickled hemoglobin and crystalized sickled hemoglobin have been examined. Rods identical to those observed in intact sickled cells were the basic structural units of the stroma-free gels. Polymers with hollow central cores resembling microtubules were not observed. Radiating elements in crystalized samples of sickled hemoglobin differed fundamentally from rods observed in gels and intact erythrocytes, supporting the concept that sickling is not due to crystalization. In stroma-free solutions, fibers of sickled hemoglobin tended to form radial arrangements rather than parallel bundles or tactoids.

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[*Illustrations follow*]

Legends for Figures

Fig 1. *(top)* Sample of cell-free sickled hemoglobin (HbS) prepared by addition of sodium phosphate buffer to hemolysate at 8.4 g% in presence of sodium metabisulfite. Dilution of hemoglobin concentration results in formation of scattered clumps of sickle gel. Tendency toward radial arrangement of filaments is apparent in small and large clusters. Phase optics. × 800.

Fig 2. *(middle)* Gel formed by drop of cell-free HbS containing 10.2 g% by sealing under cover slip for 16 hr. HbS polymers are concentrated in clusters. Radial pattern of polymers is evident in this example. Phase optics. × 1800.

Fig 3. *(bottom)* stroma-free gel of HbS prepared in same manner as sample in Fig. 2. Hemoglobin concentration was 12.8 g%. Polymers of HbS are gathered in parallel masses and produce wave-like patterns. Spindle-shaped bundles of parallel rods were not evident in these preparations. Phase optics. × 1800.

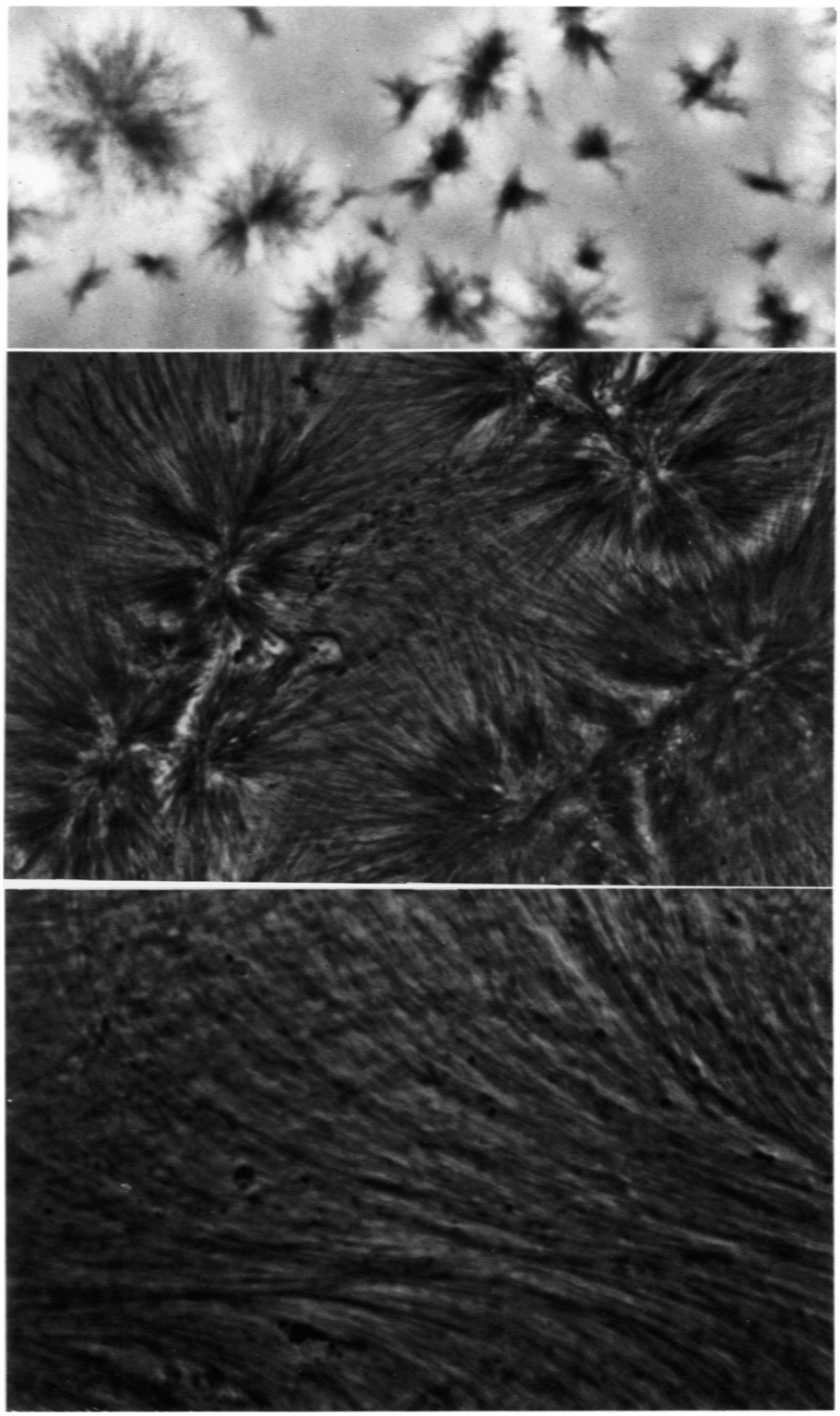
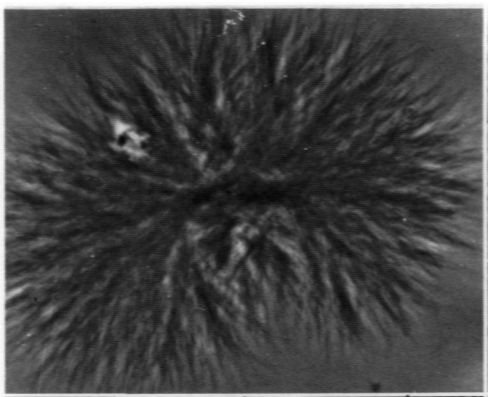


Fig 4. Examples of radial clusters of sickled hemoglobin gel and crystalized gel. Cluster in **A** has sheave of wheat pattern, while that in **B** displays complete radial configuration. Radial arrangements in **C** and **D** are crystalized gels of HbS. Phase optics. × 1800.

Fig 5. Thin section of sickled hemoglobin gel. Appearance at low magnification in electron microscope is similar to gel illustrated in Fig 4A. Polymers are concentrated in central area and radiate in all directions. × 6250.

4A



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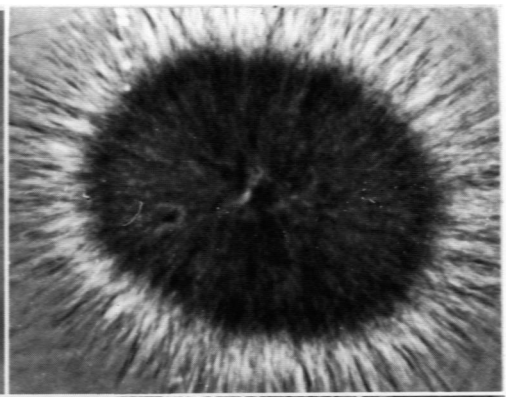
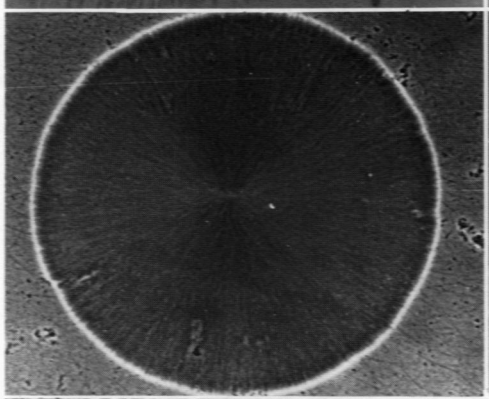
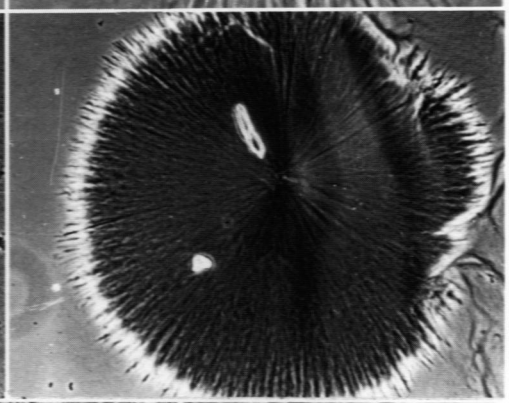


4C



4D



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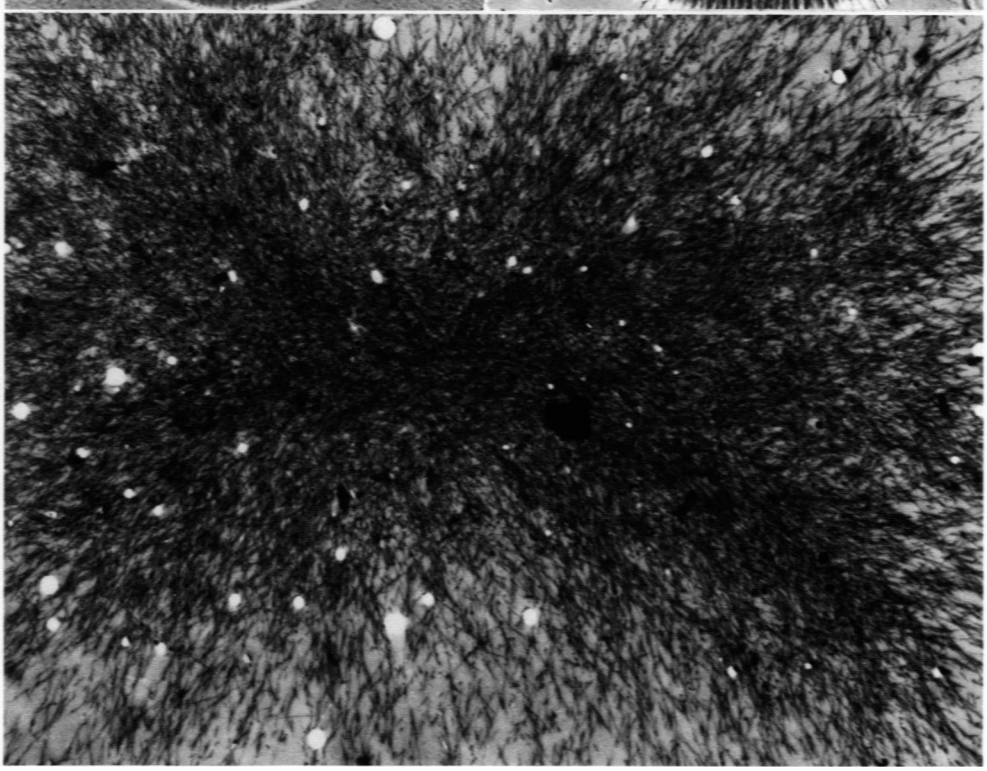
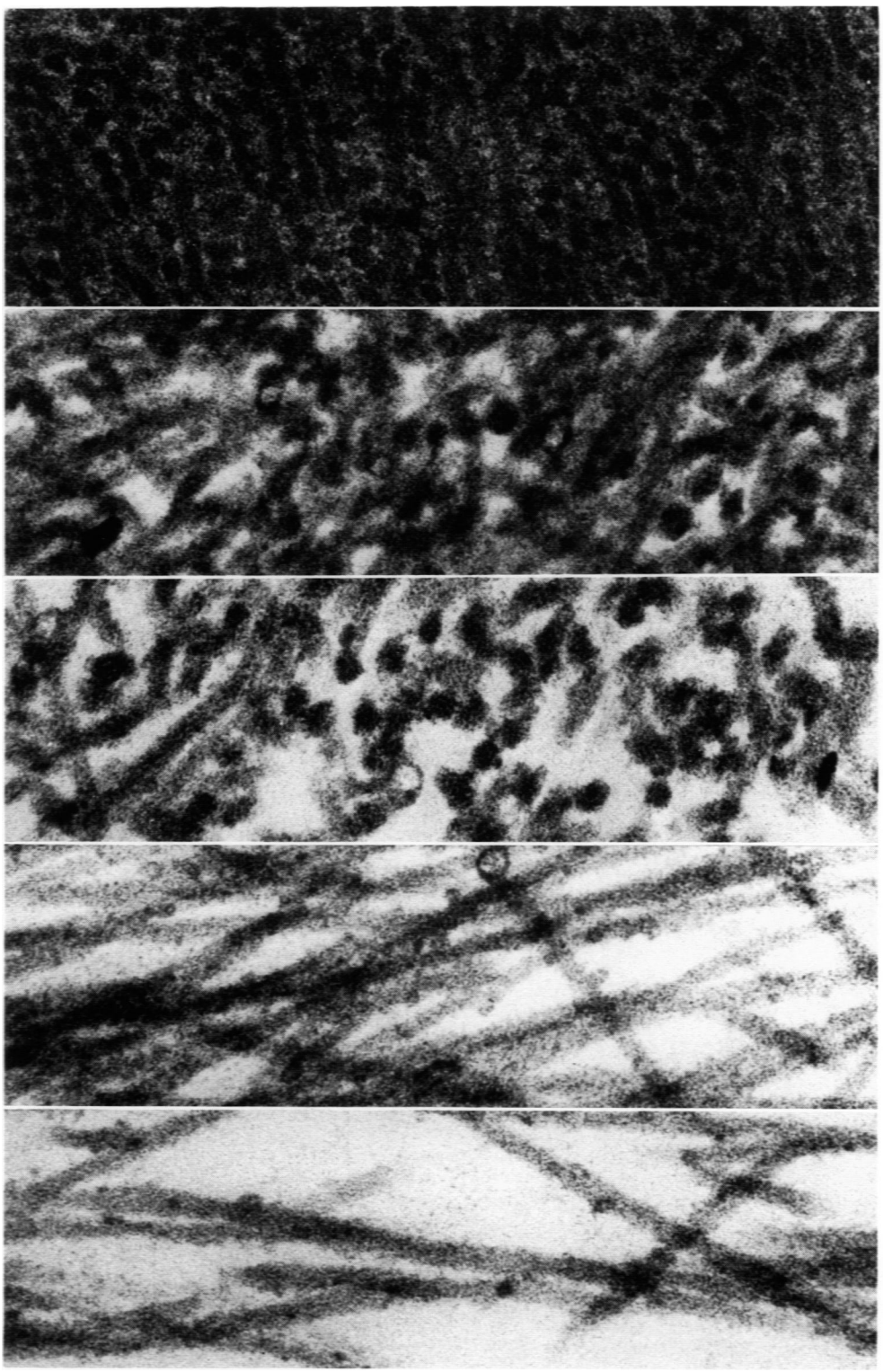


Fig 6. *(upper)* Higher magnification view of another sample of stroma-free sickled hemoglobin. Central area of cluster is shown. Polymers of HbS are cut at multiple angles as they spread outward from center. × 35,300.

Fig 7. *(lower)* At periphery of cluster shown in previous illustration, rods lie primarily in section plane. As they spread outward from center (which is toward upper right of Figure), rods become fewer in number and farther apart. Tendency toward angular interaction is apparent between rods in center and at periphery. Radial organization of rods in clusters was characteristic of all gels examined. × 39,300.

Fig 8. Compact mass of polymers in **A** is from an intact sickled erythrocyte. **B–E** are high magnification illustrations of stroma-free sickled hemoglobin representing interval steps from interior to periphery of radiating cluster of polymers. Tendency toward angular relationships rather than parallel associations appears to dominate interaction of rods of HbS. Fine structure of polymers in stroma-free gels of sickled hemoglobin is identical to that of rods of HbS inside sickled erythrocytes. **A** × 74,500; **B–E** × 129,500.



8A

8B

8C

8D

8E

Fig 9. *(upper)* Sample of sickled hemoglobin gel allowed to crystalize in presence of sodium phosphate buffer and sodium metabisulfite. Cluster is arranged in same manner as in gels with polymers radiating in all directions from central axis. Polymers of HbS are larger and more rigid in appearance and are square or rectangular rather than round on cross section. The inset shows typical radiating crystal pattern viewed in phase contrast microscope. × 7500; **Inset** × 1600.

Fig 10. *(lower)* Appearance of crystalized sickled hemoglobin at higher magnification. Periodic patterns *(arrows)* in substructure of polymers indicate crystalline lattice. × 155,400.