Sickling Reversed and Blocked by Urea in Invert Sugar

Optical and Electron Microscopic Evidence

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Optical and electron microscopic evidence is presented to support the finding that sickling of hemoglobin S can be reversed and blocked by urea in invert sugar (UIS). Erythrocytes from subjects having hemoglobin SS, AS or AA were treated with the UIS either before or after deoxygenation with $Na_2S_2O_5$. Light microscopic studies indicated that approximately one-fifth as much urea is required to block sickling as is necessary to reverse sickled poikilocytes to normal forms. Intracellular microfilaments apparent in transmission electron micrographs of sickled erythrocytes were eliminated by treating aliquots of the same deoxygenated erythrocytes with UIS. Scanning electron micrographs showed a reversion of sickled poikilocytes to a normal erythrocyte population of biconcave discs. The use of UIS was deduced from Murayama's hypothesis that the molecular mechanism of sickling clearly involves hydrophobic bonds formed between the number-6 valine substitution of the β-chain S globins and the α-chain globins of interacting hemoglobin molecules. The use of UIS to arrest the formation of such hydrophobic bonds is advocated as an evident and effective therapeutic strategy to treat sickle cell crisis. (Amer J Path 64:405–422, 1971)

The purpose of this paper is to present optical and electron microscopic evidence that sickling can be both reversed and blocked by the use of urea in invert sugar (UIS) (Urevert, Ureaphil). The molecular basis for the proposed therapeutic use of urea clinically in hemoglobin SS crisis is demonstrated and discussed.

Materials and Methods

Wet Sickle Cell Preparations for Optical Microscopy

Throughout this paper, $Na_2S_2O_5$ was used in a 2% solution to produce sickling. UIS was constituted and used according to the manufacturer's direction. All

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Supported by the Michigan Heart Association and grant GM 17367-01 from the US Public Health Service.

Accepted for publication April 14, 1971.

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molar concentrations of urea in this paper are expressed in terms of the final working solution.

Transmission Electron Microscopy (TEM)

Blood cells from 4 patients with either sickle cell anemia or sickle cell trait were fixed by the addition of 6.5% cacodylate-buffered glutaraldehyde for 4 hours.[1] Erythrocytes were fixed either promptly after venipuncture, after anticoagulation with sodium citrate or EDTA, or at the end of 1 hour of exposure to an experimental biochemical environment. After washing 2 times with buffered sucrose, the cells were collected by light centrifugation and resuspended by adding warm 3% Bactoagar.[2] The agar-erythrocyte mixture was drawn into a disposable Pasteur pipet and held there briefly until cooling permitted extrusion of a gelated cylindric mass of cells onto a glass slide. The solidified cylinder of erythrocytes was then cut into smaller cylinders for future processing. Cylinders were either stored in buffered 0.2 M sucrose overnight in the refrigerator and/or fixed for 2 hours at room temperature in 1% osmium tetroxide buffered with Veronal acetate.[3] Cylinders were dehydrated by passing them through a series of graded ethanol mixtures (50%, 75%, two passes in 95% and two passes in 100%) for 15 minutes each. The cylinders were immersed next in propylene oxide for 15 minutes with one repetition before placing in a mixture of 50% Maraglas 655 and 50% propylene oxide for about 2 hours. Cylinders were then placed in a complete Maraglas mixture overnight in the refrigerator. After the cylinders reached room temperature, fresh complete Maraglas mixture was substituted and with specimens in Beem capsules, they were cured at 60 C for 16–48 hours. Thin sections were prepared on a Porter-Blum MT-1 ultramicrotome, using a diamond knife. Sections were doubly stained with 1% uranyl acetate for 1 hour[4]; after washing 3 times in distilled water, they were finally stained with lead citrate stain applied for 10–15 minutes.[5] Viewing and photography was with an RCA EMU 4 electron microscope operated at 50 kV.

Scanning Electron Microscopy (SEM)

Fresh, whole blood anticoagulated with 3.2% sodium citrate was fixed immediately or after an experimental biochemical procedure or after a control incubation period. To 1 ml of blood, 5 ml of buffered glutaraldehyde was added. After a fixation period of 2–4 hours, cells were collected by light centrifugation, and washed 2 times with buffered sucrose for 30 minutes each, then washed with distilled water 3 times, placed in Parducz fixative for 15 minutes, [6] washed again in distilled water and finally exposed to 70% ethanol for 20 minutes to partially dehydrate the erythrocytes. Smears were prepared on glass slides and permitted to dry in air. Squares cut from the glass slide were sealed to copper specimen rods. These preparations were rotationally coated in an Edwards vacuum evaporator, first with a fine film of carbon and then by a thin coating of gold-palladium metal.

Observation and photography was made with a Jeolco scanning electron microscope operated at 25 kV with the specimen tilted at a 45° angle.

Results

Optical Microscopy

Reversal of Sickling

Sickled poikilocytes of both homozygous and heterozygous S from several patients, which were produced by the standard technic with

$Na_2S_2O_5$, were used as controls (Fig 1). An essentially total reversal of sickled poikilocytes was obtained within 10 minutes by addition of UIS to freshly drawn sickled blood (Fig 2). Cells from the same patient treated, but 2 days or more old, demonstrated the persistence of an occasional sickled poikilocyte. However, the great majority of cells in the system still reverted to the normal erythrocyte configuration after treatment. Studies were not done to investigate whether such cells, which were apparently refractory to short-term treatment with UIS, consisted of "irreversibly sickled cells." Nor were studies made on such cells to determine whether prolonged exposure to UIS would eventually reverse the sickling. The hemoglobin of the easily reversed sickled poikilocytes is a nematic liquid-crystal system when sickled and is thus susceptible to immediate response to chemical agents. The molecular state of the hemoglobin in the irreversibly sickled cells still remains to be elucidated.

The reversal of sickling by UIS was a rapid, reproducible event as observed *ex vivo* through the optical microscope.

Blocking of Sickling

UIS will also block the sickling event in susceptible cells. Lower concentrations of urea are required for blocking than for the reversal of the sickling event. Freshly drawn, homozygous S erythrocytes incubated at room temperature for 20 minutes in UIS at concentrations as low as 0.2 M regularly failed to sickle with the addition of appropriate quantities of $Na_2S_2O_5$. Untreated cells from the same individuals sickled promptly when exposed to 2% $Na_2S_2O_5$ as expected. The blocking of the sickling event in SS or SA cells by the use of UIS was a simple, regularly reproducible demonstration as observed *ex vivo* through the optical microscope.

These procedures, events and observations have been repeatedly confirmed in our separate laboratories. The use of UIS in the above investigations never resulted in hemolysis.

Transmission Electron Microscopy (TEM)

Ultrathin sections of SS erythrocytes deoxygenated by $Na_2S_2O_5$ revealed two populations of cells typified by the presence or absence of intracellular microfilaments. Many erythrocytes had relatively homogeneous and coarsely granular interiors that were indistinguishable from the fine structure of normal erythrocytes.

The other type of erythrocyte, the sickled poikilocyte, displayed microfilaments in addition to areas of homogeneity. Microfilaments

were both randomly dispersed and organized into ordered patterns of parallel fibers especially near cell surfaces (Fig 3). Such transformation of the sickled erythrocyte's interior varied in amount but rarely filled the section. Depending on the internal organization of the microfilaments and the plane of sectioning, they appeared as longitudinal elements or as cross-sectioned electron-dense structures. There was a suggestion of internal organization of elements within the microfilaments (Fig 4). Microfilaments were not found in erythrocytes from normal subjects nor from the one patient examined with sickle cell trait (hemoglobin AS).

Examination of sickled erythrocytes exposed subsequently to 1.66 M UIS demonstrated a loss of the microfilaments and reversion to a homogeneous cytoplasm (Fig 5) identical to that observed in normal erythrocytes.

Scanning Electron Microscopy (SEM)

Views of the surface detail on membranes of erythrocytes taken from 3 subjects with sickle cell anemia (hemoglobin SS), 1 subject with sickle cell trait (hemoglobin AS) and normal volunteers (hemoglobin AA) were obtained.

Treatment with $Na_2S_2O_5$ of erythrocytes from all 4 subjects with SS hemoglobinopathy resulted in a variety of sickled poikilocytes in contrast with the typical biconcave disc configuration from normal subjects. In the latter, 86% of the erythrocytes appeared as biconcave discs.

The number of sickled poikilocytes and extent of erythrocyte deformity related (1) to the time permitted for production of deoxygenated hemoglobin, and (2) to the molecular species of hemoglobinopathy. Erythrocyte shapes ranged from the normal biconcave discs through flattened banana shapes, boat shapes, holly leaf forms, crescent shapes and oat seed forms. Often among such poikilocytes, some displayed one or more surface projections that were bulbous or had long narrow strands. Some cells were swollen and the number of smooth spheres was increased especially in the specimens anticoagulated with EDTA.

A differential erythrocyte count on the basis of shape was made on the sickled specimen of patient FK (SS hemoglobin) collected in EDTA. After chemical deoxygenation, 53% of the erythrocytes were observed to have bizarre forms; 24%, smooth spheres and only 23% existed as smooth-surfaced biconcave discs (Fig 6). Some of the bizarre

forms showed roughening of membranes with the wrinkling reminiscent of underlying filamentous organization.

The sickled poikilocytosis of the erythrocytes of all 4 subjects with hemoglobinopathy was reversed by UIS *ex vivo*. Although reversion to the normal surface configuration of the sickled erythrocytes was not achieved in every single cell, the normal pattern of biconcave discs was evident in the great majority (Fig 7). For example, the sickled specimen from FK (see above) converted in the UIS environment from 53% bizarre forms to 7% while the biconcave disc population rose from 24% to 86% (the percentage observed in normals).

Thus, there is optical and/or electron microscopic evidence that the molecular mechanisms of sickling can be both reversed and blocked by the use of UIS. Our preliminary work with wet sickle cell preparations examined by optical microscopy suggests that only one-fifth as much urea and probably less is required to block sickling as to reverse previously sickled cells.

Discussion

We have confirmed by TEM the existence of both random and ordered arrays of hemoglobin microfilaments in sickled erythrocytes as previously suggested by Murayama[7] and demonstrated by White,[8] Bertles and Dobler[9] and Stetson.[10] Such microfilaments were not observed in cells sickled by $Na_2S_2O_5$ and reversed by UIS. In addition, UIS treatment converted sickled cells to normal forms as observed by SEM. The SEM is proving to be a valuable tool in considerations of protein conformation, energy supply and membrane alterations attended by changes in erythrocyte shape. Clarke and Salsbury[11] presented the first SEM pictures of sickled cells. Jensen[12] documented the changes in shape of erythrocytes as they desickled in response to oxygenation. Rebuck *et al*[13] first reported long tails on many sickled cells by using TEM on whole-mount preparations of erythrocytes. These elongated ends were again demonstrated with SEM by Farnsworth *et al*.[14] That biochemical induction of enzyme-deficiency states can alter erythrocyte shape was reported by Barnhart *et al*.[15]

Theories, principles, data and molecular mechanisms supporting the use of UIS in the treatment of SS crisis have been previously published by us elsewhere.[16–18] Briefly, our rationale for the use of UIS is based on the following analysis. Murayama's hypothesis for the molecular mechanism of sickling clearly implicates the role of the hydrophobic bond. This hypothesis was confirmed by our successful development of the Murayama Test, a simple visual method for the

detection of the hydrophobic bond. Recently, in the biochemical literature, hydrophobic bonds have been recognized to be of primary importance in maintaining the integrity of the tertiary structure of proteins in aqueous protein systems.[19] Very recently in the same literature, urea has been recognized as being capable of breaking hydrophobic bonds, its long-known capacity to break hydrogen bonds notwithstanding.[19–21] Since urea (1) has molecular dimensions small enough to pass through the intact red blood cell membrane, (2) is essentially metabolically inert, (3) is nontoxic, (4) is water soluble and (5) has the ability to disrupt hydrophobic bonds, it was predicted that urea should be an effective chemical agent for desickling if used while the sickled hemoglobin in the intact red cell was in the nematic liquid-crystal state. We have provided optical and electron microscopic evidence in this paper that such is indeed the case. UIS, in fact, both *blocked* and *reversed* sickling in homozygous S, intact erythrocytes when they were deoxygenated by $Na_2S_2O_5$.

At present, we believe that UIS is an effective chemical desickling agent because the urea molecules require less energy to form new hydrophobic bonds with the hydrogen groups of the substituted valine residues at the number 6 position of both β-chain SS globins of each hemoglobin S tetramer. In addition, we believe urea forms hydrophobic bonds with the yet unidentified amino acid residues in the reciprocal combining sites of the α-globins in the interacting tetramers. Thus, the effect of urea is to induce steric alterations in the configuration of the hemoglobin S tetramer and, consequently, steric hindrance to tetramer-tetramer interaction.

Javid and co-workers developed the use of UIS (a preparation which unlike urea alone does not cause hemolysis) for the treatment of intracerebral edema.[22–24] Dr. Javid's personal experience with UIS now exceeds 2000 cases of intracerebral edema, providing us with strong evidence for the safety of this proposed therapy for sickle cell crisis. Precautions to be observed in the use of IV urea in this preparation according to Dr. Javid include the following: (1) avoidance of cutaneous infiltration of the urea preparation, which may cause severe dermal necrosis; (2) maintainance of a recumbent posture during and after treatment to avoid headache and (3) attention to fluid and electrolyte regulation. Ancillary regimens can be added to this basic therapy.

In our search of the literature, we have found only one paper, by Ponder and Ponder,[26] recording the use of urea to inhibit sickling in intact red blood cells. They used urea as an aqueous solution in several different concentrations and successfully reversed sickling in intact

erythrocytes. However, marked hemolysis was observed. Thus, while our theories and experiments concerning the use of UIS both to reverse and to block sickling were pursued at a time when we were entirely unaware of this paper, we find our results with UIS in corroborative support of the prior observations of Ponder and Ponder. Furthermore, we have found UIS to be an excellent desickling agent which does not cause hemolysis.

There is now sufficient theoretic and experimental as well as therapeutic evidence for the careful clinical trial of UIS for the treatment of bona fide sickle cell crisis. In such studies, UIS should be the exclusive therapeutic agent used, except for fluid and electrolyte replacement.

The therapeutic elevation of blood urea levels is not the metabolic equivalent of the profound physiologic dislocation of clinical uremia. In clinical uremia and sickle cell crisis, adverse physiologic factors may inhibit and presumably override the desickling action of urea, masking its beneficial effects. While, at first glance, one may expect that our proposed treatment of sickle cell crisis by the infusion of UIS should be contraindicated in those patients with renal insufficiency, an intriguing argument to the contrary can be made. In 1953, Merrill [27] demonstrated by dialysis of uremic patients against a high urea gradient that such patients became asymptomatic while retaining their original urea blood levels. Accordingly, we suggest that a patient in SS crisis with renal insufficiency be dialyzed against a high urea gradient. As the removal of the noxious metabolites is achieved by dialysis, the endogenously retained urea at an appropriate blood level may be expected to be effective in terminating the sickle cell crisis.

Data from our bona fide cases of sickle cell crisis successfully treated by UIS [28] suggest that the effective urea blood levels are of the order of 100–150 mg%.

Based on the experience of our cases, a therapeutic lag of several hours may be anticipated in clinical trials between the onset of the infusion of UIS and the termination of pain and/or hemolysis. This therapeutic lag, we believe, is due to the slow rate of diffusion to tissures physiologically compromised by sickling in the vascular system. Until urea is sufficiently concentrated at effective levels within erythrocytes in such regions and until the majority of the hemoglobin S molecules there are combined with urea in a molar ratio of probably 1:4 or more, no reversal of sickling in the obstructed circulation, and thus clinical improvement, should be expected. Again on the basis of our

successfully treated cases, the interval of therapeutic lag appears to be from 8 to 10 or 12 hours.

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[*Illustrations follow*]

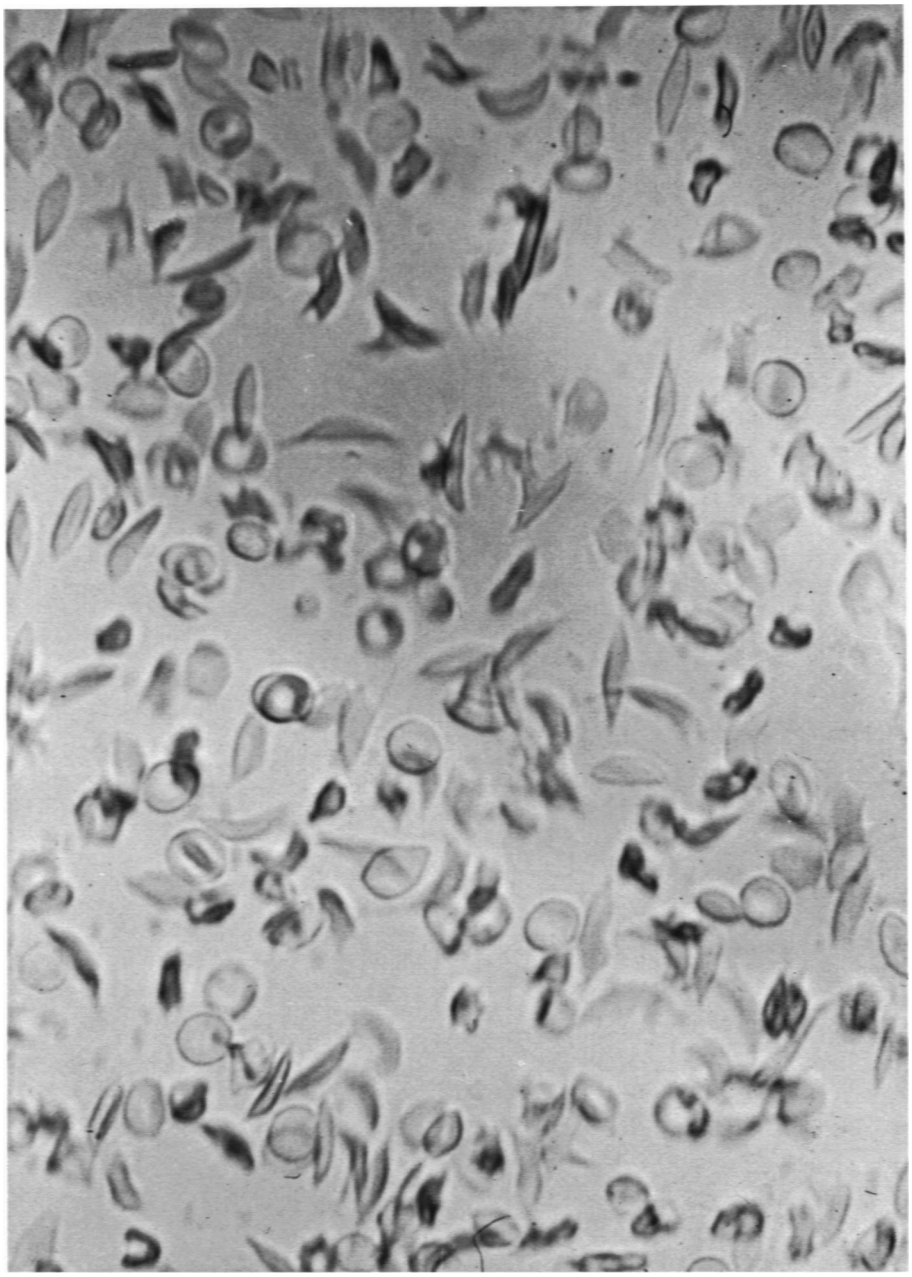

Fig 1—Typical pattern of sickled poikilocytes produced by 2% $Na_2S_2O_5$ in freshly drawn blood from a homozygous S patient (wet preparation, × 210).

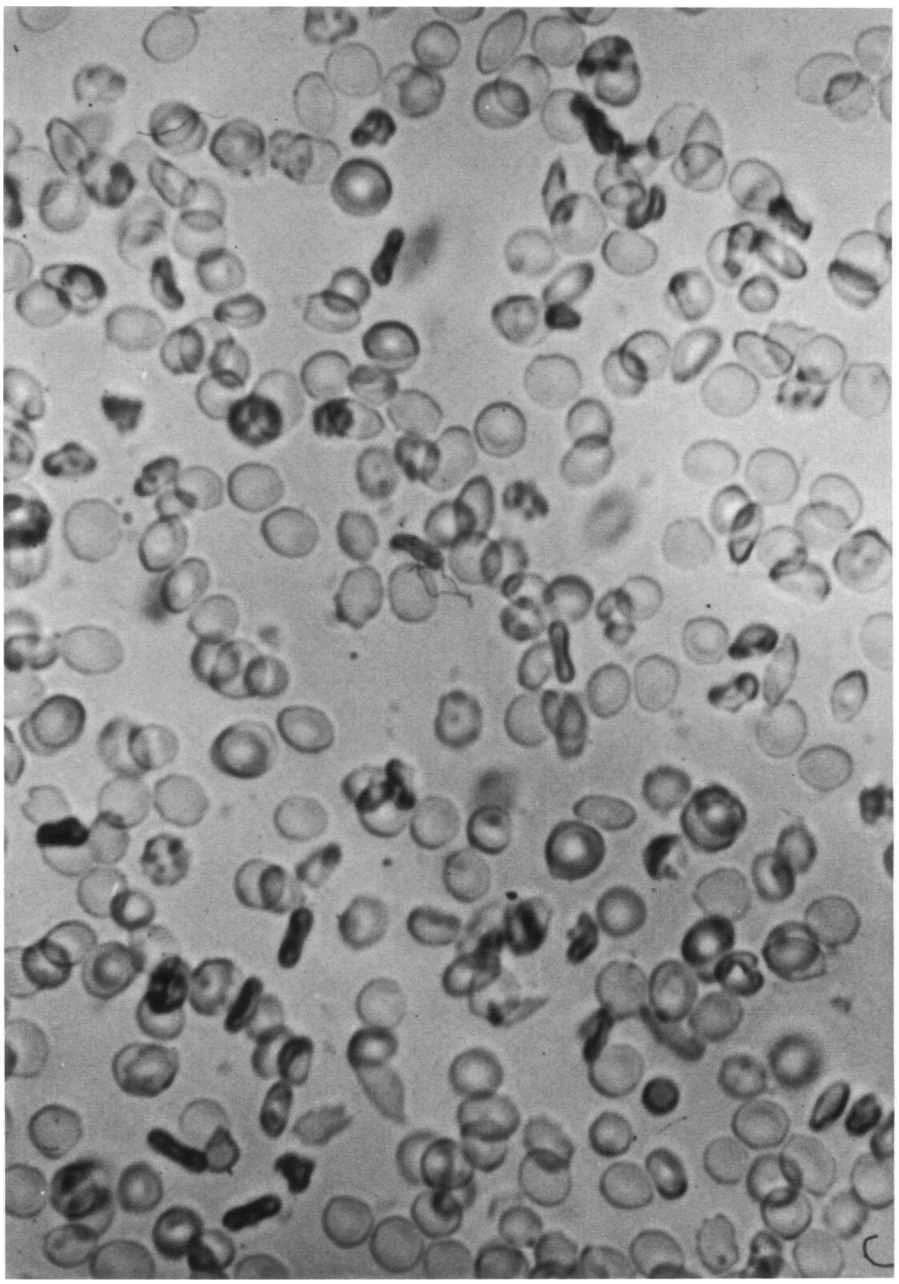

Fig 2—Previously sickled poikilocytes (Fig 1) reversed to normal biconcave discs by treatment with 1 M UIS (wet preparation, × 210).

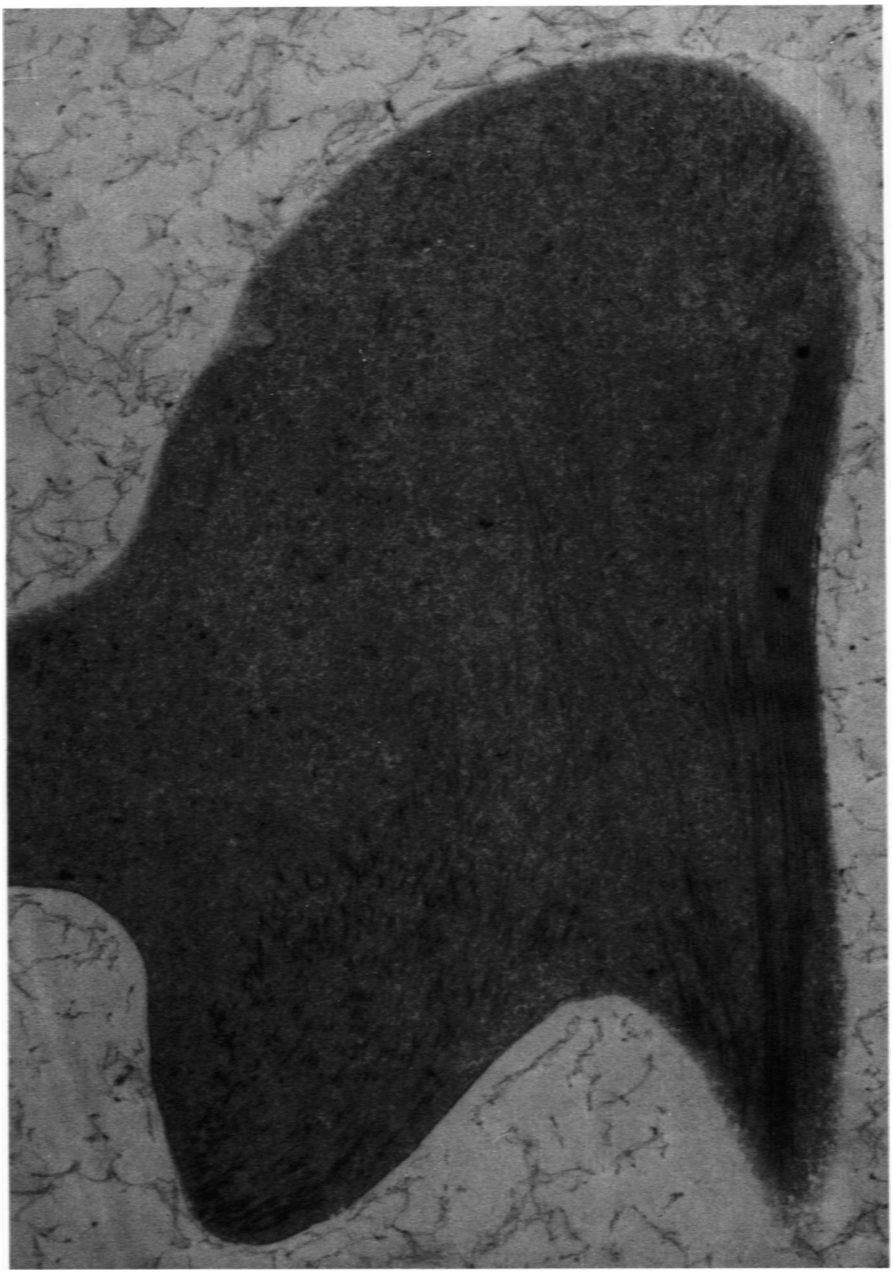

Fig 3—Microfilaments in sickled poikilocyte of patient EJ. Along upper margin of erythrocyte, the microfilaments are in ordered array (TEM, × 34,650).

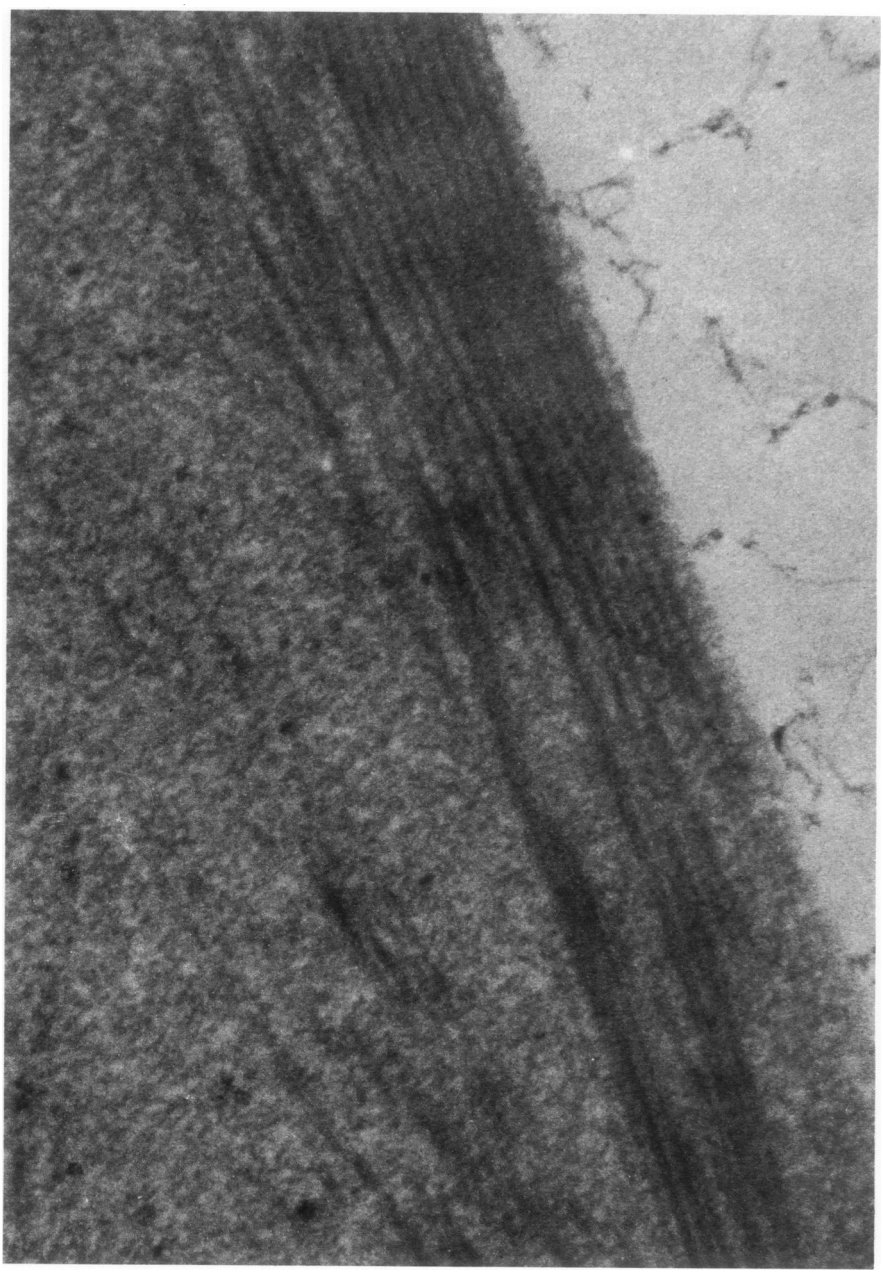

Fig 4—Surface organization of microfilaments in a sickled poikilocyte of patient EJ (TEM, × 131,380).

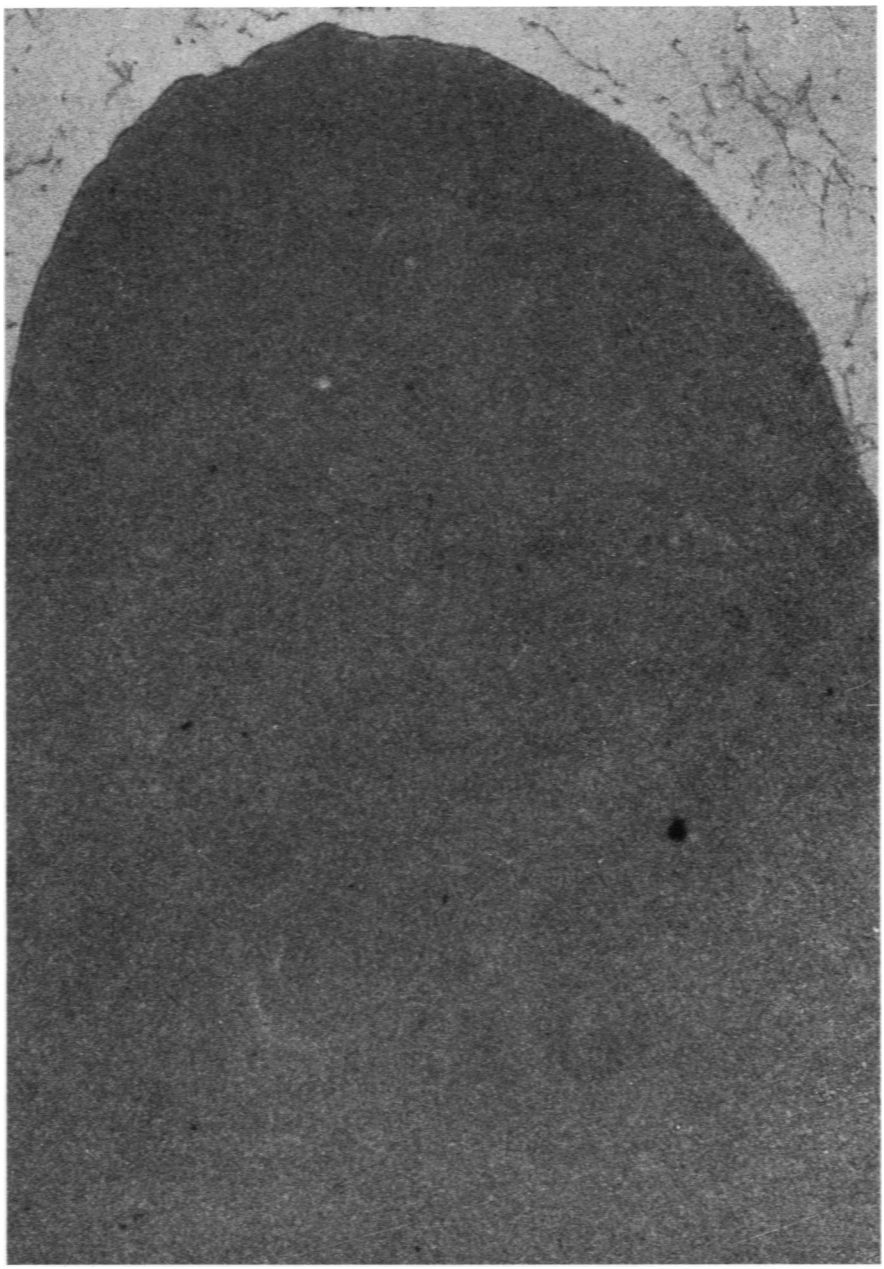

Fig 5—Erythrocyte from patient EJ after reversal of sickling by UIS. Note homogeneity of cytoplasm. No microfilaments were seen in any of the erythrocytes in this preparation (TEM, × 68,100).

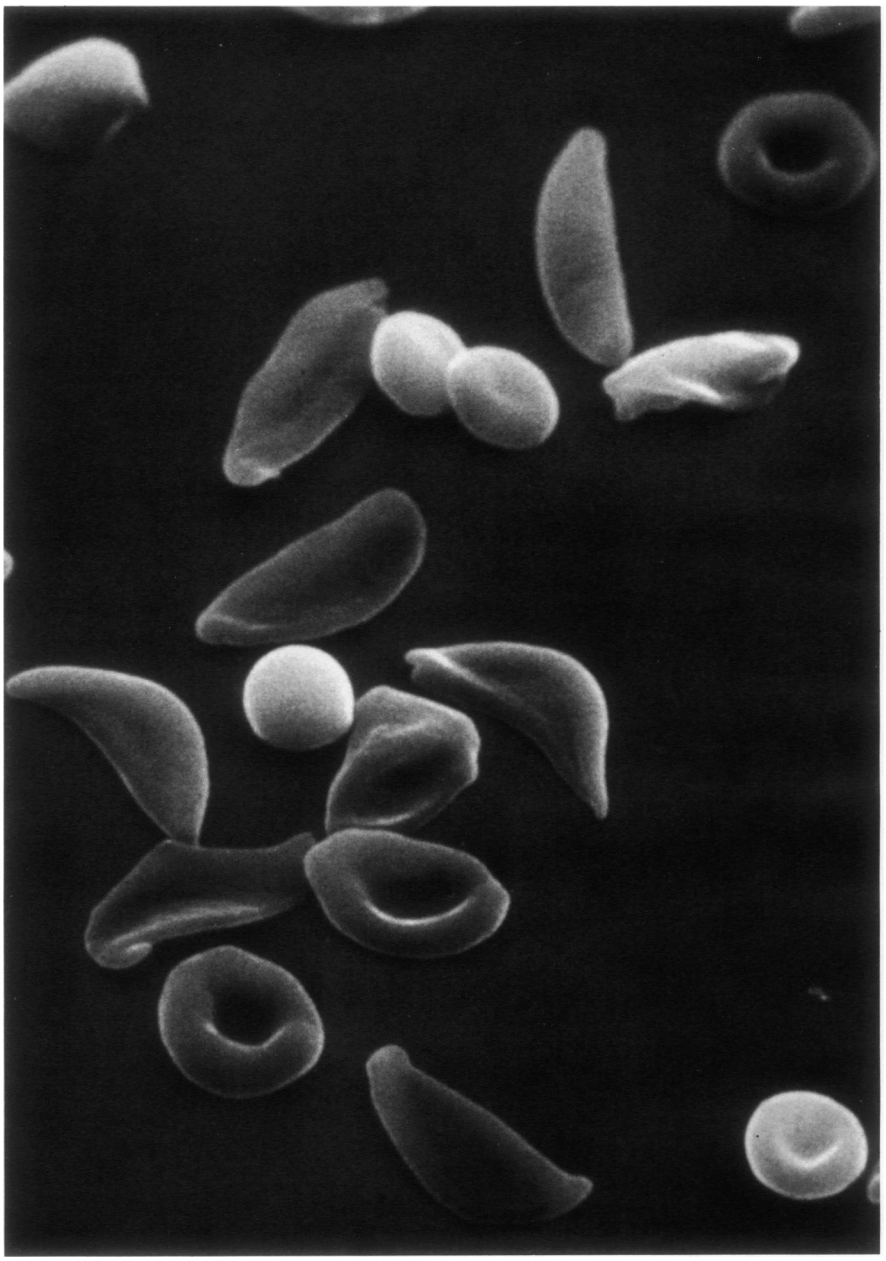

Fig 6—Erythrocytes from sickled poikilocytes of patient FK as seen by scanning electron microscopy (SEM, × 4050).

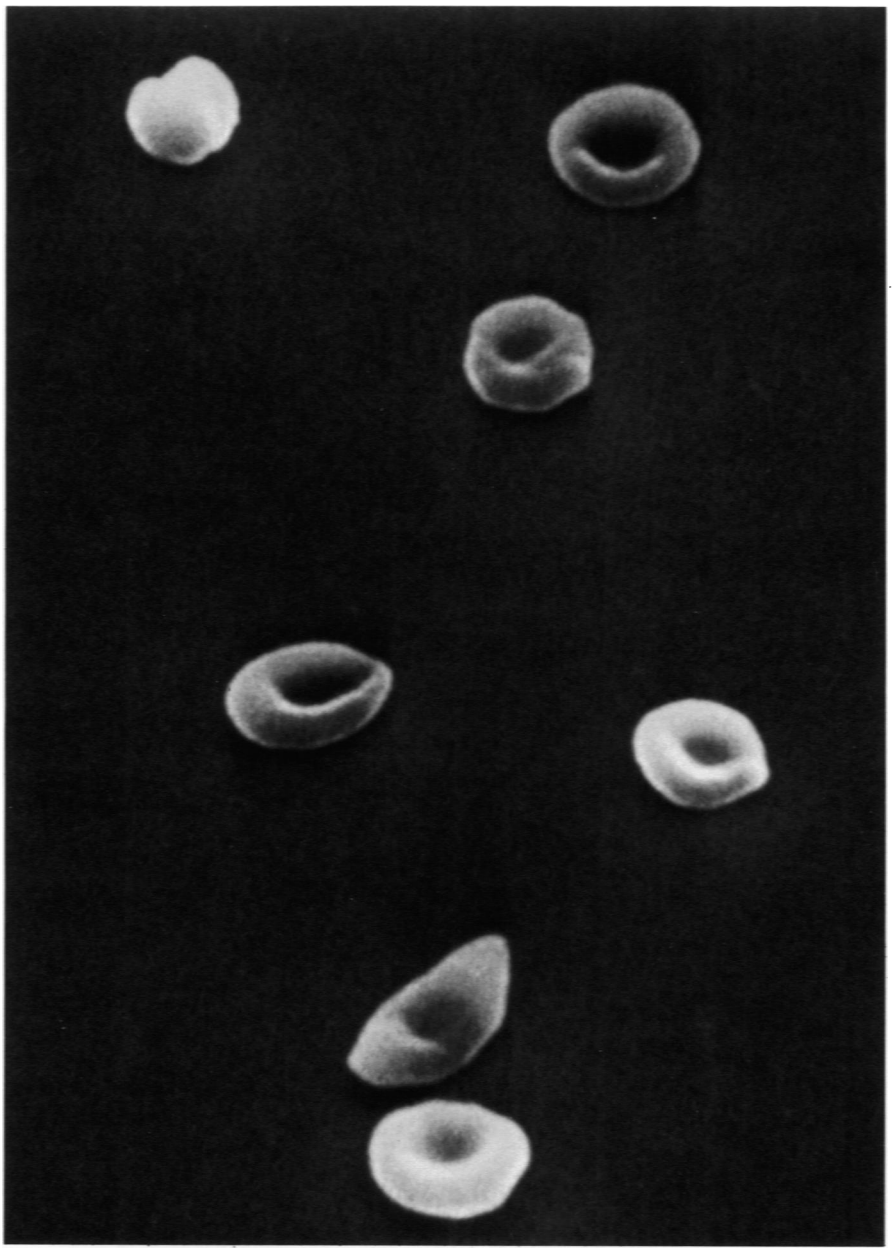

Fig 7—Reversal of sickling in erythrocytes of FK after exposure to UIS (SEM, × 4290).

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