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Research Article

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Irreversibly Sickled Erythrocytes: a Consequence of the Heterogeneous Distribution of Hemoglobin Types in Sickle-Cell Anemia

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ABSTRACT The amount of fetal hemoglobin (Hb F) in erythrocytes of patients with sickle cell anemia (Hb SS disease) was measured by two methods: (a) photometry of individual cells stained for Hb F by the Kleihauer-Betke technique; and (b) chemical assay of alkali-resistant hemoglobin in cells distributed according to specific gravity by ultracentrifugation. Irreversibly sickled cells (ISC), which could be identified directly during photometry and which were found to gather in high concentration at the bottom of ultracentrifuged cell columns, contained significantly less Hb F than non-ISC. Cell content of total Hb was constant regardless of cell size, shape, or ultracentrifugal behavior: thus absolute amounts of Hb F and S varied reciprocally from cell to cell.

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technique permitted direct visual characterization of labeled erythrocytes as ISC or non-ISC, all of which had been reticulocytes at the time of reinfusion. The transformation of non-ISC into ISC, presumably a manifestation of membrane damage, proved to begin soon after cell release from the marrow; and ISC subsequently underwent rapid removal from the circulating blood.

It is therefore apparent from these studies that, in Hb SS disease, relatively small reciprocal changes in the amounts of the two major hemoglobins carry predictive importance: (a) net synthesis of Hb F is least in erythroid cells destined to become ISC; and (b) these irreversibly deformed erythrocytes suffer preferential destruction.

INTRODUCTION

Sickle cell anemia (Hb SS disease) provides an opportunity to study in man the significance of a variability from cell to cell in the relative proportions of two similar proteins which coexist within individual members of a cell population. We report here the apparent influence of small changes in the relative proportions of two hemoglobins on the formation of morphologically obvious deviants, within the erythrocyte populations of Hb SS patients, known as irreversibly sickled cells (ISC).

Deformed erythrocytes in the peripheral blood of a patient with unexplained anemia led Herrick (1) to his original description of what is now called Hb SS disease. Herrick observed "thin, elongated, sickle-shaped, and crescent-shaped

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forms" in both fixed and wet preparations of blood. Diggs and Bibb (2) found that these "oat and crescent" shaped cells did not undergo typical sickling in sealed wet preparations, but sent out only a few filaments from the cell surface. On reoxygenation they returned to their original (deformed) shapes. Watson (3) suggested that they were old cells whose membranes, after sequestration in areas conducive to sickling, had lost their elasticity. Shen, Fleming, and Castle (4) reported that erythrocytes morphologically similar to these "irreversibly sickled" cells could be produced by sterile incubation of Hb SS blood in the absence of oxygen.

The work of Singer and Fisher (5) showed that Hb F and S are heterogeneously distributed within the erythrocyte population of patients with Hb SS disease, and their transfusion studies indicated that those erythrocytes with higher proportions of Hb F had longer life spans. Shepard, Weatherall, and Conley (6), employing direct staining of Hb F by the Kleihauer-Betke technique, confirmed the heterogeneity. If it is true that irreversibly sickled cells are relatively old, then by virtue of selective survival they should contain a higher proportion of Hb F than the general population of erythrocytes. On the other hand, a low content of Hb F would facilitate the sequestration in areas of decreased oxygen tension believed to lead to their deformation of shape, and they consequently should be relatively young.

Experiments were conducted (*a*) to resolve these conflicting predictions of Hb F content; and (*b*) to determine the age of ISC.

METHODS

Blood collection and processing

All in vitro procedures (except for microscopy and single-cell photometry) were performed within 8 hr of drawing venous blood into ethylenediaminetetraacetate (EDTA) (1 mg/ml of blood).

Patients

No hemoglobins other than A₂, F, and S were detectable by electrophoresis in polyacrylamide gel (7) or starch gel (8) in any patient used in these studies. Electrophoresis of hemoglobin in agar gel at pH 6.2 (9) and solubility studies (10) eliminated the possibility of Hb SD disease. If Hb A had been present in any patient, its proportion must have been less than 2%, the lower limit of detection by the method used. These observations,

including the appearance of the peripheral blood smears, make it unlikely that any of our patients had thalassemia-Hb S disease or other variants. None were in sickle-cell crisis at the time of study, and none had been transfused in the preceding 4 months.

Calculation of RBC indices

Cell volume (MCV) and cell hemoglobin content (MCH) were derived from the following determinations: erythrocytes were enumerated by hemocytometer (patients 10 and 11) or with a Coulter counter model A; packed cell volumes were measured after centrifugation at 10,000 *g* in capillary tubes (11); and hemoglobin was measured as cyanmethemoglobin (12).

Estimation of per cent of irreversibly sickled cells

In these investigations, irreversibly sickled cells are defined as those erythrocytes which, in cover-glass preparations made immediately after equilibration of blood with an atmosphere of 95% O₂-5% CO₂, demonstrate elongated, sickled, or double-pointed shapes. 200 cells on each of two matched cover glasses, stained with Wright's stain, were classified for each determination. Erythrocytes with characteristics of ISC were easily detected: the precision of this subjective assay was approximately $\pm 5\%$ 1 SD.

Although previous oxygenation of blood did not cause a significant difference in proportions of ISC counted, equilibration of blood samples with 95% O₂-5% CO₂ before blood films were prepared for any purpose was adopted as a precautionary measure.

Photometric estimation of Hb F in individual cells

Blood films were prepared on cover glasses and the technique of Kleihauer, Braun, and Betke (13), as modified by Shepard and coworkers (6), was used to elute hemoglobins other than Hb F. The slides were then stained for 5 min in 0.5% aqueous phloxin B-80% ethanol 1-3. Images of individual cells were cast on a ground-glass screen by transillumination, and the optical density of each was measured by placing a gun-type photocell connected to a densitometer (model 500 A, Photovolt Corp., New York) against the image. The zero optical density setting was repeatedly adjusted for background areas of the slide.

This is accepted as a semiquantitative technique only (6): stain intensity varies from slide to slide. In these experiments, photometric comparisons of ISC with non-ISC were made only within individual slides.

Alkali-resistant hemoglobin

Proportions of alkali-resistant hemoglobin were measured by the 1-min denaturation method of Singer, Chernoff, and Singer (14). At the levels assayed in these experiments, the precision of the technique was $\pm 0.7\%$ 1 SD.

TABLE I
Routine Hematologic Data on Patients Studied

Patient	Age	Sex	Hb	Hct	Reticulocytes	Hb F	Irreversibly sickled cells
			g/100 ml	%	%	%	%
1	27	M	11.9	35	20	3.4	34
2	8	F	7.2	23	22	7.0	41
3	24	F	4.5	16	20	8.5	20
4	25	F	10.0	30	24	3.8	36
5	28	M	10.5	30	14	9.4	26
6	30	M	11.2	32	6	10.7	14
7	28	F	8.7	25	16	14.7	11
8	8	M	6.5	20	6	7.1	29
9	37	M	9.5	28	6	6.6	17
10	19	F	10.3	26	20	5.0	28
11	8	F	10.5	25	19	10.6	19
12	28	M	9.0	27	17	1.9	25
13	23	F	6.0	17	20	2.9	44
14	19	M	8.2	22	26	5.4	39
15	23	M	7.7	25	23	1.9	31
16	25	M	7.1	20	12	1.5	36

Ultracentrifugation

Within 1 hr of venesection, oxygenated blood (anti-coagulated with EDTA and with packed cell volume adjusted to approximately 60) was centrifuged at 20°C in a Spinco SW 50L swinging bucket rotor for 1 hr at 40,000 rpm (45,000 *g*). Cell samples were sequentially (top to bottom) removed with Pasteur pipets from the top and bottom of the cell columns and from intermediate points and were resuspended in autologous plasma. Care was taken to prevent contamination from position to position in the tube. Each centrifuge tube, with a total volume of 5 ml, held approximately 3 ml of cells; and samples of approximately 0.4 ml of cells each were removed from the several positions in the packed cell column.

Determination of erythrocyte survival

Three methods were used to determine erythrocyte survival: two employed radioactive precursors of hemoglobin (selenomethionine-⁷⁵Se and amino acids-³H) which label reticulocytes only, and the third method used the random label ⁵¹Cr.

Method 1. 300–500 ml of Hb SS whole blood, anti-coagulated with heparin (5000 U USP/ml of heparin, 1 ml/100 ml of blood), was incubated in plastic bags for 1–2 hr at 37°C with gentle agitation in the presence of approximately 50 μ c of L-selenomethionine-⁷⁵Se (The Radiochemical Centre, Amersham, England) per 100 ml of blood. The cells were then washed with sterile isotonic NaCl, to remove the major portion of unbound radioactivity, and reinfused. Venous blood taken at approximately daily intervals thereafter was ultracentrifuged as described above, and samples from four positions in the packed cell columns of duplicate ultracentrifuge tubes

(top, bottom, and two intermediate points) were assayed for radioactivity in a well-type scintillation counter. Constant volumes of cell suspension of known hemoglobin concentration were counted to within 3% probable error, and corrections were made for plasma radioactivity.

Method 2. Blood from Hb SS patients was incubated, washed, and reinfused as in Method 1 except that a mix-

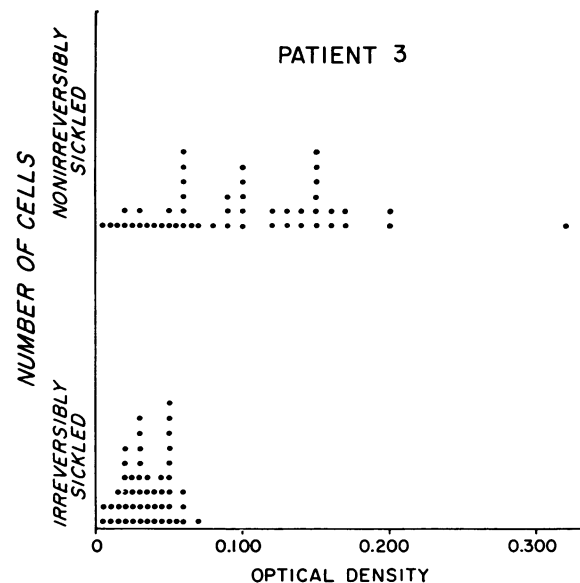


FIGURE 1 Distribution of Hb F in erythrocytes from a patient with Hb SS disease. Thin films of oxygenated blood were stained by the Kleihauer-Betke technique, and optical densities of individual cells were assayed photometrically.

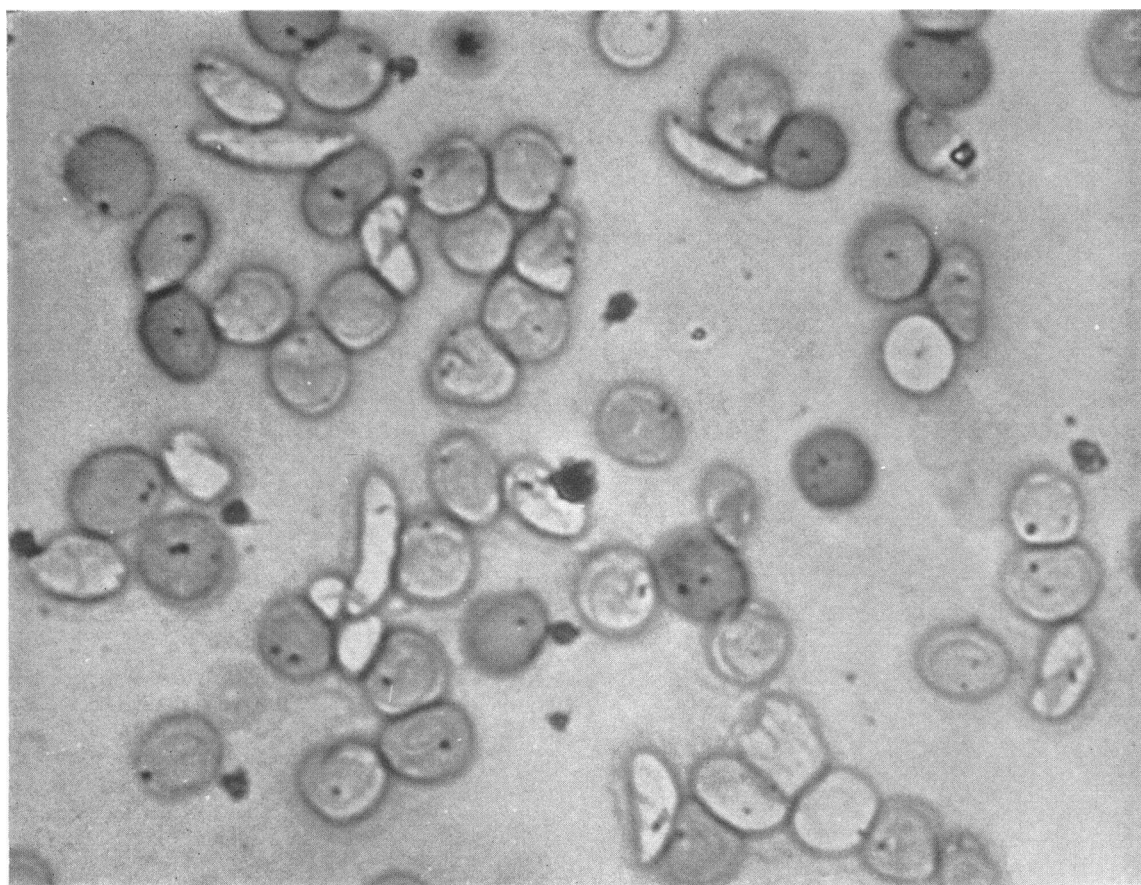


FIGURE 2 Photomicrograph of Hb SS erythrocytes stained for Hb F by the Kleihauer-Betke technique.

ture of amino acids- ^3H , approximately $200\ \mu\text{c}/100\ \text{ml}$ of blood, (reconstituted protein hydrolysate, tritiated, Schwartz Bio-Research, Orangeburg, N. Y.) was used as the isotopic precursor of hemoglobin. Cover slip preparations from oxygenated samples of venous blood removed at approximately daily intervals were fixed in absolute methanol for 10 min, mounted faceup on glass slides, dipped in Kodak NTB-2 nuclear track emulsion, and exposed for 3-5 wk. The developed, fixed, and stained preparations were then examined for grain-bearing erythrocytes.

Method 3. Incubation, reinfusion, and sampling of blood from Hb SS patients were performed as in Method 1 except that $\text{Na}_2^{51}\text{CrO}_4$, approximately $50\ \mu\text{c}/100\ \text{ml}$ of blood, was used as an isotope.

RESULTS

Clinical data and observations. Routine hematological data on the patients used in these studies are shown in Table I. In no patient did present or past symptomatology correlate with total Hb level or per cent Hb F. Scattergrams were constructed of Hb level vs. per cent Hb F,

of Hb level vs. per cent ISC, and of per cent Hb F vs. per cent ISC: no correlations were found in the first two, but per cent Hb F correlated inversely with per cent ISC ($r = 0.65$).

Photometry of single cells. Optical densities (50 sickled and 50 nonsickled cells per slide, selected at random) were measured on cover-glass preparations stained by the Kleihauer-Betke tech-

TABLE II
Optical Densities of Hb SS Erythrocytes Stained for Hb F

Patient	Irreversibly sickled cells	Nonirreversibly sickled cells	P
1	0.034 (± 0.019)*	0.068 (± 0.047)	<0.01
2	0.033 (± 0.013)	0.071 (± 0.036)	<0.01
3	0.034 (± 0.018)	0.091 (± 0.071)	<0.01
4	0.026 (± 0.019)	0.058 (± 0.035)	<0.01
Mean	0.032 (± 0.004)	0.072 (± 0.014)	<0.01

* Mean (\pm SD) of 50 cells.

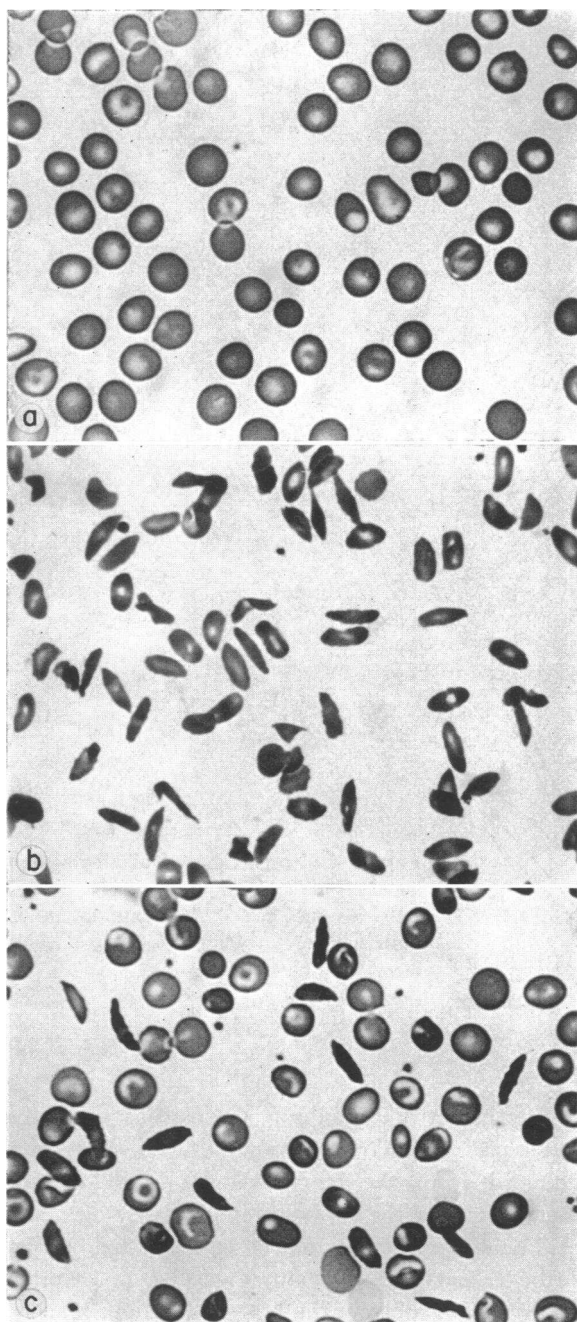


FIGURE 3 Photomicrographs of Hb SS erythrocytes (Wright's stain) (a) from the top and (b) bottom of an ultracentrifuged cell column, and (c) of whole blood before ultracentrifugation.

nique. A representative frequency-distribution plot is shown in Fig. 1. Casual inspection of this plot and of a photomicrograph of a typical area of a slide (Fig. 2) suggested that ISC contain less

Hb F than do non-ISC. Cumulated data (Table II) on patients 1 through 4 confirmed this impression: the differences between the means are highly significant. Slide preparations of Hb SS erythrocytes sickled in an atmosphere of 95% N_2 -5% CO_2 showed the same spectrum of stain intensity observed in Fig. 2: hence deformation of shape is not responsible for the staining characteristics of ISC.

Characteristics of erythrocytes separated by ultracentrifugation. Photomicrographs of cover-glass films stained with Wright's stain from top and bottom fractions of ultracentrifuged Hb SS erythrocytes and from whole blood are shown in Fig. 3: casual inspection confirms that ISC are concentrated in the heavy (bottom) fraction. When entire packed cell masses were resuspended, the proportion of ISC was identical with that in the whole blood before ultracentrifugation: thus ultracentrifugation does not artifactually alter erythrocyte morphology into a resemblance to ISC.

Proportions of alkali-resistant hemoglobin and of ISC at each of four positions in the packed cell columns are shown in Fig. 4 (patients 5 through 11). In each instance the proportion of alkali-resistant hemoglobin increased parallel with cell specific gravity, then fell sharply as the proportion of ISC increased. In the bottom fraction, where ISC predominated, per cent Hb F (mean 6.0 ± 3.5 1 SD) was significantly lower than per cent Hb F in the mid-bottom fraction (mean 12.4 ± 3.4 1 SD; $P < 0.01$) and mid-top fraction (mean 10.0 ± 2.7 1 SD; $P < 0.05$), but did not differ significantly from that in the top fraction or the whole blood.

Fig. 5 shows average size (MCV) and content of hemoglobin (MCH) of erythrocytes at each of the four positions in the packed cell columns, and of erythrocytes in whole (uncentrifuged) blood. MCV decreased with increasing specific gravity, but MCH remained constant. All values for whole blood fell within the ranges of MCV and MCH described by Diggs and Bibb (2).

Erythrocyte survival studies. Figs. 6 and 7 are representative of survival studies which employed ^{51}Cr (patients 13 and 14) and selenomethionine- ^{75}Se (patients 10 and 12). Selenomethionine- ^{75}Se , which is incorporated into hemoglobin (15), represents a cohort label in contrast to the random label ^{51}Cr . Each bottom fraction was composed pre-

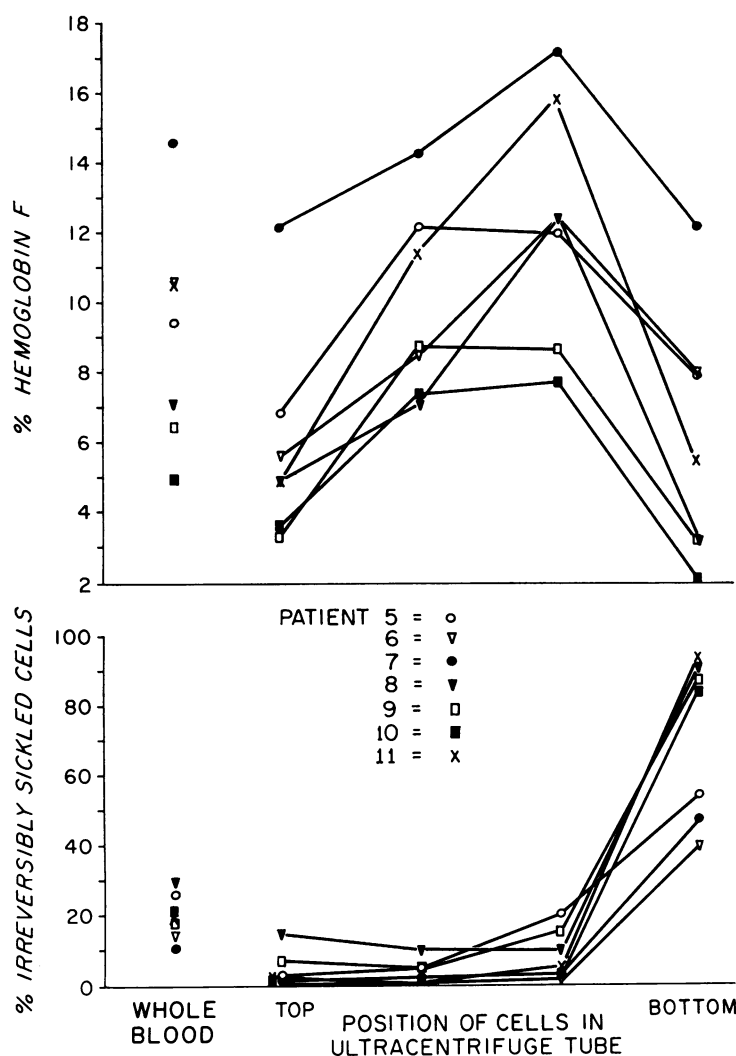


FIGURE 4 Proportions of alkali-resistant hemoglobin and of irreversibly sickled cells at each of 4 positions in ultracentrifuged cell columns. Values for whole blood are shown at the left.

dominantly of ISC; and as detailed in the figure legends the proportions of ISC in whole blood and in each sample from the ultracentrifuged columns of cells remained individually constant throughout the experiments. White blood cells and platelets made an insignificant contribution to the radioactivity of any fraction, and all counts were corrected where necessary for plasma radioactivity. Since MCH remained constant at any point in each ultracentrifuged cell column but MCV did not (Fig. 5), specific activities of hemoglobin rather than of cells are used. In both figures the rapid decrease of radioactivity in the top fraction (approximately 50% reticulocytes in Fig. 6, 40% reticulocytes in Fig. 7) presumably reflects maturation of labeled reticulocytes.

Starting at day 3 in Fig. 6, rate of loss of the randomly labeled erythrocytes was most pronounced from the bottom fraction: its specific activity ultimately fell below the specific activities of the middle fraction and the whole blood. In Fig. 7 the cohort-labeled erythrocytes began accumulating immediately in the bottom fraction, reached maximum proportions there at day 8, and subsequently the rate of loss of radioactivity from the bottom fraction exceeded that from any of the others. In both patients studied with the cohort-label ^{75}Se , the specific activity of the ISC-rich bottom fraction reached a maximum point before half of the cohort had been destroyed: this characteristic of ISC is an indication of their relative youth.

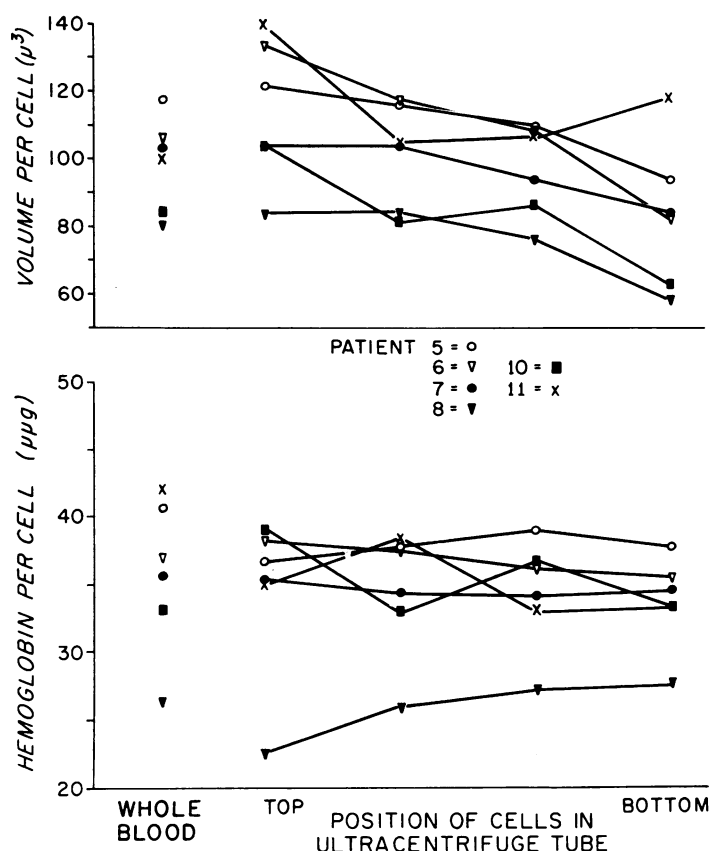


FIGURE 5 Average size (MCV) and content of total hemoglobin (MCH) of erythrocytes at each of 4 positions in ultracentrifuged cell columns. The data of both this figure and Fig. 4 were derived from the same cell samples.

Radioautography permitted direct morphologic interpretation of erythrocytes as ISC and non-ISC in blood samples taken periodically after reinfusion of Hb SS blood which had been incubated with amino acids- ^3H : the grain-bearing red cells had been reticulocytes at zero time. Time-course studies on patients 15 and 16 are shown in Fig. 8: the number of labeled ISC is expressed as per cent of total labeled erythrocytes observed. Labeled ISC, absent from the incubated blood before reinfusion, appeared *in vivo* within 1-2 days and reached maximum proportions in 4-7 days.

DISCUSSION

A low content of Hb F in ISC was demonstrated by both methods used in this study: single-cell photometry (Fig. 1 and Table II) of slide preparations stained for Hb F (Fig. 2) and direct measurement of alkali-resistant hemoglobin in ISC isolated by ultracentrifugation (Figs. 3 and 4). Variability of staining from slide to slide, and even within individual slides, make the photometric technique semiquantitative only. Ultracen-

trifugation however was found to concentrate ISC at the bottom of the packed column, thus permitting direct chemical analysis: it is apparent from Fig. 4 that ISC are responsible for the low proportion of Hb F in cells of the bottom fraction. Examination of the slide preparations of whole blood by light microscopy showed the previously observed heterogeneous distribution of Hb F (6), but further suggested that ISC are uniformly low in Hb F whereas non-ISC vary widely in Hb F content (Fig. 2). This inferential observation, rational in that non-ISC are precursors of ISC (see below), was corroborated by measurements of alkali-resistant hemoglobin along the ultracentrifuged columns of Hb SS erythrocytes (Fig. 4): proportions of Hb F varied, increasing parallel with cell specific gravity but falling sharply in the bottom fraction (predominantly ISC). The positive correlation between per cent Hb F and distance down the packed cell mass (excluding the bottom fraction) is in accord with (a) the fact that ultracentrifugation provides a better separation of normal human erythrocytes accord-

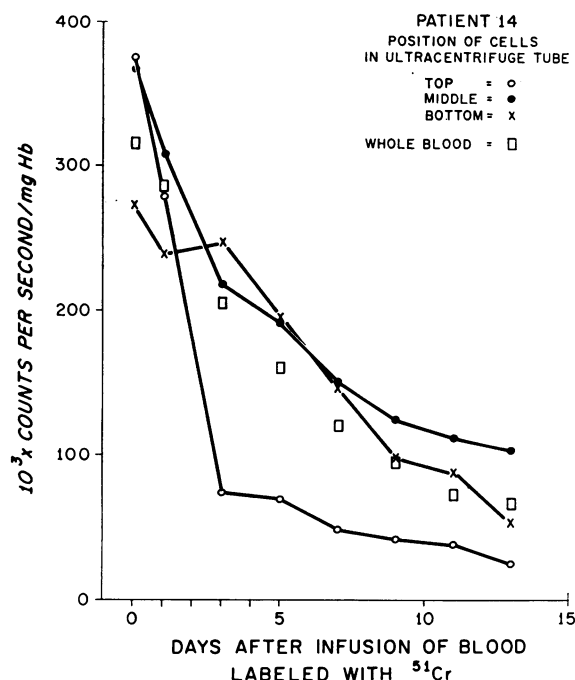


FIGURE 6 Time-course identification of randomly labeled erythrocytes as ISC and non-ISC. Hb SS blood was labeled in vitro with $\text{Na}_2^{51}\text{CrO}_4$ and reinfused. Sequential samples of venous blood were ultracentrifuged, and the specific activity of erythrocytes from several positions in the packed cell columns was determined. Proportions of ISC in each position throughout the experiment, expressed as per cent \pm SD, were: top 2.8 ± 1.5 ; middle 5.2 ± 2.8 ; bottom 84.4 ± 7.2 ; and whole blood 34.6 ± 4.6 .

ing to age than does routine centrifugation, presumably by forcing denser (older) cells down and lighter (younger) cells up through the packed cell mass (16); (b) the transfusion experiments of Singer and Fisher (5) which related erythrocyte survival in Hb SS disease to cell content of Hb F; and (c) the work of Bradley, Brawner, and Conley (17) who, using ^{59}Fe , also obtained evidence indicating preferential survival of Hb SS erythrocytes bearing the higher proportions of Hb F.

The predominance of ISC in the bottom fractions may be the result of their comparatively low MCV (Fig. 5); but regardless of mechanisms responsible, ultracentrifugation as a method of isolating ISC in high concentration permitted the studies on cell survival shown in Figs. 6 and 7. Erythrocytes were separated into three general groups: reticulocyte-rich young cells, non-ISC, and ISC. The chronological behavior of cells labeled randomly with ^{51}Cr (Fig. 6) reveals the

short life span of ISC and confirms a prediction derived from Fig. 4: the longest-lived Hb SS erythrocytes occupy the middle layer (rich in Hb F) of the ultracentrifuged cell column. In a direct approach, the half-life ($t_{1/2}^{51}\text{Cr}$) of bottom fraction erythrocytes from patient 14, labeled in vitro and reinfused, was found to be 2 days, whereas the $t_{1/2}^{51}\text{Cr}$ of whole blood was 5 days, but the possibility of cell damage by in vitro manipulation of bottom fraction cells before infusion cannot be ignored.

Fig. 7 reveals the rapid development of ISC from non-ISC, the majority of them forming early in the life of the cohort; and, as does Fig. 6, Fig. 7 shows a relatively rapid elimination of labeled cells from the ISC-rich bottom fraction. This early intravascular development of ISC is confirmed by the data of Fig. 8. Furthermore, in the two patients of Fig. 8 ISC eventually constituted 30–40% of the labeled cohort which survived longer than 5 days, in good agreement with the proportions of ISC in their whole blood (Table I). Fig. 8 also demonstrates that a negligible number of erythrocytes emerge from the marrow as ISC: therefore, non-ISC are precursors of ISC. This morphologic transformation, which maintained steady-state characteristics in our experiments, would delay the chronological loss of radioactivity from ISC-rich bottom fractions. Hence, the true survival of ISC must be shorter than that implied by Figs. 6 and 7. Another consequence of the continued transformation of non-ISC into ISC is that per cent ISC in whole blood represents the lower limit of the proportion of erythrocytes which will become irreversibly deformed before death.

Previous demonstrations (using chemical assay of Hb F) of the nonuniform distribution of Hb F and S within erythrocyte populations of individual Hb SS patients have used two preparatory methods which can be expected to alter cell size and shape: mechanical trauma (5) and hypotonic hemolysis (18). Ultracentrifugation, shown by us not to deform erythrocytes into a resemblance to ISC, confirmed the nonuniform distribution of hemoglobins: differences in specific gravity permitted separation of cells varying in size, shape, and proportional content of Hb F. However cell content of total hemoglobin (MCH) remained independent of these variables and was constant, at normal levels or above, among the samples taken

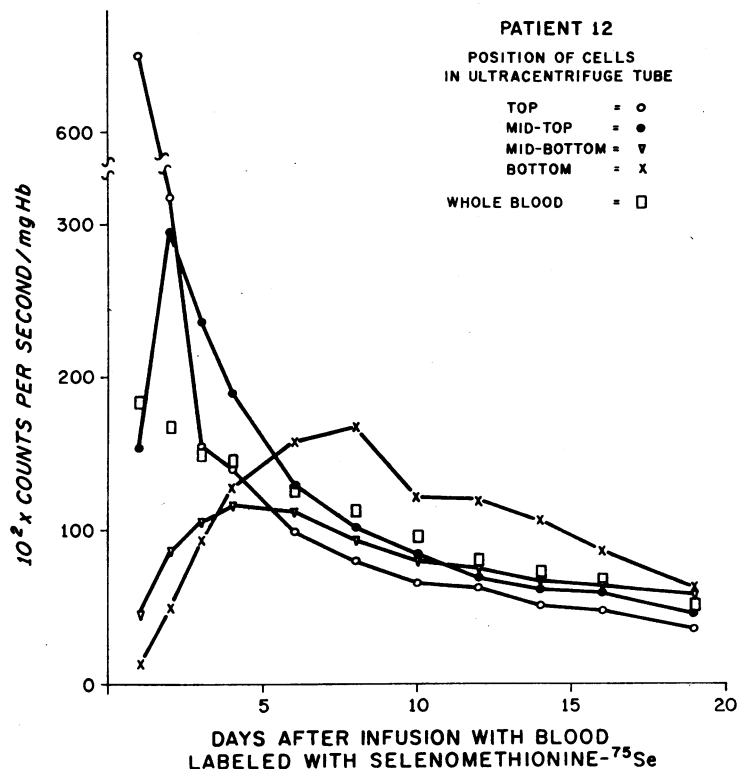


FIGURE 7 Time-course identification of cohort-labeled erythrocytes as ISC and non-ISC. Hb SS blood (17% reticulocytes) was labeled in vitro with selenomethionine-⁷⁵Se and reinfused. Sequential samples of venous blood were ultracentrifuged, and the specific activity of erythrocytes from several positions in the packed cell columns was determined. Proportions of ISC in each position throughout the experiment, expressed as per cent \pm SD, were: top 1.8 ± 1.7 ; mid-top 1.5 ± 1.3 ; mid-bottom 10.5 ± 1.2 ; bottom 91.0 ± 1.8 ; and whole blood 22.8 ± 2.7 .

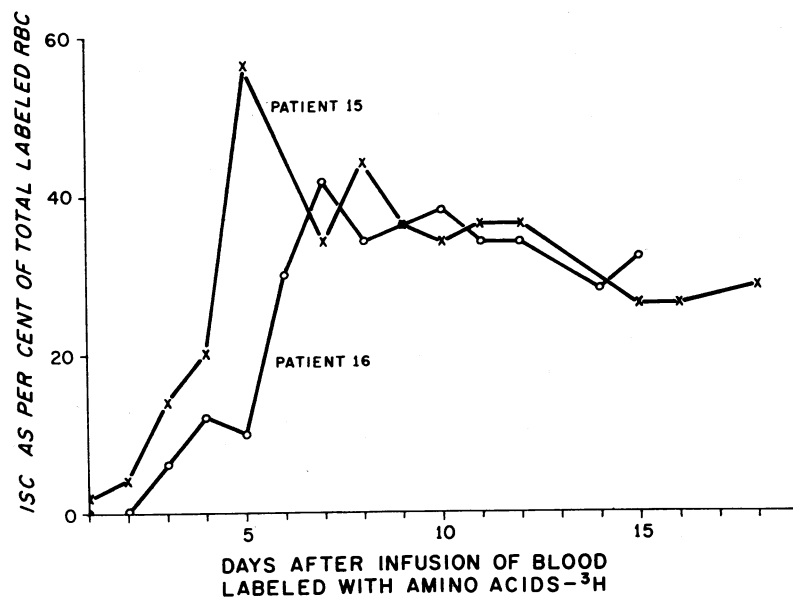


FIGURE 8 Time-course changes in proportions of ISC within cohort-labeled populations of erythrocytes. Hb SS blood (patient 15, 23% reticulocytes; patient 16, 12% reticulocytes) was labeled in vitro with amino acids-³H and reinfused. Radioautographs of sequential samples of oxygenated venous blood were prepared, 50 grain-bearing erythrocytes were located in each preparation, and the proportion of labeled ISC was plotted as per cent of total labeled erythrocytes.

along the packed cell mass: thus, absolute amounts of Hb F and S per cell varied reciprocally. In contrast to these observations on Hb SS erythrocytes, Loukopoulos and Fessas (19) found that, in centrifuged erythrocytes of patients with homozygous β -thalassemia, MCH (cell total content of Hb A, A₂, and F) was greater in denser (presumably older) than in lighter (presumably younger) erythrocytes, but the amount of non-F hemoglobin per cell was constant. Whether cells were heavy or light, their MCH was below normal. Although precise mechanisms responsible for increased cell content of Hb F remain unknown, the operational scheme proposed by Marks and Burka (20, 21) appears applicable both to our results and to those of Loukopoulos and Fessas. Thus in homozygous β -thalassemia, increased synthesis of γ -chains does not compensate fully for inadequate synthesis of β^A -chains, possibly because messenger RNA specific for γ -chain synthesis decays early in erythroid maturation, and cell content of total hemoglobin cannot achieve normal levels. In Hb SS disease, where depression of cell synthesis of β^S -chains is slight, γ -chain synthesis is adequate to achieve a normal cell content of total hemoglobin. Variable delay from cell to cell in the rate of decay of biosynthetic mechanisms responsible for γ -chain synthesis may account for the variably increased amount of Hb F per cell in both diseases.

Diggs has argued for regarding sickle cell crises as vasoocclusive episodes (22), and he has pointed out the rarity of a significant increase in hemolysis during crises (23). We suggest that, in accord with the concept of erythrosthesis advanced by Ham and Castle (24), a small select population of Hb SS erythrocytes has a predilection for occluding small vessels; and if the shortest lived red cells are most culpable in the consequent production of crises and organ damage characteristic of Hb SS disease, ISC may constitute this small select population. Barreras and Diggs (25) noted that the proportion of sickled forms in formalin-fixed venous blood tended to decrease during the course of crises, but in agreement with Jensen, Rucknagel, and Taylor (26) they found no correlation between the proportion of sickled forms and the long-term course of the disease. Here the adjective "sickled" must be employed with caution. Both groups of investigators examined venous blood anaerobically fixed in formalin, and such prepara-

tions contain two distinct types of deformed erythrocytes: ISC and reversibly sickled cells (27). Whereas reversibly sickled cells by definition contain sickled hemoglobin, electron microscopy has shown that ISC do not necessarily contain hemoglobin in the filamentous (sickled) form (27): the deformed membranes of ISC are probably the legacy of previous sequestration in the sickled state under conditions leading to metabolic and structural derangement.

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