Red Cell Filtration and the Pathogenesis of Certain Hemolytic Anemias

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There appear to be two mechanisms whereby red cells are destroyed in vivo. First, they may undergo disruption or dissolution within the general circulation, as by the action of immune, bacterial, or chemical lysins, causing the clinical picture of "intravascular hemolysis." Second, and more commonly, red cells may be trapped or sequestered in certain regions of the circulation, particularly in the spleen and liver, in which case the cellular hemoglobin is largely degraded in situ and the clinical picture is of "extravascular hemolysis." There are two proposed primary mechanisms whereby red cells may be so sequestered: by a surface adherence of the cells to the endothelial or reticuloendothelial cells lining the blood vessels, essentially a chemical or electrostatic process; or by simple filtration, essentially a physical process. Conceivably, of course, both processes may operate in unison, as by chemical or electrostatic interactions between the surfaces of reticuloendothelial cells and of physically trapped red cells. The phenomenon of phagocytosis, often written of in the literature on the reticuloendothelial system as synonymous with sequestration, is inescapably a process secondary to those described above. Sequential pathologic studies of the reticuloendothelial tissues have been made in rats during the destruction of red cells altered in various ways not in themselves lytic in vitro; erythrophagocytosis of cells so altered was a delayed, usually post-hemolytic process in vivo.

In an effort to understand the behavior of normal and altered red cells in the capillary "filter-beds" of the body, studies were undertaken of their behavior in respect to artificial filters in vitro. After crude, preliminary studies with ordinary paper fiber filters, more exact experiments were carried out employing Millipore* membrane filters. Unlike fibrous filters, the membrane filter consists of a chemically inert matrix penetrated by non-branching capillary tubes oriented perpendicularly to the filter surface, providing a structural...
analogue, albeit an imperfect one, of a living capillary bed. A preliminary report of the findings has been published.4

MATERIALS AND METHODS

The Filter Apparatus

The Millipore filter discs employed were of the SM type, having pore diameters of 5.0 ± 1.2 μ (mean ± 2 standard deviations). Filters having pore diameters of this magnitude were selected because such pores only slightly exceeded the critical dimension that would admit normal red cells. The filter discs were 25 mm. in diameter and 150 μ thick and were traversed by numerous capillary tubes, the openings of which comprised approximately 85 per cent of the filter surface. This filter may be considered as an aggregate of millions of small parallel tubes, each 5 μ in internal diameter and 150 μ long.

With homogenous fluids, pressure-flow relationships through Millipore filters may be defined by Poiseuille's law. With non-homogenous suspensions, as of blood cells, such relationships may be disturbed by a number of complexities, as by the occurrence of axial streaming and by the obstruction of capillary openings by cellular particles. Therefore the studies described below are expressed in relative and comparative, rather than absolute, terms.

The membranous filter matrix is composed of cellulose esters. According to its manufacturers, the filter possesses a weak negative charge at a physiologic pH; there is no tendency to accumulate additional charge during its perfusion with liquids. The filter pore size is unaffected by the osmolarity of the perfusing liquid. It has been shown that the filter disc does not absorb appreciable amounts of protein from solution.

In preparing for each filtration study, the Millipore disc was placed horizontally upon a glass disc with a fritted glass core and was clamped at its periphery between this and an upright cylindrical funnel, which acted as a perfusing chamber. The base of this assembly fitted into a vacuum flask which received the filtrate and which was connected to a negative pressure system and a mercury manometer. The perfusion pressure across the filter was determined as the sum of the fluid pressure in the upper chamber of the filter assembly expressed in mm. Hg. and the negative pressure in the collecting flask.

Preparation of Cell Suspensions

The red cell suspensions were derived from freshly drawn blood collected in ethylenediaminetetraacetic acid (EDTA) and obtained from suitable patients and from normal subjects. The red cells were separated by centrifuging the blood specimens and were washed 3 times with physiologic saline. The plasma was centrifuged at about 3000 g and then was prefiltered, first through Whatman No. 42 filter paper and then through a Millipore filter having an average pore diameter of 3.0 μ, before use as a suspending medium for the washed red cells.

Filtering Procedure

Preliminary experiments indicated that, under the conditions employed, an optimal flow of red cells was obtained using a 5 per cent suspension of cells in plasma. At the start of each run the filter was wetted with plasma and 4 ml. of the cell suspension was pipetted into the upper chamber of the filter assembly. At the same time, the collecting flask was subjected to the desired negative pressure and the time was noted. All experiments were conducted at room temperature.

The filterability of red cells was measured in one of two ways: (1) In some experiments the rate of flow into the collecting flask in ml./minute was recorded and the per cent passage of the red cells through the filter was measured by determining the product of the hemoglobin concentration and volume of the cell suspension which crossed the filter (Hb F X Vol F) as a percentage of that initially added (Hb o X Vol o). (No appreciable free hemoglobin appeared under the conditions of these experiments.) (2) In experi-
ments in which the filtration of abnormal red cells was to be compared to that of normal red cells, the abnormal cells were labelled with Cr\(^{51}\) before being washed and made up to a 5 per cent suspension in plasma. One part of this was then mixed with 10 parts of a 5 per cent suspension of serologically compatible normal red cells. It was then possible to determine the passage through the filter of the whole suspension (Hb\(_f\) X Vol\(_f\)) as compared to that initially added (Hb\(_o\) X Vol\(_o\)), and of the abnormal cells (Cr\(^{51}\) X Vol\(_o\)) as compared to the initial suspension (Cr\(^{51}\) X Vol\(_o\)). The passage of the normal cells could then be calculated by the difference (per cent passage whole suspension — per cent passage Cr\(^{51}\)-labelled cells). The passage of abnormal red cells was then finally expressed as a percentage of the passage of normal cells. This method of analysis tended to minimize the differences between abnormal and normal red cells since abnormal cells partially obstructed the passage of some normal cells. However, it provided each study with a reliable “internal control.”

Miscellaneous Methods

Red cell and hemoglobin concentrations were measured by standard procedures.\(^5\) Osmotic fragility was measured and corrected for pH and the osmotic activity of the cells by the method of Emerson and others.\(^5\) Red cell agglutinability was determined by both the Coombs\(^7\) and P.V.P.\(^8\) methods. Red cells were labelled with Cr\(^{51}\) in the form of Na.Cr\(^{51}\)O\(_4\) of high specific activity,\(^9\) and Cr\(^{51}\) activity was measured with a well-type scintillation counter.

RESULTS

Effect of Osmotic Swelling on Red Cell Filtration

Normal washed red cells with a mean corpuscular volume (MCV) of 86 cu. \(\mu\), were suspended to a concentration of 5 per cent in autologous plasma aliquots to which had been added an equal amount of saline of various concentrations. The resultant MCV values of the cells of each suspension were computed by the use of Emerson’s formula\(^10,11\) which relates plasma “tonicity” (osmolarity) to red cell volume. The effect of this osmotic shrinking or swelling on the filterability of the red cell at a perfusion pressure \((\Delta P)\) of 30 mm. Hg. is depicted in figure 1. Under these conditions a rather sharp fall-off in the rate of red cell passage occurred at a plasma “tonicity” equivalent to 0.70 Gm. per cent NaCl, when the MCV reached 98 cu. \(\mu\).

Effect of Sickling on Red Cell Filtration

Cr\(^{51}\)-labelled red cells from a patient with sickle cell anemia were mixed with 10 parts of unlabelled normal red cells, as described under “Methods.” The mixed suspension was equilibrated under various tensions of oxygen by repetitious flushing in tonometers with mixtures of oxygen, nitrogen, and 5 per cent CO\(_2\). Each filtration study was conducted in a closed system containing the appropriate gas. As shown in figure 2, at oxygen tensions of 100 and of 80 mm. Hg., sickle cell anemia red cells (15–20 per cent of which were “permanently sickled”) traversed the filter about 75 per cent as well as the normal red cells. The per cent passage of these cells declined at lower oxygen tensions, falling sharply below a \(P_{\text{O}_2}\) of 40 mm. Hg. to 10 per cent of normal. Although most of the red cells that passed through the filter under

\(^*\)Abbott Laboratories, North Chicago, Ill.
MEAN CORPUSCULAR VOLUME, CUBIC MICRONS

Fig. 1.—Effect of osmotic swelling on red cell filtration. The thickness/diameter ratio and M.C.V. of normal red cells was altered by suspension in plasma to which had been added various concentrations of NaCl. The rate of red cell passage through the filter was unaffected until the "tonicity" of the plasma had been rendered equivalent to 0.70 per cent NaCl, and the calculated M.C.V. had been increased from the initial 86 cu. μ to 98 cu. μ. The rate of passage is expressed as ml. of packed red cells/min.

low oxygen tensions were normal cells, the presence of sickled cells impaired the passage of normal cells, indicating obstruction of the filter pores.

The effect of perfusion pressure (Δ p) on the filterability of sickled red cells was studied by equilibrating a mixture of sickle cell anemia red cells and 10 parts of normal red cells under 95 per cent N2/5 per cent CO2. In a closed system the filterability of this mixture was then studied at perfusion pressures varying from 8 mm. to 124 mm. Hg. As indicated in figure 3, increasing the perfusion pressure over this range had no appreciable effect on the ability of sickled cells to pass through the filter. As in figure 2, the sickled cells were about 10 per cent as able as the normal cells to penetrate the filter under nitrogen, whereas under oxygen the same cells passed through the filter about 80 per cent as well as normal cells.

Effect of Agglutinins on Red Cell Filtration

"Incomplete" (Rh) agglutinins. One part of Crα1-labelled, D-positive red cells were mixed with 10 parts of D-negative red cells and the mixture was suspended in plasma containing a high-titer incomplete anti-D antibody and incubated for 1 hour at 37 C. The suspension of cells showed no agglutination, but about 10 per cent of the cells after washing in saline were agglutinated by Coombs serum or by P.V.P. As indicated in figure 4, the anti-D sensitized red cells passed the filter approximately as well as the normal (D-negative)
Fig. 2.—Effect of various diminished oxygen tensions on the filtration of sickle cell anemia red cells. As compared to the normal red cells in the same initial suspension, sickle cell anemia red cells were retained by the Millipore filter when the oxygen tension was diminished, particularly at tensions below 40 mm. Hg.

cells at perfusion pressures ranging from 4 to 124 mm. Hg. Each point represents the average of 8 separate experiments.

The mixture of sensitized and non-sensitized cells described above was suspended in the same antiserum to which had been added an equal volume of 5 per cent P.V.P. (K-44)* in isotonic phosphate buffer (.12 M, pH 7.4). This caused all of the red cells to form rouleaux, although on dilution in saline only about 10 per cent of the cells, presumably the sensitized ones, remained

*Schenley Laboratories, Inc., New York, N. Y.
Fig. 3.—Effect of perfusion pressure on the filtration of abnormal red cells. The filtration of Cr¹⁹⁵-labelled abnormal cells was related in each instance to that of tenfold the number of normal unlabelled cells in the same suspension. Type D red cells sensitized with incomplete anti-D (black circles) passed through the filter normally when suspended along with normal D-negative red cells in plasma. When suspended in P.V.P.-containing plasma and thus forming rouleaux, the sensitized cells were selectively trapped by the filter (white circles), albeit only at low perfusion pressures. Type A red cells agglutinated by anti-A were completely trapped by the filter at a low perfusion pressure (4 mm.), but partially traversed the filter at higher pressures (black squares). In contrast, sickled cells (under 95 per cent N₂, 5 per cent CO₂) were unable appreciably to traverse the filter at any physiologic perfusion pressure (white squares).
Fig. 4.—Effect of perfusion pressure on the filtration of sensitized red cells from a patient with acquired hemolytic anemia. The patient's Cr$^{51}$-labelled "Coombs-positive" red cells passed through the filter approximately as readily as tenfold the number of normal red cells present in the same plasma suspension. When the plasma contained P.V.P., however, the sensitized cells in rouleaux were selectively removed by the filter from the normal cells in rouleaux. As with red cells coated with anti-D (fig. 3) the trapping of sensitized red cells occurred only at low perfusion pressures.

in chains or clumps. Although the flow of all the red cells, including the normal (D-negative) cells, was diminished by the rouleaux-formation, that of the anti-D sensitized cells was much more strongly affected. As shown in figure 3, sensitized red cells in rouleaux passed through the filter only 10 per
cent as well as the unsensitized cells at a perfusion pressure of 4 mm. Hg. At only a slightly greater pressure (8 mm. Hg.), the passage of sensitized cells increased sharply to 33 per cent of the unsensitized cells, and there was relatively little difference between sensitized and unsensitized cells at perfusion pressures exceeding 18 mm. Hg. Similar results were obtained when rouleaux formation was induced by mixing the plasma with an equal volume of 1 per cent human fibrinogen. The addition of 50 mg. of hydrocortisonet/100 ml. plasma containing P.V.P. did not affect the filterability of the red cells added subsequently.

"Complete" (ABO) agglutinins. One part of Cr₅¹-labelled type A red cells was mixed with 10 parts of type O cells and the mixture was suspended in type O plasma. After incubation for 1 hour at 37 C., about 10 per cent of the cells were in clumps consisting of about 8 or 10 cells each. Although the flow of type O cells was only slightly diminished by the presence of the agglutinated type A cells, the passage of the agglutinated cells was markedly curtailed. At a perfusion pressure of 4 mm. Hg. none of the agglutinated cells traversed the filter and even at 30 mm. pressure only 26 per cent passed through (fig. 3). It was noted that none of the cells in the filtrate immediately after filtration at the higher pressures were agglutinated, but that agglutination reappeared as the filtrate was allowed to stand for a few minutes. Thus, the agglutinates had been disrupted by the pressure across the filter, permitting the passage of individual type A cells. Comparable results were obtained using Cr₅¹-labelled type B red cells suspended with type A cells in type A plasma.

When anti-D sensitized red cells were mixed with D-negative cells, washed, and agglutinated by Coombs serum, they manifested the same pressure-flow relationships on filtration as did type A cells agglutinated by anti-A rather than those of sensitized cells in rouleaux. When, in an analogous experiment, trypsin was employed to agglutinate the sensitized cells, the pressure-flow relationships of the agglutinated cells again resembled those of type A cells agglutinated by anti-A.

Filtration of Red Cells from a Patient with Acquired Hemolytic Anemia

Red cells from a patient with an acquired ("autoimmune") hemolytic anemia and a positive Coombs test were labelled with Cr₅¹, washed, and mixed with 10 parts of unlabelled normal red cells before suspension in compatible normal plasma. About 20 per cent of the red cells of this mixture were agglutinated by Coombs serum and 50 per cent by the P.V.P. test, indicating some transfer of antibody from the patient's cells to the normal cells. The Cr₅¹-labelled, sensitized cells in plasma passed through the filter almost as well as the "unsensitized" (or, at least, the less-sensitized) cells (fig. 4). However, when P.V.P. was added to the plasma, as described above, and the cells formed rouleaux, there was selective retention of the patient's red cells by the filter (fig. 4).

1One part of an ethanolic solution of hydrocortisone was added to 100 parts of plasma and compared to a control medium consisting of one part ethanol and 100 parts plasma.
Filtration of Hereditary Spherocytes

At perfusion pressures below 30 mm. Hg., the red cells of 5 patients with hereditary spherocytosis traversed the Millipore filter almost as well as normal red cells. Studies were made of the osmotic fragility patterns of those red cells which passed through the filter as compared to the initial suspension. In these studies the patients’ washed red cells were suspended in a medium of 1 part native serum derived from defibrinated blood and 4 parts isotonic phosphate buffer, pH 7.4; this change in protocol was made in order to obviate, as it did, the pH changes which attend manipulation of a bicarbonate-buffered (plasma) medium. Although the mean cellular osmotic fragility (i.e., 50 per cent hemolysis point) of the red cells of these 5 patients varied from normal to moderately increased, the red cells of each possessed a characteristic minor population of abnormally fragile (spheroidal) red cells. This population was prominent in two patients studied shortly after splenectomy, was moderately large in the single non-splenectomized patient studied, and was very small in the two patients studied several years after splenectomy. The osmotic fragility pattern of the majority of the red cells which had passed through the filter did not differ from the initial pattern. In each case, however, a large part of the minor population of very fragile cells disappeared during filtration (fig. 5). It was estimated that whereas less than 10 per cent of the whole cell population was retained by the filter, from 30 to 80 per cent of the minor population of abnormally fragile (spheroidal) cells were selectively retained.

DISCUSSION

These studies demonstrate that the physically abnormal red cells found in certain hemolytic anemias can be differentiated and segregated from normal red cells by an inert filter.

The physical factors which affect the filtration of particles principally include: the properties of the particles—size, shape, and viscosity; the properties of the filter—diameter and length of the openings, cross-sectional area; and the pressure difference across the filter. These factors must also apply to the red cell particle in its progress through the small openings of the capillary and sinusoidal beds of the body. Accordingly one would predict that sequestration, or filtration from the circulating blood, of red cells would be engendered by the following circumstances: (1) Physical alterations (in size, shape, or viscosity) of the red cells, as encountered in certain hemolytic states; (2) increase in the size, or change in the microanatomy, of a filtering organ, as of the spleen in the hypersplenism of methylcellulose injections in rats or of certain hypersplenic states in man; and (3) reduction in the perfusion pressure, as in that of the spleen during splenic vein or portal vein hypertension (“congestive splenomegaly”) and in the entire circulation during shock, which has been reported to precipitate extensive sickling crises. The present study has concentrated on the influence of physical alterations of red cells upon their filtration, specifically the influence of sickling, agglutination, and sphering. These observations may permit certain surmises or conclusions as to in vivo mechanisms.
Fig. 5.—The osmotic fragility patterns of hereditary spherocytosis red cells before and after filtration through a Millipore filter. Before filtration the mean osmotic fragility point of the cells was 0.46 Gm. per cent NaCl (normal 0.40 Gm. per cent), and there was a minor population (about 8 or 10 per cent of the total) of more fragile and more nearly spheroidal cells. About 90 per cent of the cells passed through the filter under a perfusion pressure of 4 mm. The filtered cells showed no change in their mean osmotic fragility but the minor population of more nearly spheroidal cells had been 70 to 80 per cent eliminated (i.e., trapped) by the filter.

Sickle Cell Anemia

At oxygen tensions below 80 mm., and particularly below 40 mm., the red cells from patients with sickle cell anemia were efficiently removed from a suspension containing tenfold their number of normal red cells. Since statistically each sickled cell was surrounded by 10 normal red cells, this indicates that capillary openings of the order of 5 μ which permit the passage of normally discoidal red cells will prevent the passage of isolated sickled cells, and are thereby themselves obstructed. Although much emphasis properly has been placed on the significance of the increased "bulk viscosity" of sickled blood in the pathophysiology of the hemolysis and vascular occlusions in sickle cell anemia, it appears that these occlusions may be initiated by the obstruction of capillaries by single cells and that the local pathologic process may devolve from the viscosity of individual cells.

Of particular interest is the finding that deoxygenated, sickled red cells
from patients with sickle cell anemia were not forced through the filter even at perfusion pressures of over 120 mm. Hg. (fig. 2). This resistance to flow far exceeds that by agglutinated red cells, and may explain the unique propensity of sickled red cells to obstruct and be sequestered by the high pressure systemic blood vessels, whereas agglutinated red cells are mainly sequestered in low pressure systems such as in the sinusoids of the liver and spleen (see below). Presumably the specific pressures needed to cause flow through a 5 μ pore filter are of comparative rather than absolute value for the precise pressure required to force a cell through an opening would depend also upon the diameter and distensibility of the opening.

Acquired (Autoimmune) Hemolytic Anemia

The difficulty of forcing red cell agglutinates composed of 8 or 10 cells through capillary tubes 5 μ in diameter is readily understood. Nevertheless, red cells agglutinated by ABO-antibodies did penetrate the filter to some extent, provided there was a sufficient pressure difference across the filter (fig. 3). Penetration of the filter occurred by disaggregation of the cells, as revealed by microscopic examination of the filtrate, and presumably the pressure required to do so provided a measure of the tensile strength of the antibody linkages between the cells.

Unlike red cells clumped by “complete” (e.g., ABO) agglutinins, cells merely coated with an “incomplete” (e.g., Rh) agglutinin passed through the filters normally. However, when these cells were aggregated into clumps and “permanent” rouleaux by agents such as P.V.P. or fibrinogen, the antibody-coated (sensitized) cells were efficiently trapped by the filter. Thus, under a low perfusion pressure (4 mm. Hg.) sensitized red cells passed through the filter only 10 per cent as well as normal cells in the same suspension. Trapping of sensitized red cells in rouleaux was sharply dependent upon the perfusion pressure, and was relatively inefficient at pressures of 18 mm. or more (fig. 3). The sensitized red cells from a patient with acquired hemolytic anemia behaved in this respect similarly to red cells sensitized by an incomplete anti-D antibody. Thus by inference, in vivo trapping of red cells coated with incomplete agglutinins would occur only in the presence of rouleaux-provoking agents and in filter beds having very low perfusion pressures.

Previous studies in man17 and animals2 have indicated that red cells coated by “incomplete” agglutinins (e.g., Rh) are preferentially trapped in the spleen, whereas red cells exposed to complete agglutinins (e.g., ABO) are trapped throughout the reticuloendothelial system, and thus mainly in the liver. An explanation has been made that this difference depends upon hemo-dynamic principles.4 Thus, sensitized red cells aggregated by fibrinogen and other rouleaux-provoking agents,8 may be trapped only in capillary beds with very slow blood flow and under low perfusion pressures, as is uniquely the case in the spleen, which possesses narrow tortuous sinusoids that conduct red cells slowly and may at times stop them completely.18,19 Such weakly aggregated clump of cells may be disaggregated by the somewhat greater shearing forces presumed to exist in the liver sinusoids. On the other hand,
red cells clumped by “complete” agglutinins may withstand this shearing force, and thereby may be arrested in the liver as well as in the spleen, albeit unable to withstand the force of the blood pressure in systemic capillaries. Direct evidence of the obstructive effects of weakly agglutinated red cells on the blood flow of liver sinusoids has been obtained from perfusion at low pressure of dog livers with dog blood treated with heterologous agglutinins. In addition, engorgement of liver sinusoids with clumped red cells has been observed in dogs following the injection of autogenous red cells agglutinated by metallic cations. The present studies substantiate the possibility, although they do not prove it, that the sites of sequestration in vivo of red cells exposed to agglutinates are determined by the tensile strength of the agglutinates and the shearing force of the pressure difference across capillary beds.

These studies do not exclude chemical interactions between antibody-treated red cells and reticuloendothelial cells, for red cells altered by either agglutinating or sensitizing antibodies, particularly those which fix complement, tend to adhere to phagocytic cells to form mixed agglutinates. Such mixed agglutination occurs rather slowly and weakly with red cells coated with incomplete agglutinins, however, and it seems probable that chemical interactions between red cells so-sensitized and reticuloendothelial cells is secondary to the physical trapping mechanism.

Hereditary Spherocytosis

Normal red cells which readily traversed Millipore filters with 5 μ pores were trapped by such filters when the cells were made more nearly spheroidal by suspension in hypotonic plasma. Red cells treated in this way are larger and thicker than normal but have a smaller diameter. The inability of such spheroidal cells to pass through the Millipore filter capillaries recalls the observations of Whipple who suggested that spherocytes are less able than discoidal cells to pass through the narrow openings of the splenic sinusoids. The spheroidal cell would be less able than a discoidal cell to fold or distort its shape under a given pressure so as to present the least cross-sectional area. Thus the spherocyte may suffer the impairment of an obese man attempting to bend at the waist.

Hereditary spherocytosis (HS) red cells differ from those produced by hypotonicity in that they are “contracted” cells having a smaller surface area than normal, rather than being swollen cells with a normal surface area. However, their spheroidal shape would render them similarly resistant to bending or distortion. In the present studies of HS cells the most nearly spheroidal cells were appreciably retained by the filter, whereas the majority of cells passed through normally.

A characteristic feature of the peripheral blood of many patients with hereditary spherocytosis, usually those with intact spleens or recently splenectomized, is the presence of two populations of cells: a major population which may have either a normal or an increased osmotic fragility; and a minor population having a markedly increased fragility. The minor population may range from a barely detectable to a relatively large fraction of the whole cell population. In spleen blood, however, the more fragile popu-
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lation constitutes a much larger portion of the whole than it does in the peripheral blood. Since the presence of a spleen promotes the occurrence of the “hyperspheroidal” population of cells,\textsuperscript{6,27} since the patients’ red cells only enter this minor population after several days in the circulation,\textsuperscript{28} it has become clear that this portion of cells has been “conditioned” by the spleen. It appears that it is these conditioned red cells which are predisposed to be trapped in the spleen, either because of their spheroidal shape or because of associated biochemical changes. Evidence has been obtained that red cells made spheroidal in other ways, as by heating\textsuperscript{21,22} or by exposure to lecithin,\textsuperscript{23} are thereby rendered vulnerable to splenic trapping, suggesting that spheroidicity \textit{per se} may provide the mechanism of sequestration.

To the contrary, Crosby and Conrad\textsuperscript{30} reported that flattening the red cells of two patients with hereditary spherocytosis by rendering the patients iron-deficient had no influence upon the rate of blood destruction. However, in those studies iron deficiency had little or no effect on the “conditioned,” hyperspheroidal population of cells. Thus, the “tonicity” at which osmotic hemolysis initially occurred before incubation was unaffected in one patient and, although decreased, remained markedly abnormal in the other, while the increased osmotic fragility of both patients’ cells after incubation was unaffected. Since it is these most nearly spheroidal, older, cells which determine the tonicity point of initial hemolysis in osmotic fragility curves and which are presumed to be the immediate precursors of the sequestered cells and to govern the rate of hemolysis,\textsuperscript{28} the character of the less spheroidal, major population of cells, which are young\textsuperscript{28} and have not been as yet critically conditioned may have no immediate bearing on the rate of hemolysis. If this interpretation is correct, the studies referred to\textsuperscript{30} do not exclude the role of spheroidicity \textit{per se} in the sequestration of HS red cells. However, the studies of Crosby and Conrad do substantiate the belief\textsuperscript{31,32} that the initial step in the hemolytic process is not related to the shape of the cell \textit{per se} but to its abnormal metabolic or structural susceptibility to normal splenic stasis\textsuperscript{33} and that the shape-change is a subsequent, albeit lethal, stage. Since it has been shown that hereditary spherocytosis red cells may show delayed mixing in the normal spleen in vivo,\textsuperscript{29} it might be suggested that this implies delayed splenic passage of these cells on the basis of an inherent surface abnormality. However, Prankerd\textsuperscript{31} has found that the red cells from a patient with hereditary spherocytosis whose cells were not abnormally spheroidal at the time were not delayed in their initial passage through a normal subject’s spleen, whereas the passage of spheroidal hereditary spherocytosis red cells was delayed. Accordingly, the following pathogenetic sequence has been proposed:\textsuperscript{32}

1. Hereditary spherocytosis red cells are abnormally susceptible to glucose deprivation.\textsuperscript{16,23,35} It is not clear at present whether this hypersusceptibility is initiated by a structural\textsuperscript{16} or a metabolic\textsuperscript{30,37} abnormality.

2. A randomly-selected minor population of hereditary spherocytosis red cells is subjected to repetitive stasis and glucose deprivation in the spleen.\textsuperscript{14,16,31}

3. This minor population of cells is continuously generated and becomes hyperspheroidal after several days of circulation.\textsuperscript{28}
4. The hyperspheroidal cells are finally trapped in the spleen, due either to changes in the physical or chemical attributes of the cell.

The present studies bear only on the fourth step presented above. They do not prove that the splenic trapping is dependent upon the spheroidal shape *per se*, but they do provide a physical analogue for that possibility.

**Summary**

Studies were made of the filterability of normal and abnormal red cells through an inert membrane-type (Millipore) filter having uniform non-branching capillary pores 5 μ in diameter. With this filter it was possible to remove selectively certain kinds of abnormal red cells from mixed suspensions of normal and abnormal red cells.

The red cells of patients with sickle cell anemia were selectively trapped by the filter under low oxygen tensions. Such sickled red cells were unable to pass through the filter even under a pressure differential across the filter (perfusion pressure) of over 120 mm. Hg.

Red cells agglutinated by anti-A were completely retained by the filter at perfusing pressures of 4 mm. Hg., but were partially disagglutinated and forced through the filter when the pressure was increased. Red cells coated with an incomplete (Rh) agglutinin and red cells from a patient with acquired hemolytic anemia were retained by the filter only in the presence of rouleaux-producing agents such as P.V.P. or fibrinogen and only at low perfusion pressures.

A small fraction of the red cells of patients with hereditary spherocytosis were trapped by a single passage through the filter; the trapped cells were largely derived from the minor population of hyperspheroidal cells.

The relevance of these findings to the mechanisms of red cell sequestration in vivo is discussed.

**Summario in Interlingua**

Esseva effectuate studios in re le filtrabilitate de erythrocytos normal e anormal a transverso un filtro del typo a membrana inerte (Millipore) con uniforme e non-brancate poros capillari de 5 μ de diametro. Con iste filtro il esseva possibile separar selectivemente certe typos de erythrocytos anormal ab miscite suspensiones de erythrocytos normal e anormal.

Le erythrocytos de patientes con anemia a cellulas falciforme esseva trappate selectivemente per le filtro sub basse tensiones de oxygeno. Tal falciformate cellulas non esseva capace de passar per le filtro mesmo in le presentia de un differential de pression trans le filtro (pression de perfusion) de plus que 120 mm de Hg.

Erythrocytos agglutinate per anti-A esseva retenite completamente per le filtro a pression de perfusion de 4 mm de Hg. Quando le pression esseva augmentate, illos esseva disagglutinate in parte e fortiate a transverso le filtro. Erythrocytos revestite de un incomplete agglutinin (Rh) e erythrocytos ab un paciente con acquirete anemia hemolytic esseva retenite per le filtro solmente in le presentia de agentes que forma rouleaus, tal como P.V.P.(K-44) o fibrinogeno, e solmente a basse pression de perfusion.
Un micre fraction del erythrocytos de patientes con spherocytosis hereditari esseva trappate per un sol passage per le filtro. Le trappate cellulas pertineva primarimente a! population, relativemente minor, de cellulas hyperspheroïdic. Es discutite le signification de iste constatationes pro le mechanismos del sequestration de erythrocytos in vivo.

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