New Thoughts on Hereditary Spherocytosis*†

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This discussion could be abbreviated by saying that if you take out the spleen, you can cure the patient with hereditary spherocytosis (HS). However, that is a clinical cure, and we know that careful measurements still demonstrate some shortening of red cell life span though well within the limits of normal marrow compensation. With the exception of intercurrent infections which may occasionally depress the marrow, patients with HS are usually asymptomatic after splenectomy. Having dealt with the treatment, however, there is much to be learned from examining the pathologic physiology of HS.

The HS erythrocyte is a fascinating cell whose pathologic changes provide us with insights into physiology of the normal red cell.

When one incubates hereditary spherocytes in a test tube, as in the autohemolysis test, for example, the changes are mimicked by normal red cells if you incubate the latter for a longer period of time. The shape change, from a disc to a sphere, which is already under way in the circulation of the patient with HS, occurs in normal blood when incubated to Adenosine Triphosphate (ATP) depletion in a test tube. The cells lose potassium and gain sodium. There is an initial period of swelling followed by shrinkage, and ultimately loss of membrane lipid and development of irreversible changes in shape. Although these changes all occur earlier in HS cells than in normal cells (Weed and Bowdler, 1966), many of the changes of this sort studied in the test tube take a period of time. However, the hereditary spherocyte is compromised within the circulation even as we find it in a sample of fresh blood. So, although *in vitro* changes are all of considerable interest, the time sequence is a matter of concern. Perhaps we have not really been examining the fundamental problem, since many of the *in vitro* alterations are late changes.

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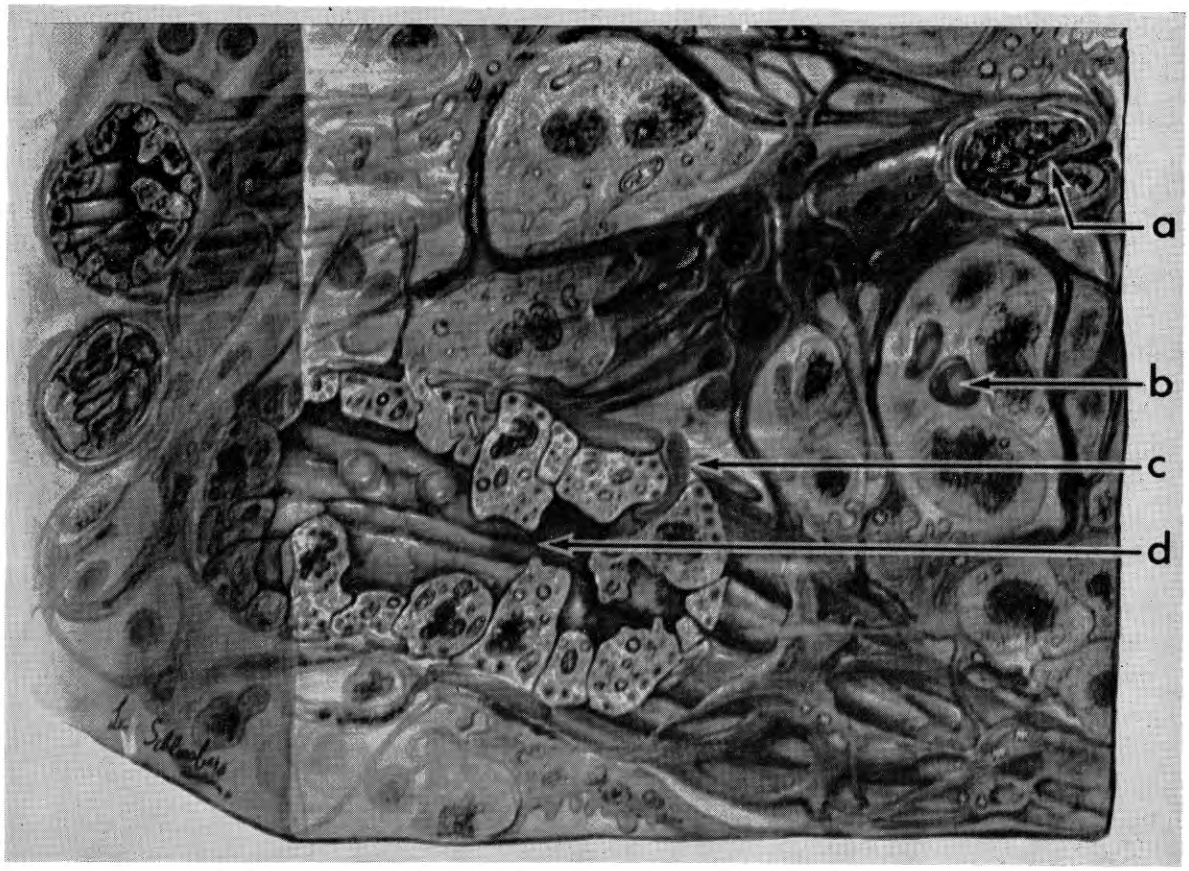

Fig 1—Fine structure of the red pulp of the spleen.
A) Terminal arteriole
B) Red cell in the splenic cord
C) Red cell traversing lumen in basement membrane
D) Splenic sinus.
(From Weiss, Amer J Anat 113: 91, 1963, with permission)

Role of the Spleen in HS

Let us look and comment on why removing the spleen is such a useful maneuver in the management of hereditary spherocytosis and examine why the splenic environment is particularly hostile to HS cells. The first point I would like to make is related to the anatomy of the spleen. As the blood enters the splenic artery, lymphocytes are filtered off into the white pulp, and the red cells which are much more deformable pass on into the red pulp. In the pulp, red cells have two possible pathways. One is a direct route from arteriole into splenic venous sinus, by-passing the pulp proper. The other is to pass through the terminal arterioles, out into the splenic cords, and then back into the sinuses and return to the general circulation.

Fig 1 is a diagramatic representation of splenic ultra-structure. As we go from A to D and follow the red cells as they make their way through the splenic pulp, we see the first hazard of the splenic circulation posed at A. Here, the high endothelial cells and the terminal arterioles deliver the first geometric requirement for deformation of the red cell. The cell must be very deformable and squeeze out in between the endothelial cells, which line the terminal arterioles just as they break up into the splenic pulp. The red cells must make their way through the cord itself. The cord is a potential space, rather than an actual anatomic space. The large hungry phagocytes which lie in the pulp (the cords) actually touch one another until they are pushed apart by the red cells making their way down. Many never make it, and get engulfed as they pass. If they are able to slip by these phagocytic cells, then the final anatomic hazard is the basement membrane (C) separating splenic cord from sinus. Here the cells must migrate through openings which are as small as from 0.5 to 5 microns in diameter. The red cell has a normal greater diameter of 8 microns but it must be able to change its shape rather dramatically to pass

through these small openings. Therefore, the first message that we can infer from looking at splenic architecture is the requirement for red cell deformation.

Determinants of Red Cell Deformability

What are the determinants of this deformability? First, the discoid red cell shape is very important with its excess of surface area in relation to volume. Any decrease in surface area or increase in volume, will produce a more spherical cell which is less able to deform. If the osmotic fragility is increased, it implies either decrease in surface area, increase in cellular osmotic contents, or cell volume in plasma. In addition to shape, the intrinsic deformability of the cell is critical. Cell contents must be fluid as illustrated by sickle cells in which hemoglobin becomes paracrystalline or crystalline under conditions of low oxygen tension. In the sickle cell, the contents and the overlying membrane become very rigid. In hereditary spherocytosis the intrinsic deformability of the membrane itself is altered as well as the shape, although contents appear unchanged in their viscosity.

Regarding shape, as mentioned, in order to develop sphering of a biconcave cell, there must either be an increase in the cell volume (which might be the consequence of abnormal permeability with swelling) without any change in surface area or, alternatively, a change in surface area (a loss of membrane material), or a change in the effective surface area by a conformational change in the membrane without any change in volume. Any one or combination of these leads to sphering and an increase in the osmotic fragility test.

Whether the HS cell swells or shrinks has been a matter of some discussion. The fact is that it does both. In terms of the autohemolysis test, which is read at 48 hours, the HS cell is in fact shrinking at the time it is hemolyzing. Fig 2 is from work done in 1940 by Drs. Ham and Castle. The volume changes and hemolysis of normal cells and those from a patient with hereditary spherocytosis are plotted against hours of incubation. Both normal and HS cells swell initially. The normal cell actually swells to about 120 percent of its zero time volume, and then shrinks. Even after

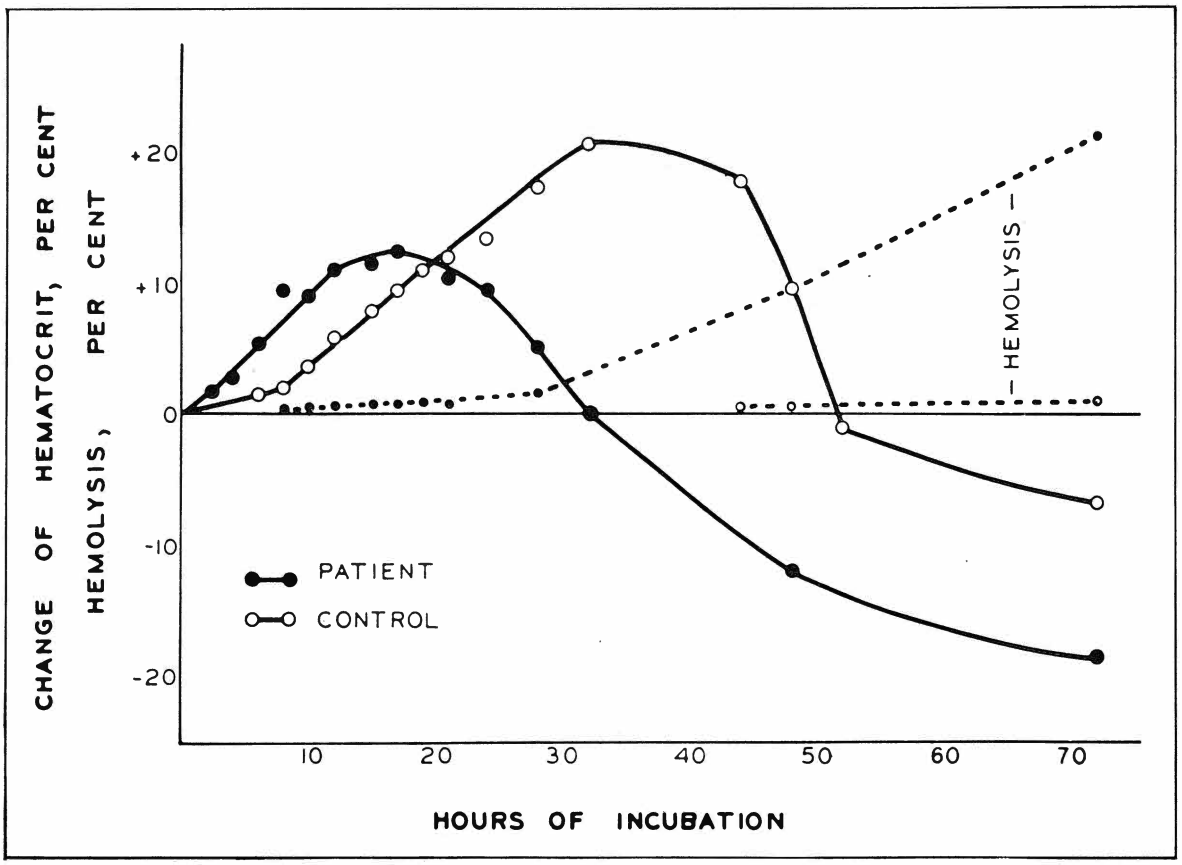

Fig 2—Sequential changes during prolonged incubation in volume and hemolysis of HS red cells (black circles) as compared with normal (white circles). Note that the pattern differs principally with respect to rate. HS cells beginning to swell sooner than normal, but do not swell as much and are shrinking when hemolysis begins.
(Reproduced through the courtesy of Dr. T. H. Ham and Dr. W. B. Castle).

about 48 hours, there is minimal hemolysis of the normal red cells. However, the HS cell swells only about 12 percent to begin with, and by 24 hours it is back to the starting volume. Yet, it is at this time that hemolysis begins, and continues as the cells continue to shrink. Thus, it is clear that hemolysis cannot be attributed to increase in volume of the hereditary spherocyte; it must be due to a decrease in the parameter which we will call *effective surface area.*

Nakao, Nakao and Yamazoe (1960) showed that normal red cells undergo a shape change from biconcave disc to sphere upon depletion of their ATP, but that if ATP is regenerated the shape change is reversible. This phenomenon can be seen in both normal and HS red cells after shorter periods of time (Weed and Bowdler, 1966). ATP depleted cells are virtually all either crenated spheres or smooth spherocytes. If one regenerates intracellular ATP with adenosine, within two hours the cells reassume their biconcave disc shape. These shape changes occur without any significant loss of membrane lipid or other membrane materials, implying a rearrangement of the cell membrane material. The process is independent of the addition of ouabain, so it is not a function of the sodium-potassium pump.

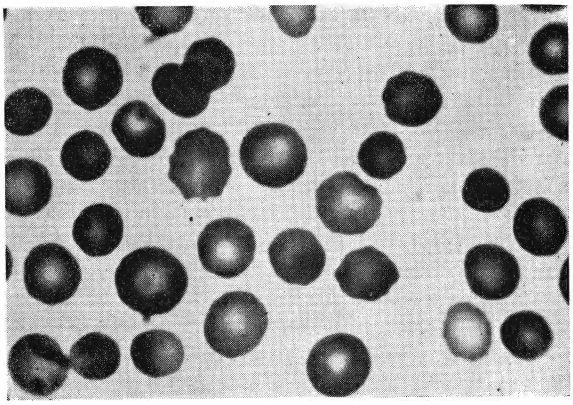

Fig 3—Peripheral blood from patient with hereditary spherocytosis, post-splenectomy. Note presence of biconcave discs, crenated discs, crenated and smooth spheres.

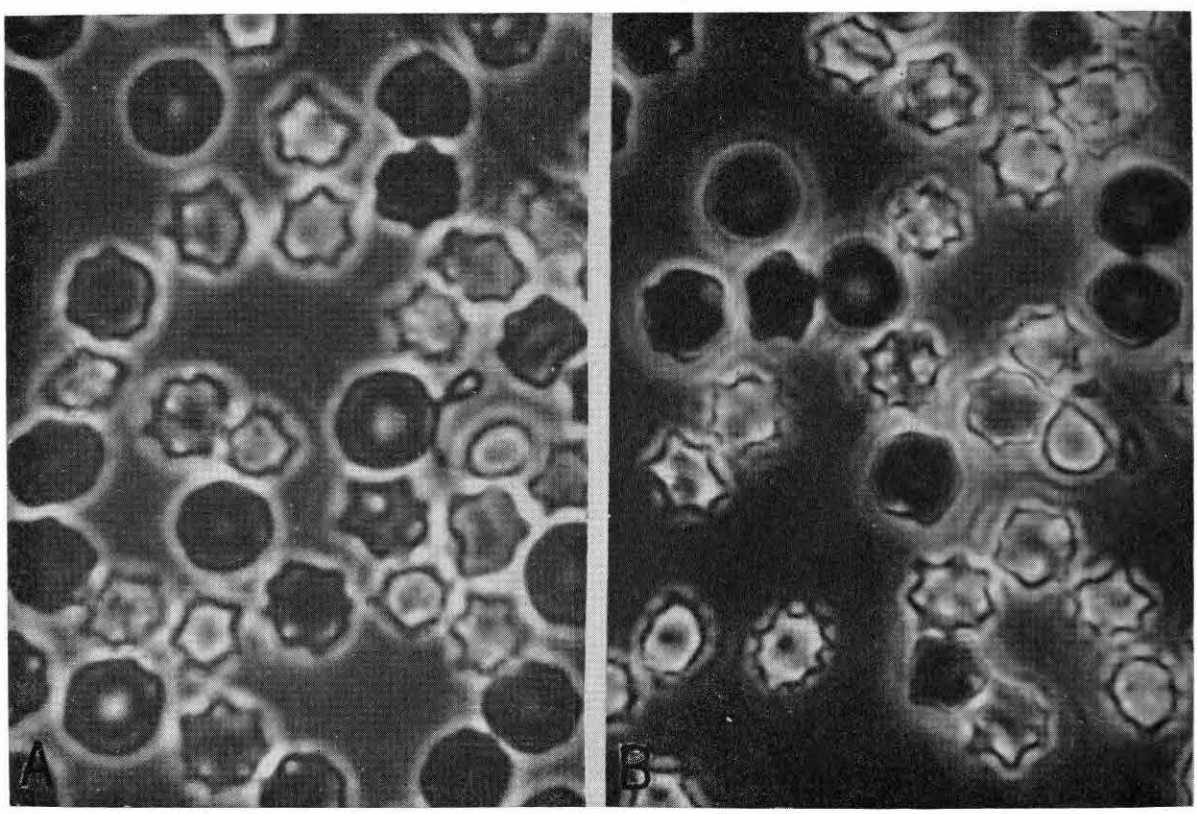

Fig 4—Phase photomicrograph of hereditary spherocytosis blood incubated 5 hours in absence of glucose. Note fragmentation. (From J Clin Invest 45: 1137, 1966, with permission).

Hereditary spherocytes, when incubated with adenosine, will also revert to a normal shape. Fig 3 is a smear from a patient with hereditary spherocytosis. In addition to the typical microspherocytes which we anticipate, one also sees biconcave discs. In fact, HS cells begin their lives as biconcave discs. The disc-sphere transformation seen in normal red cells when they are severely ATP-depleted, is already underway in the peripheral blood of the HS patient—in blood which has normal mean ATP levels. Although many attempts have been made to try to find a metabolic defect in the hereditary spherocyte, no glycolytic defect has been clearly demonstrated. Thus, we have to look elsewhere, and for this reason it has been suggested that the intrinsic defect is in the membrane itself.

If one observes HS blood from the standard autohemolysis test, in addition to the shape changes that are occuring, one can begin to see that the cell actually begins to fragment off pieces of membrane. The biochemical correlate of this is loss of membrane lipid as illustrated in Fig 4. The same phenomenon occurs in normal red cells after a much longer period of depletion. Thus, there is shape change initially, followed by this loss of pieces of membrane which may range in size from sub-microscopic aggregates of lipid, all the way up to pieces that may be as large as platelets. The consequence of breaking off pieces like this without losing contents or changing volume, is progressive increase in sphering. Decrease of surface area in relation to a constant volume leads to further sphering and the sphere so produced cannot negotiate narrow circulatory channels because of its shape. In patients who have severe hemolytic hereditary spherocytosis, Langley and Felderhof (1968) have demonstrated that there may be as much as a 40 percent decrease in membrane lipid. This is what has been called "conditioning" by the spleen. We would suggest that splenic conditioning may come about because cells that are already somewhat rigid are fragmented to some extent during their passage through the spleen, and as a result they actually suffer a real loss of surface area. They may be markedly more spherocytic when they reemerge in the general circulation and only pass around the circulation once or twice more. To recapitulate, the HS cell undergoes an accelerated shape change as a result of ATP depletion as an early phenomenon. Secondly, fragmentation loss of membrane surface area contributes to the shape change, further limiting *in vivo* and *in vitro* survival.

Turning to intrinsic deformability of the HS cell apart from shape, there is no evidence for any loss of fluidity of cell contents in hereditary spherocytes; but there are changes in the intrinsic deformability of the membrane itself. One can measure the viscosity or loss of jilterability of a packed washed cell suspension; both increase very dramatically with ATP depletion.

However, these are relatively gross ways of evaluat-

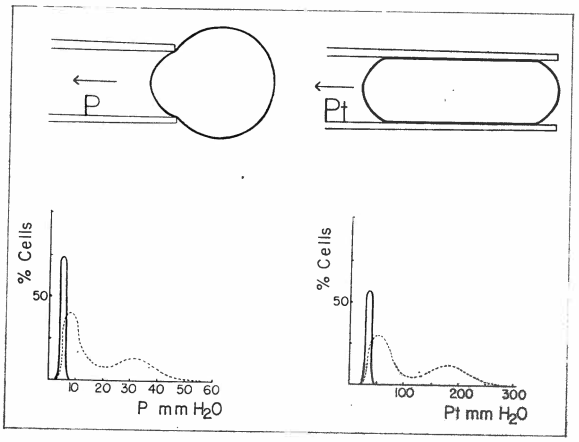

Fig 5—Distribution of individual cell deformability (P_t) and individual cell membrane deformability (P). Normal cells are represented by solid lines and cells from patients with hereditary spherocytosis by dotted lines. (From work by P. L. LaCelle, submitted to J Clin Invest)

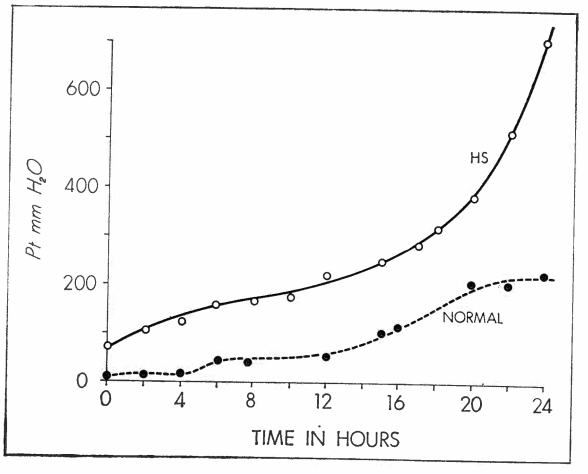

Fig 6—Changes in cellular deformability of normal erythrocytes and hereditary spherocytes during *in vitro* incubation (From work by P. L. LaCelle submitted to J Clin Invest)

ing the problem since both filterability and viscosity depend in part on shape and state of cell contents while our interest has been in the properties of the membrane itself. My colleague, Dr. LaCelle (Weed, LaCelle and Merrill, 1969; LaCelle, 1969) has used a micropipette to measure the pressure necessary to deform the membrane. With that technique, one can look at individual cells rather than the mean behavior of the population, as we do when we assay for enzymes, for example.

Normal cells will pass with ease into a 2.9μ pipette. The analogy in the microcirculation is to capillaries which have a length of about 14μ or longer. If there is a shorter length to be negotiated, the cell can get through a narrower diameter. Thus, a normal cell can negotiate the 0.5μ openings in the spleen, if the length of the passage is short enough. Capillaries in the general circulation range from 3 to 12μ in diameter, so some challenge of this kind is imposed throughout the general circulation. Canham and Burton (1968) have calculated that normal individuals or patients with hereditary spherocytosis, when the spleen is still present, have red cells in their circulation which can negotiate a hole 3 microns in diameter. This means that only smaller cells can persist in hereditary spherocytosis. When the spleen is removed, there is a 30 percent lessening of this requirement, so that they need only be able to negotiate a 4 micron diameter. It is clear that the spleen imposes a striking geometric requirement within the circulation.

The bone marrow, interestingly enough, has a similar architectural requirement. The openings between the hematopoietic cords and the sinuses in the marrow are of essentially the same dimensions (Weiss, 1965), and the architecture bears important similarities to splenic ultrastructure. The marrow ultrastructure imposes the first hazard to red cell survival; that is, the requirement to get out into the circulation in the first place. Since splenectomy is essentially curative, it implies that the hereditary spherocyte starts out life pretty normal, and as pointed out above, HS cells are biconcave discs when they enter the circulation. They escape from the marrow without any difficulty but are altered and destroyed after circulating. Many other cells which are more rigid at the beginning, eg, thalassemic cells, may undergo intramarrow hemolysis to a great extent probably because they cannot negotiate the narrow dimensions to begin their transit in the circulation.

Fig 5 illustrates two different kinds of information to be learned from micropipette measurements. If we think about the capillaries in the circulation, and the narrow passages in the spleen, the parameter that is designated P_t (or the negative pressure required to pull the whole cell into the pipette) is the appropriate measurement. This measurement, however, reflects shape and intrinsic deformability of the membrane; also if the cell contents are rigid they will be reflected

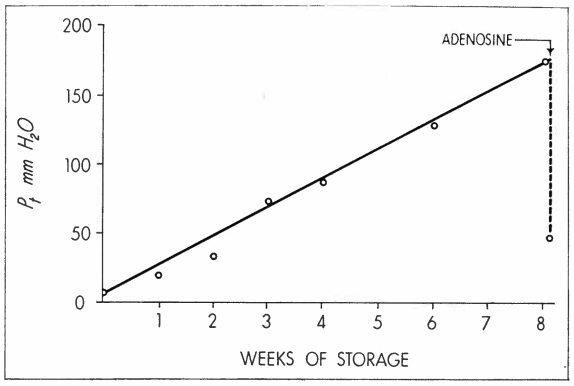

Fig 7—Alteration of P_t with duration of storage under blood bank conditions and restoration of P_t with incubation in 30 mM adenosine. (From Transfusion 9: 239, 1969, with permission).

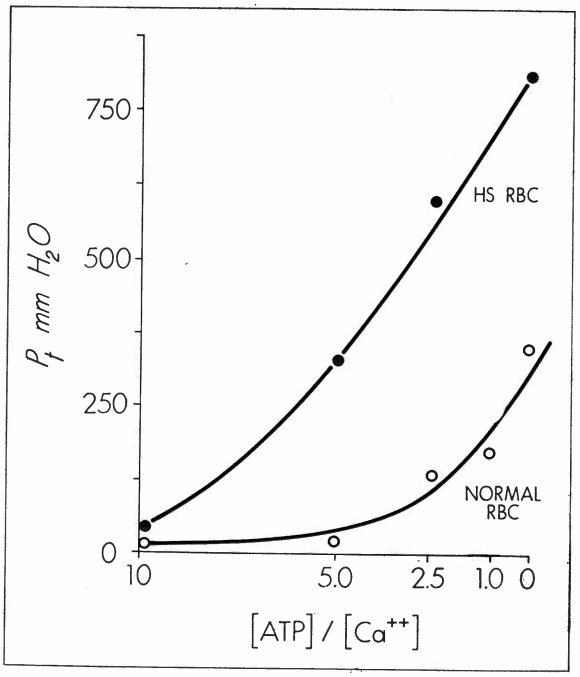

Fig 8—Deformability of normal and HS erythrocyte ghosts reconstituted to contain varying ATP/Ca ratios. (From work by P. L. LaCelle, submitted to J Clin Invest).

by an abnormal P_t value. P_t allows us to predict whether the cells will survive or not (LaCelle, 1969). The intrinsic deformability of the membrane itself, is evaluated by measuring the pressure to pull a hemispherical bulge into the micropipette. The latter pressure is independent of the shape of the cell unless it is already completely spherical, and it is independent of the contents. The data at the bottom of the figure shows solid lines which are values for normal cells, and you can see how remarkably deformable they are. 4 mm H_2O negative pressure will deform the membrane, and only 8 mm will pull the whole red cell into the pipette. For comparison, 50 mm H_2O pressure is required to deform a lymphocyte. The dotted line shows HS cells. Both P and P_t are very abnormal. In each case, there is a population which is only slightly different from normal, and a second population which is strikingly different from normal. Many of the cells in the abnormal range are not spherical, though, so there is an intrinsic abnormality of membrane deformability apart from change in shape.

The Role of ATP in HS

Fig 6 is a plot of deformability against time in an *in vitro* incubation. Normal red cells are represented by the dotted line. There is even in normal red cells, a small but significant loss of deformability between four and six hours when ATP falls about 30 percent. This occurs without any change in shape. Between six and 12 hours there is little change, but thereafter as ATP falls to about 20 percent the cells become very rigid. HS cells are somewhat rigid to start with and as they become ATP-depleted they become more rigid than normal cells similarly depleted. However, many changes occur during metabolic depletion and in order to try to factor these out, we have prepared ghosts reconstituted to contain specific metabolites. There is no difference between the deformability of a ghost and that of the intact cell from which it was prepared. Ghosts from depleted cells have the same rigidity as the depleted intact cell does itself. ATP, calcium or magnesium added outside the cell have no effect on it. They must be present inside. Incorporation of ATP or EDTA instantly restores a depleted cell to the properties of a fresh cell. Magnesium will reverse it to a significant extent, but not completely. If we take a normal fresh cell which has a value of about 4 mm H_2O for P, and incorporate 10^{-4}M calcuim, it becomes very rigid; the deforming pressure goes up to 350 mm H_2O. Of the various normal intracellular metabolic intermediates, ATP is specific; 2, 3-DPG, NAD and NADP are without effect.

Fig 7 is from studies of stored cells (La Celle, 1969) and illustrates the correlation between the P_t value, and survival. After storage for increasing periods, the cells become increasingly rigid. P_t has an excellent correlation with survival, and one can predict a 50 percent survival at P_t values of 100.

If one reconstitutes HS cells with ATP they are restored to the properties of fresh normal cells! Incubation of intact HS cells with adenosine will do the same thing and it will also restore their permeability to normal. If you prepare reconstituted ghosts, and alter the two most critical parameters—ATP and calcium—you find that at an ATP/Ca ratio which is comparable to that in normal intact red cells (a ten-fold excess of ATP over calcium), normal red cells are very deformable as seen in the lower curve of Fig 8. At an ATP/Ca ratio of 2.5/1, normal cells begin to become increasingly rigid. The HS cell is much more sensitive to a decrease in the ATP/Ca ratio as seen in Fig 8.

pH and Oxygen Tension

Returning to the relation of decreased deformability and the spleen, two additional local factors deserving of mention are pH and oxygen tension. pH has a rather dramatic effect on intrinsic membrane deformability, and Murphy (1967) has suggested that the spleen has a pH as low as 6.8. A pH of 6.8 compared to 7.4 essentially doubles the rigidity of red cells.

The other important local intrasplenic environmental factor relates to oxygen tension. A great deal of interest has been focused on oxygen dissociation curves, particularly in hemolytic states. Hematologists have once again discovered that the main purpose of the red cell is to transport oxygen to the tissues, and that this vital property may be drastically altered in disease states, either in a compensatory or a non-useful fashion. It is known now that organic phosphates (DPG in particular) will bind to deoxyhemoglobin, but ATP will also bind to deoxyhemoglobin. Potentially a cell that was sufficiently deoxygenated might have resultant binding of ATP to the hemoglobin and might become quite rigid. That, in fact, is actually what happens.

Normal mixed venous pO_2 is 40 mm Hg, which poses no threat to a normal red cell. However, when one gets down below pO_2 of 25 mm Hg, normal red cells become very rigid, just as if they either had had calcium incorporated or were severely ATP-depleted. This rigidity is instantly reversible, just by re-oxygenating the cell. Cells from patients with hereditary spherocytosis are more rigid than normal cells at arterial pO_2 values but they undergo a sharp increase in rigidity at $pO_2 < 40$ mm Hg. Thus, relative hypoxia within the pulp may be an additional very important splenic parameter. Both low pO_2 and pH may constitute two environmental parameters that the already somewhat rigid HS red cell encounters acutely upon entering the splenic pulp that will convert it from a cell that is just able to negotiate the circulation to a cell that is very rigid.

It has been suggested (Weed and Bowdler, 1966; Weed, LaCelle and Merrill, 1969) that red cell membranes contain a muscle-like protein which has sol-gel contractile potential when exposed to sufficient calcium. This would explain how calcium-membrane interaction within the cell produces a very rigid, sphered cell. Normally there is very little calcium in the red cell, and whatever is present is chelated by ATP. Magnesium would be expected to compete for the calcium site on the membrane, thereby explaining how magnesium can protect against the calcium. The ATP level, however, will be affected by the oxygenation of hemoglobin, and if the pO_2 falls below a critical level, hemoglobin will remove some free ATP and interfere with the role of the latter in preventing intracellular calcium from interacting with the membrane. Although evidence suggests the existence of a red cell membrane calcium pump which is ATP dependent, the latter operates over a long period of time, while the deoxygenation changes are acute and rapidly reversible. Based on the calcium-ATP curves for the hereditary spherocyte, it is suggested that the fundamental abnormality in HS cells may relate to an altered membrane protein with a higher affinity for calcium than that of normal red cells. Such a proposed difference in membrane affinity for calcium need be the only difference between a normal cell and hereditary spherocyte. Thus, at normal ATP levels, the HS cell would be unable to prevent calcium interaction with the membrane and it need not have higher calcium, lower magnesium, or abnormal ATP levels as it circulates. The whole sequence of events after entry into the low pH, low pO_2 splenic pulp might simply be an exaggeration of the calcium interaction seen in severely ATP-depleted normal erythrocytes.

Summary

In addition to its accentuated pattern of rigidity at decreasing ATP/Ca ratios, the hereditary spherocyte poses a special challenge within the splenic pulp because of its shape. In addition to the geometric requirement for deformability, the spleen also poses a challenge to HS cells because of its pH, and probably also the pO_2 within the splenic pulp. Thus, it is suggested that splenectomy is essentially curative in hereditary spherocytosis because it removes an organ with unique anatomic requirements for red cell passage as well as an adverse environment of lower pH and pO_2.

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