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23RD ANNUAL STONEBURNER LECTURE SERIES
Symposium on Hematologic Disorders

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Cover  Photographs of normal red blood cells (center) and of cells showing various abnormalities. Clockwise from top center: sickle-shaped cells, congenital acanthocytosis, pericellular spherules induced by heat, cell with Cabot's ring, megaloblast with Howell-Jolly bodies, erythroblast with basophilic stippling, iron-deficiency anemia, and hereditary ovalocytosis. Photographs reproduced with permission from Marcel Bessis' Life Cycle of the Erythrocyte, Sandoz, 1966.
The 23rd Annual Stoneburner Lecture Series
Symposium on Hematologic Disorders

Glucose-6-Phosphate Dehydrogenase Deficiency*

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Major attention was first focused on the problem of acute hemolytic anemia in the early 1950's, during the Korean War. Large numbers of American Negroes stationed in Korea developed this anemia after taking primaquine for treatment or prophylaxis of malaria. A study group was formed in Chicago under the direction of Alving; it was this group of investigators who contributed much of the early information which has led to our understanding of this type of anemia (Dern, et al, 1954; Dern, Beutler and Alving, 1954; Beutler, Dern and Alving, 1954). This also resulted in an upsurge of interest in red cell metabolism, and led to many of the advances we are hearing about in this lecture series.

Clinical Course

The clinical course of primaquine-induced hemolyosis is illustrated in Fig 1. Acute intravascular hemolyosis occurs two to four days after exposure to the drug, and is accompanied by hemoglobinuria. The hematocrit continues to fall for seven to 12 days and approximately 30 to 50 percent of the red cell mass is destroyed (Dern et al, 1954; Dern, Beutler and Alving, 1954). As the reticulocyte response reaches its peak in around ten to 12 days, the hematocrit begins to rise again and gradually returns to normal levels in four to five weeks, even though primaquine is continued. Radioiron red cell labeling studies have shown that it is the older population of red cells that is most susceptible to primaquine hemolysis while young erythrocytes are relatively resistant (Beutler, Dern and Alving, 1954). However, if the dosage of primaquine is increased sufficiently even the younger cells are hemolyzed.

At the time of the Korean War, the only way to detect a primaquine-sensitive soldier was to challenge him with the drug, which was not without danger. The usual hematologic studies were normal and were of no help in detecting the susceptible individual. The first significant clue came when Heinz bodies were noted on supravital staining of the peripheral blood smear during the acute hemolytic episode. This gave rise to the first in vitro test for detecting primaquine-sensitive individuals, the Heinz body test devised by Beutler (Beutler, Dern and Alving, 1955). In this test blood is incubated with the oxidant drug, acetylphenylhydrazine, and then observed for the presence of Heinz bodies. Fig 2 shows that the red cells from a susceptible individual, on the left, have many small Heinz bodies; while normal red cells, on the right, have one or two large Heinz bodies.

Mechanism of the Anemia

The formation of Heinz bodies is of major importance in the pathogenesis of this type of hemolytic anemia. The importance of deformability of the red cell as it travels through the microcirculation has been stressed throughout this lecture series. Heinz bodies, which represent precipitated denatured hemoglobin, are rigid bodies which hamper pliability of the red cell and interfere with its ability to traverse the microcirculation, particularly in the spleen. Rifkind (1965; 1966) has demonstrated this very nicely with electron microscopy of red cells containing Heinz bodies in the spleen. Fig 3 shows several red cells containing Heinz bodies. One red cell is completely devoid of hemoglobin except for the Heinz bodies which are firmly attached to the red cell membrane. In Fig 4 a red cell is shown going from a splenic cord into a sinus. It is apparent that the Heinz body in the tail will prevent either the entire red cell or a portion of it from making it through.

Although the Heinz body test was useful in detecting primaquine-sensitive individuals, it shed no light on the underlying biochemical defect. The first biochemical lead was the observation that red cell reduced glutathione (GSH) was lower in affected individuals than in normals. This difference was slight when fresh blood was used, but when blood was incubated for

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two hours with acetylphenylhydrazine there was a marked fall in GSH levels in primaquine-sensitive red cells with nearly no fall in normal cells (Beutler, 1957). (Fig 5) This formed the basis for the glutathione stability test for detecting susceptible individuals.

This observation focused attention on erythrocyte glutathione metabolism and eventually led to the delineation of the basic red cell defect. Glutathione reductase was studied first. When this proved to be normal, glucose-6-phosphate dehydrogenase (G6PD) was assayed and found to be deficient (Carson, et al, 1956).

The importance of the G6PD-glutathione system in protecting the red cell from oxidative injury can be summarized in a series of interrelated reactions (Table 1).

Table 1

| Interrelated Reactions of the G6PD—glutathione System in Protecting the Red Cell from Oxidative Injury |
|--------------------------------------------------|--------------------------------------------------|
| 1. Red Cell + oxidant \( \rightarrow \) \( \text{H}_2\text{O}_2 \) | 3. GSSG + NADPH + H+ \( \rightarrow \) 2GSH + NADP+ glutathione reductase |
| 2. \( \text{H}_2\text{O}_2 \) + 2GSH \( \rightarrow \) 2H\( \text{H}_2 \)O + GSSG | 4. G-6-P + NADP+ \( \rightarrow \) 6-PG + NADPH + H+ G-6-P dehydrogenase |
When the red cell is subjected to oxidative stress through exposure to some drug or infectious agent, hydrogen peroxide is generated. Although the erythrocyte is rich in catalase, which decomposes \( \text{H}_2\text{O}_2 \), glutathione peroxidase seems to be more important in the reduction of \( \text{H}_2\text{O}_2 \). An hereditary deficiency in catalase (Aebi, Heiniger and Suter, 1963) is not associated with hemolytic anemia while a deficiency of glutathione peroxidase is (Steinberg, Brauer and Necheles, 1970).

For glutathione peroxidase to remain effective in reducing \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \), a continuous supply of \( \text{GSH} \) must be available. This is accomplished by the reduction of \( \text{GSSG} \) to \( \text{GSH} \) by glutathione reductase, which in turn requires NADPH as a cofactor. A deficiency of glutathione peroxidase, glutathione reductase or glucose-6-phosphate dehydrogenase, or the inability to synthesize glutathione all lead to defective reduction of \( \text{H}_2\text{O}_2 \) and hemolytic anemia. When \( \text{H}_2\text{O}_2 \) accumulates, hemoglobin is irreversibly oxidized and precipitated as Heinz bodies (Mills and Randall, 1958; Cohen and Hochstein, 1961).

Tests to Detect G-6-PD Deficiency

We have mentioned the Heinz body and glutathione instability tests as methods to detect G6PD deficiency, but they are somewhat cumbersome, indicating a need for simple screening tests for large populations. We have found the methemoglobin reduction test (Brewer, Tarlov and Alving, 1960) to be simple and reliable. This test is based on the fact that there are two methemoglobin reductases in the red cell—one using NADH as a cofactor, and the other using NADPH which is derived from the G6PD reaction in the pentose phosphate pathway. Blood is incubated with sodium nitrate which oxidizes oxyhemoglobin to methemoglobin. Methylene blue is also added to stimulate the pentose shunt which leads to more efficient methemoglobin reduction. When there is G6PD deficiency the shunt is not stimulated, NADPH is not generated, and methemoglobin is not reduced properly. After two hours the brown color of methemoglobin remains, rather than the red color of oxyhemoglobin which is present after two hours incubation of normal blood. This test is simple to perform, requires only a few

Fig. 2—Heinz body formation after incubation with acetylphenylhydrazine. Primaquine-sensitive red cells on the left, normal red cells on the right. (From Beutler E, Dern RJ, Alving AS: The hemolytic effect of primaquine. VI. An in vitro test for sensitivity of erythrocytes to primaquine. J Lab Clin Med 45: 43, 1955. Reprinted with permission.)
common reagents, and can be read visually. However, it often gives equivocal results if carried out during an acute hemolytic episode, and is poor in picking up female heterozygotes.

The cyanide-ascorbate test of Jacob and Jandl (1966) is another simple screening test which can be read visually. This test has an advantage in that it is usually positive in the face of acute hemolysis and will usually detect female heterozygotes. The ascorbic acid causes generation of \( \text{H}_2\text{O}_2 \), which stresses the system described previously; the cyanide inhibits catalase. Thus, not only a deficiency in G6PD but a deficiency in the other enzymatic steps in the system also gives a positive test.

A young man whom we worked up recently for an hereditary hemolytic anemia provided a good example of the usefulness of this test. The methemoglobin reduction test was negative for G6PD deficiency; the cyanide-ascorbate test was positive, which immediately focused attention on the glutathione system. Enzyme assays for G6PD, glutathione reductase, and glutathione peroxidase were all normal or increased; but the level of GSH was found to be only ten percent of normal. Further studies showed that the patient had a deficiency of glutathione synthetase, which was responsible for his hemolytic anemia. The patient was homozygous for the deficiency, while his parents were heterozygous and had a lesser degree of red cell glutathione synthetase deficiency (Mohler et al, 1970). Although several families with this type of enzyme deficiency have been reported in Europe, this was the first such report in America. For the reasons enumerated, we now believe that the cyanide-ascorbate test is the most satisfactory screening test.

**Variants of G6PD Deficiency**

Although in the United States G6PD deficiency is seen primarily in the Negro (ten to 13 percent of Negro males), the deficiency occurs in many ethnic groups and over 50 mutations have now been described (Carson and Frischer, 1966; Kirkman, Kidson and Kennedy, 1968).

The enzyme has been purified and found to have a molecular weight of approximately 240,000 consisting...
of six peptide subunits weighing 40,000. There are two major electrophoretic patterns which are designated A and B. Table 2 shows which electrophoretic type occurs in various population groups. Normal Caucasians have only type B, while 82 percent of normal Negroes have type B and 18 percent have type A. Most Caucasians have G6PD deficiency type B, while in the Negro the common deficiency type is A and rarely B.

Yoshida (1968) and his colleagues have shown that the only difference between types A and B G6PD is that type A has aspartic acid while type B has asparagine. A great many biochemical comparisons in terms of enzyme kinetics have been made between normal and deficient type A in Negroes. The only difference which has been found is that the enzyme is degraded more rapidly in the deficient erythrocyte than in the normal (Yoshida, 1968). This explains why older red cells are more susceptible to oxidant stress than younger cells, since the G6PD content falls rapidly as the red cell ages. This also explains why it is only the mature red cell which shows a deficiency of G6PD in the Negro, as white cells have a short life span.

However, in G6PD deficiency in Caucasians from the Mediterranean, type B, the deficiency is noted in leukocytes as well as erythrocytes. Also, a kinetic difference has been observed when comparing the normal and deficient enzyme; the latter has a weaker affinity for its substrate, glucose-6-phosphate (Yoshida, 1968).

Fig 6 shows the world distribution of G6PD deficiency. It has been estimated that over 100 million people have this enzyme deficiency (Carson and Frischer, 1966; Beutler, 1966). Since the distribution follows mainly tropical areas where there is a high incidence of malaria, it has been proposed that the enzyme deficiency affords some protection against infection with the malarial parasite and accounts for its increased prevalence in such areas. However, malnutrition and other diseases with a similar geographic distribution,
Fig 5—Content of GSH in primaquine-sensitive and normal red cells before and after incubation with acetylphenylhydrazine. (From Beutler E: The glutathione instability of drug-sensitive red cells. J Lab Clin Med 49: 89, 1957. Reprinted with permission.)

![Graph showing content of GSH in primaquine-sensitive and normal red cells before and after incubation with acetylphenylhydrazine.](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>Population</th>
<th>Electrophoretic Type</th>
<th>Amino Acid Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Caucasian</td>
<td>B+</td>
<td></td>
</tr>
<tr>
<td>Normal Negro</td>
<td>B+ (82%)</td>
<td>Aspartic acid for asparagine</td>
</tr>
<tr>
<td>Deficient Caucasian</td>
<td>A+ (18%)</td>
<td></td>
</tr>
<tr>
<td>Mediterranean</td>
<td>B–</td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>B–</td>
<td></td>
</tr>
<tr>
<td>Deficient Negro</td>
<td>A–</td>
<td></td>
</tr>
<tr>
<td>Common</td>
<td>B–</td>
<td></td>
</tr>
<tr>
<td>Rare</td>
<td>B–</td>
<td></td>
</tr>
</tbody>
</table>

Fig 7 shows a family which we have reported (Mohler and Crockett, 1964) in which two first cousins were affected. The genetic transmission here, as in all cases of G6PD deficiency, is sex linked; the hemizygous sons show the full effect of the deficiency, and the heterozygous mothers show variable expression. In our two patients there was no measurable red cell G6PD activity and the mothers had borderline low values.

Fig 8 shows a comparison of G6PD levels in various groups. In our laboratory normal G6PD activity ranges from four to seven units. The patients with higher than normal levels had a variety of hemolytic anemias with reticulocytosis, the increased number of young red cells accounting for the elevated G6PD activity. The second column shows deficient Negroes who had about ten percent of normal activity. The last column shows three Caucasians with no measurable activity, and includes the two patients shown in Fig 7 plus a man of Turkish ancestry. The values in the top part of that column represent the relatives of the two cousins depicted in Fig 7, and again illustrate the borderline levels of the mothers.

**Causes of Acute Hemolytic Episodes in a G6PD Deficient Individual**

Table 3 lists some of the most common oxidant drugs; more complete lists are also available (Carson and Frischer, 1966; Beutler, 1966). Most of these drugs are aromatic amines; all of them act as electron carriers, hence having the ability to generate H₂O₂ and act as oxidants. Some are potent oxidants, such as the sulphonamides and nitrofurans. Others, such as aspirin and chloramphenicol, are relatively weak and cause hemolysis only in Caucasians with very low G6PD levels or when they are used in high dosages.

It is well known that a variety of drugs produce acute hemolysis in a G6PD deficient patient, but it has not been sufficiently emphasized that infections alone also cause hemolysis. Burka and his associates (Burka, Weaver and Marks, 1966; Burka, 1969), reviewing a large number of hemolytic episodes in G6PD deficient patients seen at Columbia-Presbyterian Hospital, found that infections alone were a more common cause of hemolysis than drugs alone. Acute hemolytic episodes occurred after bacterial pneumonia and a variety of viral infections, particularly viral hepatitis.

They also pointed out in their study that many women had hemolytic episodes. Approximately two percent of Negro women are homozygous for G6PD deficiency and would be expected to react to oxidant stress; but heterozygous female carriers, or about 20 percent of Negro women, were not thought to be susceptible. This study pointed out that there is enough variation in enzyme levels in female heterozygotes, in keeping with the Lyon hypothesis, that a significant
GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

number will hemolyze when exposed to the oxidant stress of drugs or infection.

In summary, G6PD deficiency should be suspected and tested for not only in Negro men who are taking oxidant drugs, but also in Negro women and during acute infections whether drugs are used or not.

References
DERN RJ, BEUTLER E, ALVING AS: The hemolytic effect of

Fig. 6—The distribution of glucose-6-phosphate dehydrogenase deficiency. (Reprinted from Human Biology 32: 50, 1960: Metabolic polymorphisms and the role of infectious diseases in human evolution, by Motulsky AG, by permission of the Wayne State University Press. Copyright 1960 by Wayne State University Press.)
D. N. MOHLER

Table 3

Some Common Drugs which Produce Hemolysis of G6PD Deficient Red Cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonamides</td>
<td>Sulfamethoxypridazine (Kynex®, Midic®)</td>
</tr>
<tr>
<td></td>
<td>Sulfisoxazole (Gantrisin®)</td>
</tr>
<tr>
<td></td>
<td>Salicylazosulfapyridine (Azulfidine®)</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Nitrofurantoin (Furadantin®)</td>
</tr>
<tr>
<td></td>
<td>Furaltadone (Altafur®)</td>
</tr>
<tr>
<td></td>
<td>Nitrofurazone (Furacin®)</td>
</tr>
<tr>
<td>Analgesics</td>
<td>Acetylsalicylic acid</td>
</tr>
<tr>
<td></td>
<td>Acetanilide</td>
</tr>
<tr>
<td></td>
<td>Acetophenonetidin (Phenacetin)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>Thiazides (Diuril®, Hydrodiuril®)</td>
</tr>
<tr>
<td></td>
<td>Acetazolamide (Diamox®)</td>
</tr>
<tr>
<td>Hypoglycemic Agents</td>
<td>Tolbutamide (Orinase®)</td>
</tr>
<tr>
<td></td>
<td>Chlorpropamide (Diabinese®)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Chloramphenicol (Chloromyacin®)</td>
</tr>
<tr>
<td></td>
<td>Para-Aminosalicylic Acid (PAS)</td>
</tr>
<tr>
<td></td>
<td>Vitamin K (Hykinone®)</td>
</tr>
<tr>
<td></td>
<td>Naphthalene (Moth Balls)</td>
</tr>
<tr>
<td></td>
<td>Fava Beans</td>
</tr>
</tbody>
</table>


Fig 8—Glucose-6-phosphate dehydrogenase activity of red cells from normal subjects, and from enzyme deficient Negro and Caucasian individuals.
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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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 spherening 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 spherering 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

![Graph](image)

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.
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 auto-hemolysis test, in addition to the shape changes that are occurring, 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 spherering. Decrease of surface area in relation to a constant volume leads to further spherering 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 re-emerge in the general circulation and only pass around the circulation one 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-
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 2.9 µm pipette. The analogy in the microcirculation is to capillaries which have a length of about 14 µm 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 0.5 µm openings in the spleen, if the length of the passage is short enough. Capillaries in the general circulation range from 3 to 12 µm 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, (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, with duration of storage under blood bank conditions and restoration of P, 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 $P_i$ value. $P_i$ 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$_2$O negative pressure will deform the membrane, and only 8 mm will pull the whole red cell into the pipette. For comparison, 50 mm H$_2$O pressure is required to deform a lymphocyte. The dotted line shows HS cells. Both $P$ and $P_i$ 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$_2$O for $P_i$ and incorporate $10^{-4}$M calcium, it becomes very rigid; the deforming pressure goes up to 350 mm H$_2$O. 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 $P_i$ value, and survival. After storage for increasing periods, the cells become increasingly rigid. $P_i$ has an excellent correlation with survival, and one can predict a 50 percent survival at $P_i$ 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, spheroid 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 $pO_2$.

References


LANGLEY GR, FELDERHOF CH: Atypical autohemolysis in hereditary spherocytosis as a reflection of two cell populations: relationship of cell lipids to conditioning by the spleen. Blood 32: 569, 1968


Paroxysmal Nocturnal Hemoglobinuria—New Thoughts*

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Introduction

The illness I am going to discuss bears some relationship to what we have been discussing previously—that is, basically the problem is one of hemolysis brought about by a defect in the red cell (Dacie, 1967). However, it differs in several major respects from the other hemolytic anemias under discussion. First, the defect is not congenital but rather acquired. Also, there may be 100 million to 200 million people with a glucose-6-phosphate dehydrogenase deficiency, but mercifully paroxysmal nocturnal hemoglobinuria (PNH) is rarer. The incidence is probably not as low as has been suspected, but increases as one looks for the disease. In our own clinic we have, within the last three years, found 16 patients with PNH, some by referral to be sure. It is important to identify the patients with PNH because of the implications that are inherent in treating these patients.

Clinical Presentations of PNH

PNH is probably one of those diseases that is badly misnamed. With its present name, people tend to think of PNH only when the patient comes to the physician complaining of passing dark urine in the morning which clears by noon. Under this definition, hardly twenty percent of those patients with PNH would be ordinarily identified.

It has become apparent that there is a great deal more wrong with the hematopoiesis of patients with PNH than was originally thought. This defective hematopoiesis has two characteristics which may be manifest in different proportions in different patients. On the one side, the predominant problem may be marrow hypoplasia, not simply of the red cells but also of the white cells and of the platelets (Lewis and Dacie, 1967). The other hematopoietic problem is that the cells that are made are defective. The manifestation easiest to investigate is, of course, the defect in the red cell which renders it sensitive to the lytic action of complement. This is traditionally demonstrated by the acidified serum test (Ham and Dingle, 1939). This defect in the red cells leads to hemolysis, which then leads to hemoglobinuria. It has become apparent in recent years, though, that the white cells and platelets are likewise defective (Aster and Enright, 1969).

The classic clinical presentation of the patient with PNH is, of course, hemoglobinuria (Table I); when it is paroxysmal and nocturnal, it is a striking finding. But, as I have said, if we relied upon this symptom we would detect, I suspect, not more than twenty percent of the patients with the disease. When the symptom is present, the morning urine may be very dark indeed and the evening urine may not be clear. The amount of hemoglobinuria may vary greatly from day to day. One of our patients who was also a wine connoisseur, reported his urine in terms of the wine it most nearly resembled. If the color resembled the color of Chablis, he was pleased but when it looked like Port, he became apprehensive.

The more usual presentation of PNH is chronic hemolysis without, at least to the patient’s eye, hemoglobinuria. In any patient with chronic hemolysis, one ought to look for the presence of PNH.

As we mentioned, the bone marrow hypoplasia may be a major part of the illness, and PNH may grow out of patients with bone marrow hypoplasia as a primary event. The bone marrow hypoplasia may be due to drugs, as it has become apparent that some patients taking chloramphenicol may, as they recover, show the manifestations of PNH (Quagliana, Cartwright and Wintrobe, 1964). The cytopenia in PNH patients may be simply leukopenia, and/or thrombocytopenia, without much anemia. But when the patient is carefully observed, and blood is carefully examined, the defect in the red cells may be demonstrated.

Iron deficiency is a common presentation of patients with PNH. Usually the reticulocytosis is higher than one would expect for a similar degree of iron deficiency in patients not hemolyzing. This is one of

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the few causes of hemolysis in which the loss of iron is a major problem, and, therefore, iron deficiency is not uncommon.

Venous thrombotic phenomena are not uncommon in patients with PNH, possibly because of the release of thromboplastic substances during intravascular hemolysis. These thromboses are not simply the usual arteriosclerotic variety, such as coronary thrombosis or cerebral thrombosis. In PNH the thrombi are usually venous and result in unusual syndromes. Abdominal venous thrombosis may occur in the portal vein, the splenic vein, or most characteristically the hepatic veins, leading to the Budd-Chiari syndrome. The presentation of the hepatic vein thrombosis may be very insidious in its onset, but many patients that die of PNH, have evidences of hepatic vein thrombosis. Cerebral vein thrombosis can lead to very bizarre neurological syndromes due to cerebral venous or sinus thromboses.

Finally, a most interesting complication of PNH has been the occurrence of acute leukemia. Dr. Dameshek predicted many years ago that acute leukemia might well occur in these patients, and indeed it has been reported in three patients (Dameshek, 1967). In all three, the leukemia is acute myelogenous and the PNH defect in the red cells disappeared with the onset of leukemia.

**Diagnosis: Demonstration of the Red Cell Defect**

How do we make the diagnosis of PNH? We now define the disease in terms of the red cell defect which is manifest as an abnormal sensitivity of the red cells to lysis by complement. It is present in patients with PNH, by definition, and leads to the problems of the hemolysis on which we will now base most of our remaining discussion.

The sensitivity to complement lysis may be detected in several ways. In Fig 1, I have illustrated the way we do this (Rosse and Dacie, 1966). To generate this graph, cells are sensitized with an antibody, anti-I cold agglutinin in this case, and are lysed with different amounts of normal human serum as a source of complement. In the ordinary complement titration using sheep cells, differences in the amount of lysis reflect not differences in the red cells, since sheep cells are assumed to be always the same, but rather differences in the amount of complement present. In the complement sensitivity lysis test illustrated here, the amount of complement present at a given dilution of serum is the same since normal human serum is used, so that differences in lysis will therefore reflect not differences in the amount of complement, but differences in the sensitivity of the cells to lysis by complement in the presence of antibody.

The cells from a normal patient form a single straight line in this test when one plots the amount of complement present on a logarithmic scale against

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**TABLE I**

<table>
<thead>
<tr>
<th>Clinical Presentation and Complications of Paroxysmal Nocturnal Hemoglobinuria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Nocturnal hemoglobinuria.</td>
</tr>
<tr>
<td>2. Chronic hemolytic anemia.</td>
</tr>
<tr>
<td>3. Pancytopenia - aplastic anemia.</td>
</tr>
<tr>
<td>leukopenia</td>
</tr>
<tr>
<td>thrombocytopenia</td>
</tr>
<tr>
<td>5. Acute myelogenous leukemia.</td>
</tr>
</tbody>
</table>

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Fig 1—The lysis of normal and PNH cells by anti-I and normal human serum. The single population of normal cells and the two populations of PNH cells are seen.
the logarithm of the fraction lysed over the fraction unlysed (please forgive that way of expressing the data; it has been a tradition since 1916, at least, for plotting this sort of thing in order to obtain a straight line). The straight line with normal cells indicates that there is a single population of cells with respect to complement sensitivity. The graph also illustrates that it requires very large amounts of complement in order to lyse these cells. When PNH cells are examined in the same system, a double curve is obtained. One population of cells is nearly normal, although almost never entirely normal with respect to their complement lysis sensitivity. Another population of cells, called the complement-sensitive cells, are extremely sensitive to lysis by complement; that is, they require very small amounts of complement to lyse the cells. The proportion of cells in each population will vary from patient to patient.

It does not really make any difference what antibody one uses in order to demonstrate this phenomenon. In Fig 2 the same thing is demonstrated using a rabbit antihuman antibody. Here again, the normal is a single straight line, and both sensitive and insensitive populations in PNH cells are demonstrated. In this instance, the difference between the complement sensitive and the complement insensitive populations is not quite so striking as with the anti-I, but is nevertheless apparent. From this and other data (Rosse and Dacie, 1966), we have concluded that the increase in immune lysis under these circumstances is not an effect of antibody but rather is due to the fact that the cells have increased sensitivity to the effect of complement.

What do we mean by increased sensitivity to the effect of complement? We mean simply that the completion of the complement sequence is more efficient. In the normal human red cell, the complement sequence appears to be very inefficient; i.e., for every complement sequence that is begun, few go through to completion—probably less than 1 in 500 on the normal cell. In the PNH cell, the chances of a complement sequence, once begun, going through to completion are very much greater although there is still a considerable amount of inefficiency in the system. The nature of the defect which leads to this complement sensitivity is, at the present time, totally unknown. There is a very interesting set of compounds which can render cells which are normal to appear somewhat similar to PNH cells. These are the compounds that contain the reactive thiol groups such as glutathione, cysteine, and so on. The concentrations of these compounds that are required are often very high, however, and the exact relationship of the effect of these compounds to the defect in a PNH cell is not at all certain at the present time.

What is the relationship of this complement sensitivity to the phenomena with which we are more familiar, i.e., the diagnosis of PNH from the in vitro tests that we use, or the clinical syndrome that we observe in the patient? The diagnosis of PNH is not, under ordinary circumstances, made by generation of the complex curves which I have shown. Rather, at least historically, this diagnosis has been made by the Ham test, or the acidified-serum lysis test. In the Ham test, one simply acidifies normal serum and incubates the cells of the patient to observe whether hemolysis takes place. It was early observed, by Ham himself, that anything that destroyed complement (that is, heating the serum, adding agents which would chelate calcium, and so on) would destroy the ability of that serum to hemolyze the cells of patients with PNH. Further evidence that there is a relationship between the complement lysis and lysis observed in the Ham test is obtained from electron microscope pictures. When the cells of patients with PNH are lysed in acidified serum and examined on the electron microscope, small, round, 100 A defects with the heaped-up edge which are characteristic of destruction of the membrane by complement are observed (Rosse, Dourmashkin and Humphrey, 1966).

What initiates the lysis in the Ham test is not at all certain. There is no demonstrable antibody present in the normal serum. It is thought that fluid-phase activation of complement may be sufficient to bring

Fig 2—The lysis of normal and PNH cells by rabbit antihuman red cell antibody and human serum.
about the lysis of the cell. In the early days, it was proposed that another immune system related to properdin was responsible for the initiation of complement lysis, and this may still have a certain amount of validity although the exact nature of this system is not at all certain (Hinz, 1966). Nevertheless, the difference between the normal cells and PNH cells is probably not a difference in the amount of complement that is added to the cell, but in the efficiency with which the complement sequences are able to be completed in order to bring about lysis of the cell.

It was early observed by Dacie and others that the amount of lysis in the Ham test was highly variable from patient to patient. When we examined the relationship between the amount of lysis in the Ham test and the proportion of complement-sensitive cells present in patients with PNH, we found that, in general, the percentage lysis in the Ham test is less than the proportion of complement-sensitive cells. In addition, different specimens of normal serum may tend to lyse different numbers of cells. In most instances the complement-sensitive population is not entirely lysed on the first exposure to normal acidified serum. However, on exposure a second or third time, the entire complement-sensitive population may be lysed. If the complement-sensitive population is very small, the lysis may not be detected in the Ham test; this has occurred in three patients in our experience. In general the Ham test is a reasonably good screen, although it is rather cumbersome when done with all controls. It does detect most patients with PNH, but does not quantitate very well the proportion of the complement-sensitive population.

In addition to the false negative test, there is a "false-positive" acidified serum lysis test. This occurs in a group of patients, first described in detail by the Crookstons in Toronto (Crookston, et al, 1969) and by Vervilghin in Belgium, who have a congenital defect in erythropoiesis, characterized by a dyserythropoietic anemia, multi-nucleated red cell precursors, and a membrane defect. The membrane defect is manifest in three ways. First, it makes the cell more complement-sensitive than normal, but the complement sensitivity is distributed in the single population, and the complement sensitivity increase is five to six times normal rather than 30 times normal as seen in PNH. Second, an antigen is exposed on the red cell surface which is not present on normal cells, and to which normal people have isoantibodies. The reaction of a naturally occurring antibody present in most normal serum, together with a modest increase in complement sensitivity, renders the acidified lysis test positive with these cells. However, the serum of these patients does not contain the antibody, and therefore their own cells will not lyse in their own serum, despite acidification.

More recently, Drs. Hartmann and Jenkins at Nashville have introduced the sucrose lysis test, which likewise appears to depend upon complement lysis (Hartmann and Jenkins, 1966). The reduction in ionic strength increases the efficiency of several of the steps in the complement sequence. Therefore, by incubating the cells in the presence of normal serum, the complement-sensitive cells in PNH will lyse. With this test there is a much better correspondence between the percentage of cells in the complement-sensitive population, and the amount of lysis that one obtains, although the degree of variability is somewhat greater than in the acidified serum test. To date, we have not found any false negatives in patients with PNH having small complement-sensitive populations which do not lyse in the sucrose lysis test. There are, on the other hand, instances of false-positives of which one must be aware. The cells of patients with immune hemolytic anemia in which the antibody can fix complement may lyse, if the cells are incubated in their own serum and sucrose. However, if the cells are incubated in someone else's type compatible serum, lysis will usually not occur.

We have related the in vitro phenomena that we commonly associate with PNH to the complement-sensitive population. In order to relate these to in vivo phenomena, we have investigated the life span of the red cells in PNH. If the complement-sensitivity has anything to do with the hemolysis that one observes, then there should be a difference in the life span of the complement-sensitive cells, compared to the complement-insensitive cells.

If the life span of the red cells is determined using glycine-2-C14 as a cohort label, the complement-sensitive cells are seen to be destroyed extremely quickly in a random fashion. The half-time of this random destruction is in the order of four to six days, indicating that the life span of an individual complement-sensitive cell may be extremely short. The complement-insensitive cells are also lysed in a random fashion to a far greater extent than normal. Ordinarily, less than five percent of normal cells are lysed before reaching senescence. In PNH, probably 25 percent of the complement-insensitive cells are lysed. Those cells that miss the random destruction process are destroyed at approximately 110 to 120 days. The complement-sensitive cells do not become complement insensitive, since as the complement-sensitive population is lysed and falls, the amount of label in the whole population falls, indicating that the cells in the complement-sensitive population are born bad and die young.

We have also studied the red cell life span of both populations of cells with DFP32. Again, the life span of the complement-sensitive cells was much shorter than that of the complement-insensitive cells, reaffirming that the complement-sensitive cells are de-
stroyed more rapidly than are the complement-insensitive cells. However, the life span of the complement-insensitive cells is shorter than normal, again demonstrating that these cells are not normal. So, we can see then that the lysis of the complement-sensitive cells is an important in vivo correlation to an in vitro demonstration of complement-sensitivity.

Determinants of the Rate of Hemolysis

One might ask, on what does the daily rate of hemolysis depend? In Table II, we have listed some of the things which appear to determine the amount of hemolysis in patients with PNH. In the first place, probably the most important parameter is the proportion of complement-sensitive cells. Among most patients with PNH, the sensitivity to complement of this population is nearly the same. In general, those patients with a high proportion of complement-sensitive cells have a higher rate of hemolysis. On the other hand, an occasional patient does have a complement-sensitive population which, instead of being 15–25 times more sensitive than normal, is 5–7 times more sensitive. These patients have less hemolysis and less difficulty with the disease. Thus, on rare occasions, the decreased sensitivity of the complement-sensitive population may spare the patient some of the trial and tribulations of the disease.

On occasion, the complement-insensitive cells may also be markedly abnormal. If this population is more sensitive to complement than usual, then the rate of hemolysis may in part depend upon destruction of cells in the population.

Another important determinant of the amount of hemolysis is the rate of erythropoiesis. The proportion of precursors of cells in the complement-sensitive population is far greater than the proportion of these cells in the peripheral blood. Therefore, each day a large number of complement-sensitive cells are delivered to the circulation. If the ability to make red cells is increased, the number of complement-sensitive red cells that are delivered to the circulation, and thus the number that are susceptible to hemolysis is increased. Thus patients who are iron deficient with PNH may have a bout of hemoglobinuria when given iron because of the increase in erythropoiesis.

Fig 3 illustrates a patient with iron deficiency who, after initial studies, was given intravenous iron. The reticulocyte count did not increase until four to five days later. It was only when the reticulocyte count began to increase that there was an increase in urine hemoglobin. As the reticulocyte count rose, the amount of urine hemoglobin rose precipitously. During this time, the hematocrit rose and the percentage of complement-sensitive cells rose somewhat. At this point, the patient began to have abdominal pains, a fairly frequent complication of a hemolytic episode in patients with PNH, probably due to minor thromboses in the mesentery and the gut wall. The patient was transfused, and the reticulocyte count and the urine hemoglobin fell. He was next given iron dextran and did not have a hemolytic response because erythropoiesis had been suppressed by elevation of the hematocrit by transfusion. Thus we were able to demonstrate that the hemoglobinuria which follows iron

### TABLE II

Determinants of Hemolysis in Patients with Paroxysmal Nocturnal Hemoglobinuria

1. **Cellular Abnormality.**
   a. Proportion of complement-sensitive cells.
   b. Sensitivity of complement-sensitive cells.
   c. Sensitivity of complement-insensitive cells.

2. **Rate of Erythropoiesis.**
   a. Response to iron therapy.
   b. Response to androgen therapy.

3. **Initiating Mechanisms.**
   a. Immunologic phenomena.
      i. infections—especially viral
      ii. vaccinations
   b. Drugs—? heparin.
   c. Unknown.
      i. nocturnal-acting.
      ii. others.
therapy is not due to a toxic effect of the iron, but rather due to a sudden increase in erythropoiesis. We have seen the same effect in patients given androgens, but the hemolytic episode is not so abrupt or so dramatic as in iron therapy.

Other determinants initiating hemolysis are not well known. Some are immunologically related, since the red cells are susceptible to immunologic lysis. One would expect that if immunologic reactions were going on in the patient, hemolysis might be increased. This is, in fact, true. Certain virus infections, especially the Hong Kong flu, vaccinations, especially typhoid vaccinations, and some bacterial infections have been incriminated as precipitating crises in hemolytic episodes.

The cause of the nocturnal variation is totally unknown. It was originally thought that maybe there was an accumulation of acid during sleep which would then mimic the acidified serum lysis test. This is probably not true. It has also been suggested that the diurnal variation might be related to diurnal variations in erythropoiesis. This is difficult to demonstrate, but it may be true. Most of the time, we do not know why patients with PNH have a paroxysm of hemoglobinuria.

Summary

I have tried to summarize some of the facts we know, and some of the questions we need to ask in a disease which, although it is not common, probably is not as rare as we once thought. Once the diagnosis is made, one must be very careful in tending to the patients, since there are instances in which they react much differently than would normal people—either by the hemolytic episode or with other complications. This is especially true with regard to surgery, which may be extremely dangerous in these patients. The post-operative course may be complicated by thrombosis, infections, and other forms of morbidity. Identification of these patients is important, simple, and relatively helpful in their care.

References


DAMESHEK W: Riddle: What do aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and "hypoplastic" leukemia have in common? Blood 30: 251, 1967


ROSSE WF, DACIE JV: Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. II. The role of complement components in the increased sensitivity of PNH red cells to immune lysis. J Clin Invest 45: 749, 1966

The New Hemoglobinopathies*

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In 1910 a Chicago physician, Dr. J. B. Herrick, called attention to a peculiarly formed red cell which he observed in the blood smear of an anemic West India Negro student (Necheles, Allen and Finkel, 1969). Linus Pauling, a physical chemist, demonstrated in 1949 that the protein portion of the hemoglobin molecule found in patients having sickle cell disease differed from that found in normal adults. Ingram (1957) further defined the molecular defect in that sickled hemoglobin differed from normal adult hemoglobin in one amino-acid residue per half molecule. The specific molecular identification of the defect in sickle cell anemia was that of valine replacing the normally occurring glutamic acid in the number 6 position of the beta chain. This finding provided inspiration and technical knowledge whereby over 100 different hemoglobinopathies have now been described. The clinical explorations which have resulted from studies of mutant hemoglobin have allowed definition of a variety of syndromes whose manifestations require explanation in terms of the effects of molecular distortion on physiological processes. The complex patterns of inheritance which lead to amino-acid substitution in either the alpha or beta chain dictate a variety of structural alterations which may modify hemoglobin affinity for oxygen, afford protection against falciparum malaria, and provoke changes in microcirculation which influence renal and splenic function. Whether abnormal hemoglobins induce these changes is determined at least in part by the site of replacement, changes on external surfaces exerting little effect, and the co-existence of abnormalities elsewhere in the molecule. The discovery of various hemoglobinopathies has demonstrated that specific molecular defects may express themselves in various pathophysiological manifestations. For instance, sickle cell anemia, a single molecular defect of the beta chain in which valine is substituted for the number 6 amino-acid glutamine, presents a wide spectrum of clinical manifestations. A marked dissociation between the degree of anemia and the occurrence of other complications may be found. More detailed pathophysiological studies of patients with hemoglobinopathies should be performed to gain a better understanding of the expression of molecular disease.

Classification of Hemoglobinopathies

A better understanding of the hemoglobinopathies can be obtained if they are broken down into various categories. Table I shows these different types of hemoglobinopathies. The first is a defect in the rate of synthesis of a specific polypeptide chain. The most frequently considered clinical disease in this category is that of thalassemia. In this group of hemoglobinopathies, there is either a defect in the synthesis of one or more alpha chains or one or more beta chains. These molecular defects result in specific clinical syndromes. Beta thalassemia minor may range in severity from completely asymptomatic elevations of one of the minor hemoglobin components to a syndrome consisting of splenomegaly, intermittent jaundice, and mild anemia. The peripheral blood in patients with thalassemia minor shows hypochromia, microcytosis, anisocytosis, and poikilocytosis. Target cells are quite common and the red cells show a decreased osmotic fragility. Beta thalassemia major is a more severe disease and is characterized by hypochromic anemia, splenomegaly, and normoblastemia. These patients are severely affected and have signs of severe anemia and phenotypically show expanded facial bones and increased pigmentation. Alpha thalassemia is usually incompatible with life and results in hydrops fetalis.

The second major categorization of the hemoglobinopathies are those hemoglobins in which a biochemical alteration in hemoglobin structure has occurred. The first significant subdivision within this category consists of patients with sickle cell disease, hemoglobin C, hemoglobin D, and combinations of hemoglobin S with other hemoglobinopathies such as hemoglobin SC, SD, and AS.

The next sub-category includes the unstable hemoglobin hemolytic anemias (UHHA). The unstable hemoglobin hemolytic anemias are characterized by the

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presence of hemolytic anemia from infancy. Heinz bodies are usually seen in the red cells of patients with this type of anemia. Splenomegaly is usually present and when splenectomy is performed the peripheral blood shows a marked increase in the number of red cells containing Heinz bodies. Dacie (Dacie et al, 1964) reported studies on five patients with mild anemia which he characterized as hereditary Heinz body anemia. A distinctive characteristic of this group of patients with hemolytic anemia was the presence of Heinz bodies in a blood suspension stained with methyl violet. When hemolysates prepared from washed red cells of all five patients were heated to 50 C for one hour, easily visible precipitates developed. Since this time, numerous hemoglobinopathies characterized by hemolytic anemia and thermal lability of the hemolystate have been demonstrated. On paper or starch gel electrophoresis at an alkaline pH, the abnormal hemoglobin band is seen as a minor component moving more slowly than hemoglobin A. It is extremely difficult to demonstrate these hemoglobinopathies on conventional paper or Agar gel electrophoresis. Carrell and Lehmann (1969) have reviewed in detail the unstable hemoglobin hemolytic anemias. It is felt that the globin of the unstable hemoglobins precipitates because it is no longer stabilized by firm heme to globin bonding. Hemoglobin Zurich is an example of the unstable hemoglobin hemolytic anemias in which there is a replacement of the non-heme linked histidine beta 63 by an arginine amino-acid. This hemoglobinopathy is characterized by an increased susceptibility of the individual to hemolysis as a result of taking sulfonamides. Other examples of the unstable hemoglobin hemolytic anemias consist of Ube-I disease. This was described in 1963 in a fifteen-year-old Japanese girl. This has subsequently been

<table>
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<td><strong>HEMOGLOBINOPATHIES</strong></td>
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</table>

I. A defect in the rate of synthesis of a specific polypeptide chain (Alpha and Beta Thalassemia)

II. A biochemical alteration in hemoglobin structure
   A. Sickle cell disease, Hb. C, D, and combinations of S with other hemoglobinopathies
   B. The unstable hemoglobin hemolytic anemias (U H H A)
      Hb. Sabine, Zürich, Köln, Ube-I, Gunhill, and Freiburg
   C. Hemoglobins with increased affinity for oxygen
      J Capetown, Chesapeake, Yakima, Kempsey, Rainser, Hiroshima
   D. Hemoglobins with decreased oxygen affinity - Hb. Seattle and Kansas
   E. Hemoglobins unable to combine with oxygen - methemoglobin
      1) Hb. m disorders - Boston, Saskatoon, Iuate, Milwaukee
      2) Oxidant drugs
      3) Diaphorase enzyme deficiency DPN dependant system

III. Defect in polypeptide synthesis with structural alterations (sickle/β- Thalassemia disease)
shown to be similar to hemoglobin Koln which is characterized by blocking of the 93 amino-acid cysteine in the beta chain.

Schneider (Schneider et al, 1969) described hemoglobin Sabin disease in which there is a substitution of the beta chain at the 91 amino-acid of a lucine amino-acid by a proline residue at helical position F7 of the beta polypeptide chain. Other hemoglobinopathies in this group are those of hemoglobin Sydney (Raik, Hunter and Lindsay, 1967; Carrell et al, 1967) in which variant a substitution of valine for alanine at the beta 67 position occurs. Santa Ana (Opfell, Lorkin and Lehmann, 1968), Gun Hill (Bradley, Wohl and Rieder, 1967), Torino (Beretta et al, 1968), Hammersmith (Dacie et al, 1967), Genova (Sansone, Carrell and Lehmann, 1967), and numerous other hemoglobinopathies have been described in this category. On a molecular basis, the instability of hemoglobin molecules is thought to result from either a defect in the heme-binding site, a change in the overall globin confirmation, or a defect at the site of subunit interaction where the alpha and beta chains react together (Carrell and Lehmann, 1969). It has been pointed out that the concentration of amino-acid substitutions of the unstable hemoglobins in the hemoglobin binding site is striking. Of the 11 positions cited by Perutz (1965) as being most closely related to the heme group, seven are found to be replaced in the unstable hemoglobins. A molecular change which would alter the stability of the hemoglobin binding sites would result in instability of the hemoglobin molecule.

Substitutions of amino-acids lining the heme pocket or crevice by others that are too large, alter the distribution of electric charges, or permit formation of abnormal cross links within the molecule, decrease its stability with resultant precipitation of hemoglobin within the red cell and associated hemolytic anemia. Another group of changes consists of the substitution of proline for lucine residues. This changes the overall globin conformation with loss of stability of the molecule. Another aspect leading to stability of the globin heme molecule is the site of subunit interaction. It appears that it is necessary that heme and globin be bound together for normal solubility and function of hemoglobin.

The instability of hemoglobin H and Barts which are tetramers of normal beta and gamma chains respectively, has been previously demonstrated. Substitutions in the area of subunit contact promote dissociation to monomers which are inherently unstable.

The third sub-grouping within this category of alterations in hemoglobin structure consists of those hemoglobins associated with increased affinity for oxygen. Charache (Charache, Weatherall and Clegg, 1966) investigated hemoglobin Chesapeake, an alpha chain variant, which was found to have an increased oxygen affinity. This hemoglobin is characterized by a substitution at the 92 residue of the alpha chain of lucine for arginine. The amino-acid substitutions in hemoglobins with increased oxygen affinity have been found in two areas. One is the area of contact between the alpha 1 and the beta 2 subunits in the tetramer; the other is near the C-terminal portion of the beta chain. Hemoglobin Rainier (Stamatoyanopoulos, Yoshida and Adamson, 1968) is the substitution of histidine for invariant residue 23 tyrosine in the beta hemoglobin polypeptide chain. Hemoglobin Chesapeake, Yakima, Cape Town, and Kempsey all have changes in the region between the F and H helical segments (Jones et al, 1967). These areas provide the end chain contact between the polar groups of the alpha and beta chains and are involved in alpha-beta chain interaction. Substitution in these areas seems to alter the normal respiratory movements of the subunits such that oxygen affinity is increased. It should also be noted that four of these hemoglobinopathies have remarkably decreased n values with nearly normal effects.

The fourth sub-grouping within this major category is that of hemoglobins associated with decreased oxygen affinity characterized by hemoglobin Kansas (Reissmann, Ruth and Wohours, 1961) in which there is a substitution of asparagine at the 102 residue of the beta chain by threonine. It is also a result of a substitution in the area of alpha 1, beta 2 contact.

The heme and globin components of the polypeptide are linked together by the iron of the heme binding to a specific histidine residue. This occurs at position number 87 in the alpha chain and number 92 in the beta chain. In addition, the histidine residue at number 58 in the alpha chain and number 63 in the beta chain are bound to the iron of the heme by either an oxygen molecule in oxyhemoglobin or a water molecule in desoxyhemoglobin (Necheles, Allen and Finkel, 1969).

Four of the five hemoglobin M's are produced by substitutions of the invariant histidine residues on either side of the iron atom in the heme area (Conley and Charache, 1969). The presence of oxygen tends to change the iron to the ferric form producing methemoglobin, a compound that cannot combine reversibly with oxygen. The substitution of tyrosine for histidine renders ferric iron so stable that the usual intracellular enzymes cannot reduce it to the functional ferrous (oxygen binding) state.

The maintenance of heme iron in a functional (Fe ++ ) state requires metabolic machinery for reducing iron in ferric form (Fe+++). This is accomplished by a dipyridine nucleotide. DPN (NAD) dependent methemoglobin reductase is a means by which the diaphorase enzyme acts to convert methemoglobin (Fe+++Hb) to functional hemoglobin (Fe++Hb). Conversion of one or more of the four ferrous atoms in the hemoglobin molecule to ferric category leads to
an increased affinity of the remaining ferrous atoms for oxygen.

Thus, amino acid substitutions and heme-globin interactions are important in the molecular behavior of hemoglobin. In addition, cell constituents influence the oxygen affinity of hemoglobin. Benesch (Benesch and Benesch, 1967) has shown that the affinity of hemoglobin may be increased or decreased by its interaction with organic phosphates. Increased intracorpuscular levels of 2-3-Diphosphoglycerate (2, 3-DPG) combine reversibly with deoxyhemoglobin, shifting the oxygen dissociation curve to the right. Charache (Charache et al, 1970) has shown that the concentration of 2-3-DPG is increased in hemoglobin SS erythrocytes as compared to hemoglobin AA red cells. This may partially explain the decreased oxygen affinity of hemoglobin SS. In addition, increased levels of 2-3-DPG have been found in low oxygen tension states and may be an important compensatory mechanism in hypoxemia.

The third major category of the hemoglobinopathies are those in which there is a defect in polypeptide synthesis with a structural alteration combined with an inability of the individual to synthesize certain of the alpha or beta polypeptides. An example of this is sickled-beta thalassemic disease.

Recently we described an example of methemoglobinemia associated with a Coombs positive hemolytic anemia. This patient (G.R.) was found to have increased levels of methemoglobin in his red cells during a severe hemolytic episode. However, the majority of his methemoglobin was extracellular consisting of hemoglobin which had been converted to methemoglobin. The patient showed multisystem degeneration and died in hepatic and renal failure. Methemoglobin has a characteristic spectral absorption curve which can be employed in its identification. Fig 1 shows a spectroscopic analysis of this patient's plasma. The methemoglobin is treated with cyanide to form cyano-methemoglobin and the change in light absorption at 632 mµ is measured. This method is quite specific except for the M hemoglobins.

Fig 1—Spectroscopic analysis of plasma from patient G. R. with hemolytic anemia and free methemoglobin.
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which have anomalous spectroscopic properties. A simple screening method for this hemoglobin can be performed by obtaining three tubes of hemolysate or plasma. One tube is shaken with room air and if it does not turn red with adequate oxygenation, this is an indication of an abnormal hemoglobin. Potassium cyanide should be added to the second tube and if it turns red this indicates the presence of methemoglobin. Sulphhemoglobin produces a dark band at 618 m\(\mu\), which is difficult to distinguish from that of methemoglobin in the hand spectroscope. However, it can be recognized by the fact that it is not removed by the addition of cyanide. Hydrogen peroxide (3 percent) causes both of these bands to disappear. Addition of potassium cyanide and hydrogen peroxide to hemolysates is a simple screening procedure. A more specific identification can be obtained with a spectroscopic examination as demonstrated in Fig 1.

Methemoglobin reduction in normal cells may be accomplished by four known systems. These are ascorbic acid, glutathione, DPNH diaphorase, and TPNH diaphorase. Glutathione is maintained in the reduced state by glutathione reductase, an enzyme chiefly active with TPNH but also capable of using DPNH as a substrate. The DPNH diaphorase is by far the most active reducing methemoglobin.

Another new hemoglobinopathy which has enabled us to gain insight into both the molecular and pathophysiology of sickle cell disease is that of hemoglobin Memphis/S disease. This was initially discovered by Lorraine and Alfred Kraus at the University of Tennessee (Kraus et al, 1966; Kraus et al, 1967). This hemoglobinopathy is characterized by a normal and mutant alpha chain with an alpha \(\alpha_2^a/\beta_2^a\) and hemoglobin Memphis/S consisting of \(\alpha_2^m/\beta_2^a\) in two subjects. These patients, homozygous for hemoglobin S and heterozygous for hemoglobin Memphis, have very mild sickle cell disease suggesting that the serious consequences of some hemoglobinopathies may be ameliorated by structural changes at other chain positions. Following the initial investigations by Kraus, we found a third patient with homozygous hemoglobin S heterozygous for hemoglobin Memphis who exhibited a similar profound dissociation between the degree of his anemia and the presence of serious complications. Special attention has been directed toward elucidating the effect of this hemoglobin on blood viscosity in an effort to account for the benignity of the process (Cooper et al, 1969).

Our interest in this area was stimulated by (J.P.) a 53-year-old Negro male who has had episodic abdominal and musculoskeletal pain since childhood and has been repeatedly hospitalized for treatment of anemia (Fig 2). Despite shortness of breath and need for frequent blood transfusions, he worked as a farm laborer until four years ago when pulmonary insufficiency forced his retirement. He is 5' 1" tall, weighs 105 lbs., and his blood pressure ranges from 130–190 systolic and 85–100 diastolic. Funduscopic examination reveals slightly distended retinal veins. Slit lamp studies have shown no comma-shaped or curlicue segments of conjunctival vessels and no sludging. He exhibits signs of mild pulmonary emphysema and a Grade IV ejection systolic murmur was heard at the apex. The liver was smooth and nontender and was felt 5 cm below the right costal margin. The spleen was nonpalpable. Double distal interphalangeal palmar creases were present in each middle finger and on the left fifth finger.

Fig 2—Patient J. P., 53-year-old Negro man with hemoglobin Memphis/S disease.

Admission laboratory data shows a urine specific gravity of 1.010; hemoglobin 9.5 gm/100 mg; hematocrit 27 vol percent; RBC 3.1 million/cu ml; MCV 100 \(\mu\); MCHC 33 vol percent; reticulocytes 5–24.6 percent. The creatinine clearance was 59 ml per minute. The serum folate levels were 19 ng/100 ml; serum iron 149 mcg/100 ml and iron binding capacity 244 mcg/100 ml. Hemolysis began at 0.34 percent sodium chloride and was complete at 0.28 percent in
an unincubated sample; with incubation, marked resistance to hemolysis was exhibited at lower concentrations of sodium chloride (Fig 3).

Hemoglobin electrophoresis revealed no hemoglobin A with hemoglobin either in the S or F forms (Fig 4). Alkali denaturation showed fetal hemoglobin to be 6 percent. Bone marrow aspiration yielded a marrow which was 100 percent cellular showing marked erythroid hyperplasia and many sickled red blood cells (Fig 5). Peripheral blood smears contained many sickled cells and nucleated erythrocytes (Fig 6). Poikilocytosis was prominent and Howell Jolly bodies were present. Liver function studies disclosed no evidence of abnormality. An oral cholecystogram demonstrated many small gallstones and a chest film showed mild left ventricular hypertrophy. Radiographic bone survey revealed diffuse osteoporotic and osteosclerotic changes as well as concavity of the vertebral bodies consistent with sickle cell anemia. No bone infarcts were seen and at no time could the spleen be visualized by X-ray. Serum bilirubin was 2.6 mg/100 ml of which 2.0 mg/100 ml was conjugated. The lactate dehydrogenase was 1980 units and the direct and indirect Coombs tests were negative.

Routine hematological data were derived by standard techniques. Sickling was ascertained using Sherman's test and minimal concentration gelling of hemoglobin solution was determined according to the method of Singer. Numerous isotopic studies consisting of $^{51}$Cr labeled erythrocytes, $^{51}$Cr labeled platelets, and heat-treated $^{51}$Cr labeled erythrocytes were employed. Hemoglobin electrophoresis was done on starch gel and cellulose acetate using a discontinuous buffer at pH 8.6. Individual erythrocytes were stained for fetal hemoglobin which was then quantitated by alkali denaturation using Betke's method. After electrophoresis, hemoglobin was characterized by chain separation, tryptic digestion with peptide mapping, and amino acid analysis of aberrant polypeptides according to Kraus (Kraus, Miyaji and Iuchi, 1966). Whole blood viscosity was determined with the falling ball viscometer. Oxygenated and deoxygenated blood viscosities were also determined by using a modified Brookfield viscometer. Renal function studies were determined and the effect of hyperosmolar solution on the patient's hemoglobin was also studied.

Family studies were performed and the results showed a co-dominant inheritance pattern. The Sherman test (Table II) demonstrated 4.5 percent sickled cells in arterial blood and 21.5 percent in venous blood. Venous blood from homozygous SS patients contains 30–60 percent sickled forms and 5–20 percent sickled forms in the arterial blood. A minimal gelling concentration of 27.2 gm/100 ml was obtained for $S$ hemoglobin and 35.6 gm/100 ml for hemoglobin Memphis/S (Table III). The patient's deoxygenated blood viscosity was determined by the

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**Fig 3—Incubated osmotic fragility of J. P.'s RBC.**

**Fig 4—H. J. Electrophoresis with cellulose acetate strips pH 8.6.**
HEMOGLOBINOPATHIES

Fig 5—Bone marrow aspiration showing hemoglobin Memphis/S sickled erythrocyte.

falling ball method (Fig 7). The result showed that the viscosity was 3.7 centipoise units in contrast to 2.77 centipoise units for hemoglobin AA blood and 4.9 centipoise units for hemoglobin SS blood. For oxygenated blood, comparable values were 3.2, 2.73 and 3.71 centipoise units.

When the Brookfield viscometer was used, no difference in viscosity of oxygenated and deoxygenated hemoglobin AA blood was found, while patients with sickle cell trait displayed an increase of $1 \pm 0.46$ centipoise units in deoxygenated blood. Blood viscosity increased approximately 27 centipoise units from the oxygenated to the deoxygenated state at all shear rates for homozygous SS and SC disease (Fig 8). Although the propositus is homozygous for hemoglobin SS, his blood viscosity increased by only $1.1 \pm 0.1$ centipoise units with deoxygenation. The difference in viscosity between oxygenated and deoxygenated blood from hemoglobin AA subjects was not significant. For sickle cell trait, the difference at all shear rates was $>0.1$ and $<0.5$ significance level. The difference for hemoglobin Memphis/S was $>0.01$ and $<0.001$. The difference in viscosity of deoxygenated blood between hemoglobin Memphis/S and sickle cell anemia was significant at $>0.001$ percent level (Fig 9). However, the difference between hemoglobin Memphis/S and AS disease blood both with oxygenation and deoxygenation was not significant.

Renal function studies were performed during Mannitol diuresis, sodium loading, and water diuresis (Table IV). During Mannitol infusion $\text{Cosm Tc}_{60}, \text{UV}$, and urine flow were decreased in comparison to normal subjects and individuals with hemoglobin SS. With sodium loading, glomerular filtration and renal plasma flow were abnormally low and the filtration fraction was high reflecting a proportionately greater decrease of the latter. Because of diminished glomerular filtration, filtered sodium was decreased but $\text{U}_{24\text{h}} \text{V}$ was not suppressed indicating a decreased tubular re-absorption of sodium. Filtered sodium was decreased secondary to suppressed filtration during water loading but $\text{U}_{24\text{h}} \text{V}$ was greater than in normal hemoglobin SS subjects. $\text{T}_{24\text{h}}$ was considerably depressed. $\text{Tc}_{60}$ was decreased, indicative of restriction of tubular diluting activity.

Hemoglobin gelling point of the propositus required a much higher concentration of hemolysate than that which was necessary with hemoglobin SS. The quantity of hemoglobin S necessary for gel formation is decreased by the presence of hemoglobin A and C. Because our patient demonstrated a high gelling concentration in contradistinction to subjects with sickle cell anemia or sickle cell trait, the gelling point must be altered as a result of the structural change in the hemoglobin molecule resulting from the alpha 23 glutamine substitution.

Changes in blood viscosity revealed by using the
Brookfield viscometer appear more accurate and graphic than rheologic activity defined by the falling ball viscometer (Wells, Denton and Merrill, 1961). Blood is a non-Newtonian fluid (the relationship between shear stress and shear rate in such a fluid is non-linear), so the uniform shear rate during measurement provided by the Brookfield device permits more precise determinations. With the falling ball, shear rate varies continuously from 0 at the capillary tube and within the fluid not in contact with or near the ball to some maximal value at the surface of the ball or at the wall of the tube. In addition, the shear rate under these conditions is dependent on the dimensions of the instruments necessitating correction for all but the lowest degree of non-Newtonian behavior. Viscosity is also temperature dependent. Our studies were performed at 37°C.

With the Brookfield instrument we found the viscosity in hemoglobin Memphis/S disease to be similar to that found in sickle cell trait disease but significantly lower than in normal subjects when the hematocrit was adjusted to 42–49 vols percent. The difference found in viscosity of oxygenated and deoxygenated blood in these two conditions was significant but the differences between sickle cell trait and hemoglobin S/Memphis were not. Thus, the alpha chain variant decreases the viscosity of hemoglobin S to the range found in sickle cell trait.

Sickle cell disease may result in a variety of renal lesions: impairment in urine concentrating ability, medullary necrosis, hematuria, and the nephrotic syndrome. Frequent examinations of the urine from our patient failed to demonstrate erythrocytes, the typical features of medullary necrosis were not delineated by intravenous pyelography, and proteinuria was less than 100 mg/day. The renal concentrating defect in sickle cell disease is characterized by a failure of TcH2O to increase progressively with hypertonic saline loading with retention of the capacity to attain maximal levels in response to Mannitol infusion. Our patient was unable to increase the TcH2O during Mannitol diuresis, probably reflecting chronic renal disease. This was additionally manifested by depressed glomerular filtration and marked diminution in tubular sodium re-absorption in response to sodium loading and water diuresis. Diluting capacity response following oral or parenteral water loading was also impaired, a feature characteristic of chronic renal disease. While it could

Fig 6—Peripheral blood from patient J. P. with hemoglobin Memphis/S disease.

Fig 7—Viscosity of Hb. AA, Memphis/S, and SS—Falling Ball Viscometer.
Table IV. Renal Function Studies.

### TcH$_2$O DURING 10% MANNITOL DIURESIS

<table>
<thead>
<tr>
<th>Hb</th>
<th>V* (ml/min)</th>
<th>UV* (mOs/m/min)</th>
<th>Cosm* (ml/min)</th>
<th>TcH$_2$O* (ml/min)</th>
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<tbody>
<tr>
<td>AA</td>
<td>16.7</td>
<td>6,434</td>
<td>21.63</td>
<td>4.9</td>
</tr>
<tr>
<td>SD</td>
<td>± 4.13</td>
<td>± 1,130</td>
<td>± 3.56</td>
<td>± 0.39</td>
</tr>
<tr>
<td>SS</td>
<td>16.0</td>
<td>6,170</td>
<td>20.2</td>
<td>4.3</td>
</tr>
<tr>
<td>SD</td>
<td>± 3.04</td>
<td>± 1,030</td>
<td>± 3.15</td>
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<tr>
<td>M/S</td>
<td>12.2</td>
<td>4,300</td>
<td>13.7</td>
<td>1.46</td>
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<tr>
<td>SD</td>
<td>± 0.88</td>
<td>± 326</td>
<td>± 0.84</td>
<td>± 0.28</td>
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### EFFECT OF SODIUM LOADING ON TUBULAR SODIUM TRANSPORT

<table>
<thead>
<tr>
<th>Hb</th>
<th>V (ml/min)</th>
<th>GFR* (ml/min)</th>
<th>FNa* (meq/min)</th>
<th>UNa* (meq/min)</th>
<th>TNa* (meq/min)</th>
<th>RPF* (ml/min)</th>
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<tbody>
<tr>
<td>AA</td>
<td>15.3</td>
<td>134.4</td>
<td>17.0</td>
<td>1.31</td>
<td>15.7</td>
<td>—</td>
</tr>
<tr>
<td>SD</td>
<td>± 4.24</td>
<td>± 6.31</td>
<td>± 2.60</td>
<td>± 0.40</td>
<td>± 1.76</td>
<td>—</td>
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<tr>
<td>SS</td>
<td>16.5</td>
<td>170</td>
<td>22.09</td>
<td>1.22</td>
<td>20.87</td>
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<tr>
<td>SD</td>
<td>± 3.56</td>
<td>± 5.0</td>
<td>± 0.93</td>
<td>± 0.27</td>
<td>± 1.70</td>
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<tr>
<td>M/S</td>
<td>12.2</td>
<td>59.9</td>
<td>7.9</td>
<td>1.33</td>
<td>6.5</td>
<td>196.6</td>
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<tr>
<td>SD</td>
<td>± 0.98</td>
<td>± 4.2</td>
<td>± 0.89</td>
<td>± 0.07</td>
<td>± 1.1</td>
<td>± 15.7</td>
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### EFFECT OF WATER DIURESIS ON TUBULAR SODIUM TRANSPORT

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<th>Hb</th>
<th>V (ml/min)</th>
<th>GFR* (ml/min)</th>
<th>FNa* (meq/min)</th>
<th>UNa* (meq/min)</th>
<th>TNa* (meq/min)</th>
<th>TNa*/FNa* (%)</th>
<th>CH$_2$O* (ml/min)</th>
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<tbody>
<tr>
<td>AA</td>
<td>14.8</td>
<td>123.8</td>
<td>17.09</td>
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<td>16.71</td>
<td>97.8</td>
<td>13.4</td>
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<td>± 2.1</td>
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<td>± 0.41</td>
<td>± 0.5</td>
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<tr>
<td>SS</td>
<td>19.6</td>
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<td>0.41</td>
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<td>18.9</td>
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<tr>
<td>SD</td>
<td>± 3.9</td>
<td>± 17.2</td>
<td>± 1.7</td>
<td>± 0.10</td>
<td>± 2.1</td>
<td>± 0.11</td>
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<tr>
<td>M/S</td>
<td>13.03</td>
<td>59.8</td>
<td>7.7</td>
<td>0.73</td>
<td>6.75</td>
<td>88.5</td>
<td>5.6</td>
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<tr>
<td>SD</td>
<td>± 2.1</td>
<td>± 3.9</td>
<td>± 0.40</td>
<td>± 0.13</td>
<td>± 0.25</td>
<td>± 1.8</td>
<td>± 1.2</td>
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**TABLE IV:** Renal function studies of J. F. P. with Hb M/S disease compared to previously reported studies of patients with Hb. AA and SS Hatch et. al. J.C. I.Vol. 46, No. 3, pp. 336-345, 1967.

*Abbreviations - V= urine flow; UV= solute excretion; Cosm= osmolar clearance; TcH$_2$O= free water reabsorption; GFR= glomerular filtration rate; FNa= filtered sodium load; UNa= urinary sodium excretion; TNa= tubular sodium reabsorption; RPF= renal plasma flow; TNa*/FNa*= fraction of the filtered load of sodium reabsorbed; CH$_2$O= free water clearance. Values corrected to 1.73 M$^2$ BSA.
be argued that these changes could result from Memphis hemoglobinopathy or that hemoglobin SS patients of comparable age might present similar findings, the weight of clinical evidence seems to favor chronic renal disease. Renal biopsy failed to reveal the vascular congestion and the accumulation of sickled cells usually found in glomerular capillaries. Instead, glomerular hypercellularity with endothelial proliferation and arteriolar nephrosclerosis with arteriolar thickening were found. Thus, it is not possible to assume that the alpha chain variation modifies the renal concentrating defect of sickle cell disease, although these studies are suggestive and typical clinical features of sickle cell disease have not been observed. Younger and older subjects with hemoglobin Memphis/S (with and without hemoglobin S) without chronic renal disease will have to be examined to clarify this point.

The pathological changes of sickle cell disease are thought to be a result of a formation of sickled cells under conditions of deoxygenation with increased viscosity, vascular stasis, and occlusion of the microcirculation. Charache and Conley (1964) demonstrated that the viscosity of deoxygenated blood varied directly with the percentage of sickle cells and exponentially with the hematocrit, concluding that the clinical manifestations of sickle cell anemia could be attributed to intravascular sickling. To account for the occasional patient who has a more benign course even with marked sickling and increased viscosity, protective extracorporeal factors were postulated. Recent studies by Murayama (1964; 1966) and Perutz (1965; Perutz and Lehmann, 1968) of effects of amino acid substitution on the functional activity of hemoglobin may afford explanation of these clinical variations. Murayama proposed that the substitution of a glutamic acid residue at the number 6 position by a valyl residue results in an intramolecular hydrophobic bond with the N-terminal valine; a cyclic structure on the beta chain is formed which can lock into the alpha chain. Muirhead and Perutz (1963) demonstrated that the beta chains of reduced hemoglobin are 7 Angstrom units further apart than those of the oxygenated form. Thus, the reduced beta chains of hemoglobin/S would fit into the alpha chains which are not affected by decreased oxygen tension, forming a rigid tactoid. Murayama postulated that the binding sites produced by cyclization at the N-terminal part of the beta chain must fit precisely with the binding sites on the alpha chain. When the binding sites on the beta chain move closer together on oxygenation, the key of the beta chain no longer fits the lock of the alpha chain. Murayama demonstrated by polarized light, magnetic orientation, and electron microscopy that microtubules of hemoglobin/S were linearly arranged with resulting stacking up of molecules. The previous patients reported with hemoglobin Memphis/S disease have been found to have sickled red cells, but our patient has been shown to have normal viscosity with deoxygenation. This molecular defect on the alpha chain must modify the lock so that less rigid tactoids form.

Perutz (Perutz, Kendrew and Watson, 1965) has suggested that the presence of histidine, glutamic acid, or aspartic acid is necessary for the formation of corners or non-helical regions on globin molecules, and has found that one of these amino-acids is a constituent of every corner and occurs in an adjacent area of every non-helical region. Perutz's model of hemoglobin...
bin describes the 23rd residue of the alpha chain interacting with Number 21 histidine, stabilizing the corner between the alpha and beta helical regions. The loss of stability at this position with glutamine substitution for glutamic acid must result in the change in tertiary conformation. Thus, the instability of the alpha Memphis and beta chain interaction appears to decrease rigidity of the “lock and key” mechanism—resulting in normal viscosity, increased gelling concentration and decreased sickling in the deoxygenated state. Finding whether or not the alpha chain defect can completely modify sickle cell disease must await the discovery of homozygous hemoglobin Memphis/S disease. The heterozygous alpha chain defect by itself is not symptomatically expressed.

Additional studies have been performed measuring the effect of hyperosmolar solutions on the viscosity of various hemoglobinopathies. Perillie and Epstein (1963) reported the effect of hypertonic solutions on the various hemoglobinopathies. They reported that sickling occurred within a few seconds in all patients with sickle cell anemia and sickle cell variants when their blood was mixed with hypertonic saline. An increase in the sickling in the blood of patients with sickle cell anemia (SS disease) was first observed in osmolality of 600 mosm/kg. As the concentration of salt solution increased, the percentage of sickle cells reached a maximum at 600 to 1,000 mosm/kg. It did not usually increase with further increase in extracellular toxicity. The same phenomena was observed in patients with sickle cell trait, but the sickle cells were less numerous than in subjects with sickle cell anemia. They proposed that in patients with sickle cell anemia, sickling may take place in the capillaries of the renal medulla but that this is partly related to another property of this tissue—its hypertonicity. Thus, sickling would increase the viscosity of blood entering the medulla and, therefore, the resistance to blood flow through the medullary capillaries would be increased. They postulated that by decreasing intermolecular distances within the red cell, intracellular dehydration induced by hypertonicity might be expected to enhance the additional tendency to sickling produced by hypoxia or acidity. Ham (Ham et al, 1968) reported on a series of studies investigating the effect of various solutions on the viscosity of red cells. His group found that red cells fixed in glutaraldehyde or formaldehyde were more viscous than normal cells and the flow properties were more Newtonian in behavior, thus being less dependent on shear rate. These authors showed that hyperosmolar solutions increased the rigidity of the red cells with an increase in viscosity. They reported that the increase in viscosity, decreased filtration, and resistance to packing and morphological changes were identical for oxygenated blood for dogs, normal humans, sickle cell trait blood, and sickle cell anemia erythrocytes. They found no increase in sickling as the osmolar solutions were increased. When red cells from sickle cell trait and homozygous SS disease were suspended in 600 mosm sodium chloride and then reduced, the viscosity was maximal or became unmeasurable because the cells behaved like a gel. They postulated that the hypertonicity of sodium resulted in increased viscosity of all cells but did not cause sickling of oxygenated blood. This increase in viscosity could result in decreased flow in the *vasa recti* with rising concentrations of the sodium chloride. The decreased rate of flow would lead to lowered p02 and pH and these in turn could result in sickling in both homozygous and heterozygous hemoglobin S patients. Hatch (Hatch, Culbertson and Diggs, 1967) stated that although vascular damage undoubtedly plays an increasingly important role in sickle cell nephropathy with advancing age, it does not explain adequately the renal concentrating defect that is present almost from birth. He proposed that indirect evidence suggests a possible increase rather than a decrease in medullary blood flow because of an inability of patients with sickle cell disease during hypertonic saline diuresis to exceed the maximum clearance of water as obtained during Mannitol diuresis. Thus an increase in medullary blood flow would produce a loss in renal concentrating ability through a decrease in medullary solute concentration.

We measured the viscosity of the various hemoglobinopathies at different osmolalities with a Brookfield viscometer (Table V). The viscosity was measured at 6, 12, 30, and 60 rpm on blood suspended in plasma, in 300 mosm sodium chloride solution, 600 mosm solution, 800 mosm solution, and 1,000 mosm solution concentrations (Table VI). Oxygenated cells suspended in plasma with a hematocrit of 33 vols percent showed no significant difference between the viscosity of hemoglobin AA and AS. However, there was a significant increase in the viscosity of hemoglobin AA, AS, and HS blood at the 0.01 level when compared to Memphis/S and SS hemoglobin. Oxygenated blood at 300 mosm concentration showed a significant increase of hereditary spherocytosis erythrocytes and hemoglobin SS blood as compared to Memphis/S and hemoglobin AS and AA. At 600 mosm, hemoglobin SS and hereditary spherocytosis blood showed a significant increase at the 0.01 level in viscosity as compared to hemoglobin AA, AS, and Memphis/S. With 800 mosm solutions, hemoglobin SS and hereditary spherocytosis (HS) showed a significant increase in viscosity over hemoglobin Memphis/S and hemoglobin AA and AS. At 800 mosm solution, Memphis/S showed increase in viscosity over hemoglobin AA and AS at the 0.05 level. At a 1000 mosm solution, hemoglobin AA and AS showed significantly less increase in viscosity than Memphis/S, SS, or hereditary spherocytosis. There was no significant difference between Memphis/S, SS, and hereditary spherocytic red cells.
### Table V.

<table>
<thead>
<tr>
<th>RPM</th>
<th>OXYGENATED</th>
<th>DEOXYGENATED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AS</td>
</tr>
<tr>
<td>6</td>
<td>4.0</td>
<td>4.2</td>
</tr>
<tr>
<td>12</td>
<td>3.8</td>
<td>3.9</td>
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<tr>
<td>60</td>
<td>2.6</td>
<td>3.0</td>
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### Table VI.

<table>
<thead>
<tr>
<th>RPM</th>
<th>OXYGENATED</th>
<th>DEOXYGENATED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
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<td>3.8</td>
<td>4.0</td>
</tr>
<tr>
<td>60</td>
<td>3.8</td>
<td>3.8</td>
</tr>
</tbody>
</table>
In the oxygenated blood at varying osmolalities from 300 to 1000 mosm solutions, hemoglobin SS and HS blood behaved in a similar manner. Hemoglobin AA and AS did not show increasing viscosity with increasing osmolar concentrations, as previously reported by Hamm and his group. Hemoglobin Memphis/S did show an increasing viscosity with increasing osmolalities. Hemoglobin SS and red cells from patients with hereditary spherocytosis showed a similar pattern which was of a much greater magnitude than that of Memphis/S. The same type of studies were performed with deoxygenated blood. The hemoglobins listed in order of ascending viscosity suspended in plasma were hereditary spherocytic red cells, hemoglobin AA, hemoglobin AS, Memphis/S, and SS disease. At 300 mosm solution under deoxygenated conditions hemoglobin AA and AS showed significantly less increased viscosity as compared to Memphis/S or as compared to HS or SS blood. At 300 mosm solutions, hemoglobin HS and SS behaved similarly and showed a significantly higher viscosity than the other hemoglobinopathies. Hemoglobin Memphis/S showed increasing viscosity with increasing osmolar solutions and at 600, 800, and 1000 mosm concentrations was significantly higher than any of the other hemoglobinopathies. Sherman tests were performed on the various hemoglobin solutions at varying osmolar concentrations and showed that there was no increase in the number of sickled cells in hemoglobin AS or SS disease at increasing osmolar concentrations, similar to the results obtained by Ham and his group at Case Western Reserve. Hemoglobin Memphis/S showed an increased number of sickled cells in hyperosmolar solutions, but this was a constant number throughout all of the osmolar solutions. The consistent finding was that the red cells were spherical and crenated with numerous spicules.

These data suggest that there may be an association between hereditary spherocytosis and sickle cell disease. A group of investigators from Japan (Matsuda et al, 1969) reported a case of nephrotic syndrome in a ten-year-old Japanese boy with hereditary spherocytosis. They pointed out that spherocytosis and sickle cell anemia have some features in common such as abnormal shape, short erythrocyte survival, persistent icterus, and occurrence of crises. In addition, there may be some association between hereditary spherocytosis and sickle cell anemia in reference to a marked increase in viscosity with hyperosmolar solutions in oxygenated blood. It has been pointed out by Jensen (1969) that the repeated sickling and unsickling of a cell may cause repetitive fragmentation with proportionally greater membrane than volume loss which eventually will result in the production of a spherocyte. This situation would be most likely to happen during a sickle cell crisis in which the cells would be subjected to maximal damage. This may also explain the varying
results reported in patients with hemoglobin SS when subjected to hyperosmolar solutions.

Some investigators have suggested the use of low molecular weight dextran in the therapy of patients with sickle cell anemia. We have measured in an in vitro situation the effect of various dextran 40 and dextran 70 concentrations on the viscosity of hemoglobin SS disease (Fig 10, 11, 12). Our findings suggest that dextran actually increases the viscosity of hemoglobin SS disease. Oski (Oski et al, 1965) reported that low molecular weight dextran was of no benefit in the therapy of sickle cell crisis.

Eisenberg (1969) reported that low molecular weight dextran had no specific effect on blood viscosity. However, it did prevent erythrocyte rouleau formation and aggregation. It is also known that the infusion of low molecular weight dextran is associated with acute renal failure in at least fourteen patients. Mailloux (Mailloux et al, 1967) produced acute anuria in experimental animals with low molecular weight dextran. They postulated that a reduction in filtration pressure combined with a marked increase in urinary viscosity generated by the dextran may lead to tubular stasis and subsequent tubular blockade.

Laszlo (Laszlo, Obenour and Saltzman, 1969) studied the effect of hyperbaric oxygenation on erythrocyte sickling during sickle cell crisis. His group at Durham studied five patients during crises. They found that the percentage of circulating sickle cells did decrease during hyperbaric oxygenation. However, there was no improvement of the sickle cell crisis and there was no tendency toward improved renal concentrating ability during the hyperbaric oxygenation. A possible explanation for the ineffectiveness of hyperbaric oxygenation on sickle cell crisis in this study may be that with hyperoxygenation in hyperosmolar solutions, the viscosity of sickle cell hemoglobin is actually increased.

Further studies are needed to elucidate the pathophysiology of the various hemoglobinopathies. Physicians involved in the care of patients with sickle cell anemia as well as the other hemoglobinopathies should try therapeutic programs which have been proven. The use of clinical dextran and hyperbaric oxygenation should be carefully studied under experimental conditions before their widespread use is advocated.

References


Charache S, Conley CL: Rate of sickling of red cells during deoxygenation of blood from persons with various sickling disorders. Blood 24: 25, 1964


Fig 12—Viscosity of Hb. SS-oxygenated and deoxygenated RBC using Dextran 70-75.
HEMOGLOBINOPATHIES


KRAUS AP, MIYAJI T, IUCHI I, ET AL: Hemoglobin memphis/S a new variant of sickle cell anemia. Trans Ass Amer Physicians 80: 297, 1967


STAMATOYANNOPOULOS G, YOSHIDA A, ADAMSON J: Hemoglobin rainier \((\beta^{146\text{Tyrosine}^{146}}\text{Histidine})\): Alkali-resistant hemoglobin with increased oxygen affinity. Science 159: 741, 1968

New Concepts in the Management of Neonatal Jaundice: Use of Enzyme Induction and Phototherapy*

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In the last two years two new methods for treatment of neonatal hyperbilirubinemia have been successfully introduced. The first consists of administration of phenobarbital to newborn infants and to pregnant women for at least two weeks prior to delivery. The second consists of exposing the neonate to light. Before these treatments were available, only with exchange transfusions (which are time consuming, expensive, and carry a risk of about one to 55 percent even in experienced hands) or, very rarely, with dialysis could one predictably lower the concentration of serum bilirubin.

Why do newborn infants have hyperbilirubinemia, and why are we so concerned about controlling the bilirubin level? The life span of a newborn infant's red blood cell is shorter than that of an adult. As senescent cells and their fragments become sequestered in the reticulo-endothelial system the hemoglobin molecule is split into two fragments: globin which is brought into the protein metabolic pool, and heme which is further catabolized. Microsomal heme oxidase, a rate limiting enzyme, then acts on heme to form biliverdin as the principal product. Biliverdin is further reduced and degraded to indirect-reacting unconjugated, lipid soluble bilirubin. Most of the bilirubin is reversibly bound to albumin. In this form it can be distributed in blood to a variety of tissues. A small fraction of circulating bilirubin is unbound in a dissociation equilibrium with albumin bound bilirubin. Normally, it is the unbound bilirubin fraction that continuously diffuses across the surface of the liver cells. The bilirubin is taken by the cell membrane lipids and transferred to proteins within the liver cell. Similar processes occur in fat cells, epidermal cells and neurons. Bilirubin can be pulled out of cells by increasing the albumin concentration of plasma. Within the liver cell bilirubin is separated from protein and conjugated. Normally, the conjugation process helps to maintain the diffusion of biliru-

* Presented at the 23rd Annual Stoneburner Lecture Series, February 20, 1970, at the Medical College of Virginia, Richmond.
NEONATAL JAUNDICE

... has long been realized that such factors as asphyxia, hypoglycemia and hypoproteinemia may increase susceptibility to kernicterus.

There is considerable evidence that bilirubin causes decreased cellular oxygen uptake; it also inhibits oxidative phosphorylation while producing characteristic morphologic changes in large neurons in tissue culture, such as enlargement of the mitochondria. The search continues for a reliable guide to define what constitutes a dangerous concentration of serum bilirubin. As a working guide to the need for exchange transfusion, these figures continue to stand up well: 20 mg/100 ml for the full-term infant with erythroblastosis, 18 mg percent for the premature infant with erythroblastosis, and 20–25 mg/100 ml for the non-erythroblastotic infant. It continues to puzzle and frustrate clinicians that kernicterus can occur sporadically in premature infants with low serum bilirubin concentrations, and that some full-term infants with serum concentrations above 30 mg percent are amazingly resistant to its development.

Control of Hyperbilirubinemia by Enzyme Induction

Yaffe and associates (1966), and Crigler and Gold (1966) independently reported the first clinical trials of phenobarbital for treatment of congenital non-hemolytic jaundice. In Yaffe’s patient, treatment with 15 mg of phenobarbital three times daily lowered the serum bilirubin concentration and jaundice disappeared. When treatment was stopped, the serum bilirubin concentration rose to its original high levels; reinstitution of therapy again decreased serum bilirubin levels and jaundice disappeared. Parallel studies by Yaffe on salicylamide, metabolized like bilirubin, showed that the defective capacity to conjugate glucuronide before phenobarbital became normal after treatment. In Crigler’s case, the size and rate of turnover of the bilirubin pool was measured before and during phenobarbital treatment. Results indicated that phenobarbital enhanced the excretion of the bilirubin rather than causing its redistribution into extra-vascular pools.

A large body of data showing the stimulatory effects of drugs such as phenobarbital on the metabolism of normal body constituents led to its trial in the above two patients. Studies in the past decade have shown that the activity of enzymes in liver microsomes is markedly increased when animals are treated with various hormones, drugs, insecticides, and carcinogens (Conney, 1967). This increased activity appears to represent an increased concentration of enzyme protein and is referred to as enzyme induction. The induction of liver microsomal enzymes is important pharmacologically, for it leads to an accelerated transformation of drugs in vivo and so alters the duration and intensity of drug action in animals and man.
TABLE 1

<table>
<thead>
<tr>
<th>Stimulation of Microsomal Enzyme Activity</th>
<th>Pharmacological Action</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypnotics and sedatives</strong></td>
<td>Barbiturates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutethimide (Doriden)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorobutanol (Chloreton)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urethane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carbromal (Adalin)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyridione (Persedon)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methyprylon (Nodular)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloral hydrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paraldehyde</td>
<td></td>
</tr>
<tr>
<td><strong>Anesthetic gases</strong></td>
<td>Nitrous oxide</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methoxyflurane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Halothane</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ether</td>
<td></td>
</tr>
<tr>
<td><strong>Central nervous system stimulators</strong></td>
<td>Nikethamide (Coramine)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bemegride</td>
<td></td>
</tr>
<tr>
<td><strong>Anticonvulsants</strong></td>
<td>Methylphenylethylhydrantoin (Mesantoin)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diphenylhydantoin (Dilantin)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paramethadione (Paradione)</td>
<td></td>
</tr>
<tr>
<td><strong>Tranquilizers</strong></td>
<td>Phenaglycodol (Ultran)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Meprobamate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlor Diazepoxide (Librium)</td>
<td></td>
</tr>
<tr>
<td><strong>Antipsychotics</strong></td>
<td>Chlorpromazine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triflutpromazine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Promazine</td>
<td></td>
</tr>
<tr>
<td><strong>Hypoglycemic agents and related sulfonamides</strong></td>
<td>Tolbutamide (Orinase)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carbutamide</td>
<td></td>
</tr>
<tr>
<td><strong>Anti-inflammatory agents</strong></td>
<td>Phenylbutazone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Orphenadrine</td>
<td></td>
</tr>
<tr>
<td><strong>Muscle relaxants</strong></td>
<td>Carisoprodol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zoxazolamine</td>
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</tr>
</tbody>
</table>

More than 200 drugs, insecticides, carcinogens and other chemicals are known to stimulate the activity of enzymes in the liver. Examples are shown in Table 1. Enzymes can be stimulated by different types of drugs: barbiturates and other hypnotics and analgesics, tranquilizers, antihistamines, oral antidiuretic agents, and uricosuric agents. The characteristic pharmacological actions of these compounds on the organism are extremely diverse, and there is no apparent relationship between their actions or structure and their ability to induce enzymes.

The quantity of inducer necessary to have an appreciable effect on the enzymes varies considerably. It has been shown that phenobarbital given to lactating rabbits increases the levels of enzymes in the nurslings at doses that do not effect the behavior of the offspring. Phenobarbital acts by stimulating varied pathways of metabolism through liver microsomes, causing oxidation-reduction reactions, glucuronide formation and de-esterification. When the enzyme that acts on a drug or substrate is induced, the drug or substrate is metabolized more rapidly. Enzyme induction alters not only duration but also intensity of drug or substrate action.

Several effects of enzyme induction in man have been noted and are listed in Table 2. Phenobarbital stimulates the enzymatic metabolism of coumarin, dilantin, griseofulvin, doridren, digitoxin and aminopyrine. Further, it stimulates bilirubin metabolism and enhances the urinary excretion of 6-beta-hydroxycortisol.

Recent studies have shown that phenobarbital facilitates hepatic metabolism and biliary excretion of bilirubin in animals, as seen in Table 3. The administration of phenobarbital stimulates: 1) bilirubin glucuronyl transferase activity in liver microsomes, 2) the disappearance of exogenously administered bilirubin from plasma, 3) hepatic uptake of bilirubin, 4) bile flow and biliary excretion of bilirubin, and 5) the proliferation of smooth membranes of the endoplasmic reticulum. These observations suggested to Yaffe et al (1966), and Crigler and Gold (1966, 1967) that phenobarbital might have therapeutic value in human diseases of hyperbilirubinemia.

Encouraged by these studies in congenital non-hemolytic jaundice, we investigated the effect of phenobarbital on neonatal jaundice (Maurer et al, 1968).
Bilirubin formed in utero by the fetus can cross the placenta and be excreted by the maternal liver. After birth, however, the infant's hepatic bilirubin clearance is not sufficient to prevent its accumulation during the first week of life. We wanted to know whether careful treatment of pregnant women with phenobarbital for at least two weeks before delivery could enhance the metabolism of bilirubin in the newborn infant and reduce neonatal serum bilirubin levels. We chose to treat the pregnant women rather than the newborn infants with phenobarbital, to determine whether enzyme induction could be accomplished in the fetus in utero and to avoid a delay in induction of enzyme activity which might be anticipated if treatment was started at birth.

Thus, 12 pregnant women were treated with sodium phenobarbital, 30–120 mg per day for two weeks or longer prior to delivery (Table 4). Subsequently, concentrations of total serum bilirubin in their offspring and in 16 control babies were compared during the first four days of life. Premature babies and those sensitized by maternal-fetal Rh or ABO incompatibility were excluded from this study. All but one of the women received injection of 1.5 percent mepivacaine hydrochloride for continuous epidural analgesia during labor and delivery. Medications normally administered to some of the pregnant subjects were not controlled, but evaluation of the medications given to the control and the phenobarbital groups showed no significant difference.

Table 4 lists the dose and duration of phenobarbital treatment received by each of the pregnant women, and shows the maximum level of serum bilirubin observed in their infants in the first four days of life. The highest level of neonatal serum bilirubin in the phenobarbital group was 4.7 mg percent, whereas 12 of 16 control babies had peak serum bilirubin levels above this value. Since all but two of the women were treated with the same daily dose of phenobarbital, it was not possible to determine what dosage regimen most effectively lowers the concentration of serum bilirubin.

### Table 3

**Effect of Phenobarbital on Bilirubin Metabolism in Animals**

1. Stimulates bilirubin glucuronyl transferase activity in liver microsomes
2. Stimulates clearance of exogenously administered bilirubin from plasma in animals
3. Stimulates hepatic uptake of bilirubin
4. Stimulates bile flow and biliary excretion of bilirubin
5. Stimulates proliferation of smooth membranes of the endoplasmic reticulum

### Table 4

<table>
<thead>
<tr>
<th>Group and dose of phenobarbital (mg/day)</th>
<th>Duration of treatment (days)</th>
<th>Maximum neonatal serum bilirubin level (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls:</td>
<td>0</td>
<td>1.3–10.4</td>
</tr>
<tr>
<td>Phenobarbital:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>14</td>
<td>4.2</td>
</tr>
<tr>
<td>60</td>
<td>14</td>
<td>2.8</td>
</tr>
<tr>
<td>60</td>
<td>14</td>
<td>2.7</td>
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</tr>
<tr>
<td>120</td>
<td>105</td>
<td>1.2</td>
</tr>
<tr>
<td>60</td>
<td>11 yr.</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.7 ± 0.4)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.9 ± 0.5)†</td>
</tr>
</tbody>
</table>

* The value of serum bilirubin for each subject represents the maximum obtained for that subject during the first 4 days of life.
† Mean ± S.E.

### Table 5

**Effect of phenobarbital received during pregnancy on concentration of total serum bilirubin in the neonatal period**

<table>
<thead>
<tr>
<th>Serum bilirubin (mean ± S.E.) (mg/100 ml)</th>
<th>Day</th>
<th>Control</th>
<th>Phenobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3.8 ± 0.4</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.0 ± 0.6</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.7 ± 0.7</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.2 ± 0.8</td>
<td>1.8 ± 0.4</td>
</tr>
</tbody>
</table>

* Each value represents the mean and S.E. obtained on each day for the control and phenobarbital groups.
In Table 5, the mean daily concentrations of serum bilirubin are compared in control infants and babies of phenobarbital treated women. Daily levels of serum bilirubin were significantly low in the phenobarbital group. The maximum mean value for serum bilirubin in these infants was 2.5 mg/100 ml; this concentration occurred on the second day of life. In control babies, however, the maximum mean level of serum bilirubin was 5.7 mg/100 ml; this value was found at three days of age. The level of serum bilirubin in each group was not related to the type of feeding received.

All babies in the study were vigorous at birth and thereafter.

These data suggest that the administration of phenobarbital for two weeks or longer before delivery altered the usual course of physiologic hyperbilirubinemia. Serum bilirubin levels were reduced and maximum serum bilirubin values occurred earlier in babies born to women treated with phenobarbital. The low concentration of serum bilirubin in these babies suggests that phenobarbital, acquired by the fetus transplacentally, enhanced the hepatic metabolism and biliary excretion of bilirubin in the fetus and the newborn infant. Our findings were confirmed by Trolle (1968).

One question that immediately comes to mind is whether phenobarbital may be harmful to the pregnant woman or to her newborn infant. A large number of women with epilepsy or pre-eclampsia have been treated with phenobarbital for at least two weeks during pregnancy without recognizable difficulty. Regarding the danger phenobarbital may cause a newborn infant, in our series no difference was detected in the behavior of infants of treated and untreated mothers. Similarly, no differences have been detected in other series. Trolle (1968) measured the serum phenobarbital level in some of his babies, and in no case did the concentration exceed 21 mg/ml, which is well below the sedative level.

Another consideration is the possibility that phenobarbital could displace bilirubin from albumin. This could lead to a diffusion of dissociated bilirubin from the blood and interstitial fluid to the intracellular fluid, thus increasing the risk of development of kernicterus. In this regard we did not see increased skin jaundice in those babies born to phenobarbital treated women, nor did Trolle in his series. In addition, we made serial measurements of the reserve binding capacity of serum for bilirubin, using the HABA dye method, and found no difference in the serum binding capacity between the control and phenobarbital treated groups. Our findings, in conjunction with those reported by Yaffe and Crigler, indicate that the decrease in serum bilirubin concentration after phenobarbital treatment cannot be considered dangerous. All the observations suggest, therefore, that phenobarbital may be of therapeutic value in controlling neonatal hyperbilirubinemia.

Control of Hyperbilirubinemia by Phototherapy

Lucey, Ferreiro and Hewitt (1968) reported a controlled study which showed that early prolonged exposure of premature infants to artificial light might prevent or at least modify the degree of hyperbilirubinemia. Cremer, Perryman and Richards (1958) of Australia first demonstrated that serum bilirubin concentrations of some newborn infants could be reduced by exposure to sunlight or artificial blue light. Several groups of English, South American, French and Italian workers subsequently reported very favorable experiences with this method of treatment. This therapy had not found acceptance in the United States—probably due to doubts as to its effectiveness, concern that the photodecomposition products might be toxic, and unawareness of many reports in the foreign literature.

Lucey (1968) tested the effectiveness of artificial blue light in preventing hyperbilirubinemia among 111 premature infants. Treated infants from 12 to 144 hours of age were placed in light; serum bilirubin concentrations were then carried out. The control and treated infant groups were comparable with respect to birth weight, gestational age, fluid intake and weight loss. The results showed a statistically significant difference between the two groups on the fourth and sixth days of life. No differences were noted in the sleeping and feeding habits of the infants in the light treatment group.

How does light affect bilirubin metabolism? The level of indirect reacting bilirubin present in a solution of human albumin and bilirubin decreases when it is exposed to light of certain wavelengths. As the level of bilirubin decreases, first biliverdin and then a series of not yet fully characterized related substances appear in the solution. The best available evidence suggests that the derivatives of photo-oxidation are in part pyrrolic pieces of bilirubin resembling dipyrrole. There is to date no published animal data supplying any convincing evidence of the toxicity of the products of photo-decomposition. The studies of Ostrow (1968) using radio-labelled C14 bilirubin in Gunn rats clearly demonstrate that these products are rapidly excreted in the bile and urine, and that they are indistinguishable from the normal products found in Gunn rat bile and urine. It appears, therefore, that photodecomposition is a normal alternate route of excretion of bilirubin and is increased or activated in newborn infants by the use of light.

In jaundiced infants exposed to light, the presence of icterus in shielded areas of skin suggests that the photo-oxidation occurs in the skin. Too, biliverdin which is produced in the test tube, when a bilirubin-albumin solution is exposed to light is not observed in the skin or plasma of light-treated infants.
Potentially, light may have an effect on bilirubin metabolism at several different points in its in vivo metabolism (Behrman and Hsia, 1969). Light might affect the microsomal heme oxidase system, which might increase the amount of bilirubin carried in the serum and presented to the liver. It might alter the albumin binding sites for bilirubin, making it easier for bilirubin to escape in the tissue. It might affect the protein receptors for bilirubin within the liver cells or other cells. Light might stimulate a variety of mitochondrial enzymes, including UDPGT. It might affect the yet poorly characterized bilirubin and other excretory mechanisms in the liver.

Other biological effects of light have also been noted. Ultraviolet light causes capillary dilation as in sunburn, activates tyrosinase which results in skin darkening, and causes a photo-chemical transformation of ergosterol to active forms of vitamin D. Further, there are indirect effects that, though less well known, are of potential concern. The duration of light exposure in young animals can turn on or markedly delay the onset of puberty. Light has a profound effect on biologic rhythms such as body temperature, food consumption, physical activity and adrenal cortical secretion. In animals, light has a profound influence on gonadal weight and ovulation. Newborn piglets exposed to light without the use of eye shields have developed retinal detachment.

Although no obvious acute clinical toxicity has been recognized in light-treated newborn infants, no organized retrospective or prospective follow-up study of change in neurologic and behavioral sequelae is available. There is still considerable doubt and concern about all the potential biological effects of light on bilirubin metabolism in humans and in animals. Additional knowledge of these matters is critical in evaluating the potential dangers of phototherapy.

Most investigators have started phototherapy soon after birth; others have started at 12 to 72 hours after birth or when jaundice was first noted. Therapy has been continued for varying periods of time, continuously or intermittently through the sixth day of life. At the Medical College of Virginia, the light is placed over the isolette containing the infant and treatment is continued for 96 hours. The light chamber consists of ten GE#20 daylight bulbs attached to an aluminum and steel frame. The lamps emit most strongly in the blue-yellow wavelengths from 420 to 440 mU. Blue lamps and cool white lamps, with slightly different wavelength emissions, are also used. Red lamps emitting wavelengths above 600 mU are not effective; lamps that emit the lower ultraviolet wavelengths are less effective and more dangerous to the eyes than visible light. The infant’s eyes are covered with a simple bandage; no clothing is placed on the infant so as much skin as possible is exposed to light. The intensity, duration, and pattern of light exposure has varied considerably among the groups of infants who have been subjected to phototherapy by different investigators. The intensity of light has ranged from 100 to 500 footcandles. At the Medical College of Virginia 200 footcandles are used.

During a symposium on bilirubin metabolism in the newborn infant held in Chicago in June, 1969, an attempt was made to delineate tentative guidelines in light therapy (Behrman and Hsia, 1969). The following guidelines were formulated:

1. The etiologic diagnosis for jaundice should be established, as far as is practical, before starting any therapy including phototherapy.
2. Phototherapy should not be used prophylactically in term infants.
3. Phototherapy should be used for infants in whom the risks of hyperbilirubinemia are thought to out-weigh the risks of phototherapy.
4. Infants who, when first seen, have sufficient indications for an exchange transfusion should not have their transfusion delayed for trial of phototherapy.
5. Phototherapy should not be started until an abnormal rise in serum bilirubin has been demonstrated. In general, the serum indirect bilirubin concentration should be at least 10 mg percent at the time phototherapy is initiated.
6. Wavelengths between 300 and 600 mU from 200 to 400 footcandles should be effective in reducing serum bilirubin concentrations in many premature infants.
7. Eyes of the infant should be shielded with patches to protect the developing macula. In order to avoid corneal ulceration, it is also critical to make sure that the eye is closed and remains closed when patched.
8. Body temperature should be monitored to minimize the risk of over heating.

A number of questions with respect to phototherapy still remain unanswered. There is no evidence that jaundiced premature infants treated with light have fewer developmental abnormalities than untreated infants, that there is a decreased need for exchange transfusion in premature babies with hyperbilirubinemia due to isoimmunization or sepsis, or that light therapy is effective at high bilirubin levels. No organized follow-up data are as yet available regarding sequelae of light therapy. No information is available to evaluate optimal duration of light exposure or determine whether exposure should be continuous or intermittent. No good comparison is available to judge the relative effectiveness and safety of blue versus white or daylight lamps in photo-oxidizing bilirubin in humans. Data available comparing the effectiveness of light in the Negro and in the Caucasian is limited and inconclusive. No data is available comparing the effec-
tiveness of light versus phenobarbital versus a combination of light and phenobarbital for treatment of hyperbilirubinemia.

With these questions in mind, we (Dr. David Draper, Dr. Orestes Valdes and myself of the department of pediatrics; Dr. Ali Hossaini of the Blood Bank; with the cooperation of the pediatric house staff at the Medical College of Virginia) have embarked on a comprehensive study of phenobarbital and light therapy. The study is designed to compare light versus phenobarbital versus a combination of light and phenobarbital for the prevention and treatment of hyperbilirubinemia in low-birth-weight infants. The comparisons will include the effectiveness of each on Negroes compared to Caucasians; effectiveness of each in hyperbilirubinemia due to hemolysis and other causes; effectiveness of each when hyperbilirubinemia is complicated by respiratory distress syndrome, intestinal obstruction and hypoglycemia; and effect of each on the need for subsequent exchange transfusions in infants who have previously received such transfusions. In addition, a long-term follow-up to determine possible sequelae of these treatments is planned.

In the study, low-birth-weight newborn infants are treated with either phenobarbital alone at a dosage of five mg/kilo/gram per day for five days, exposure to light for 96 hours at 200 footcandle intensity, or a combination of phenobarbital and light. Only preliminary data are available at the present time and are shown in Table 6. Of seven untreated control patients, six had serum bilirubin levels over 10 mg percent during the first five days of life. However, three of eight phenobarbital-treated infants, none of the seven light-treated infants, and none of the seven phenobarbital and light-treated infants had serum bilirubin levels above this value. The Figure shows the mean daily serum bilirubin levels in each group. Beginning with the second day of life there is a marked difference in serum bilirubin levels between the controls and the two light-treated groups. Infants who received phenobarbital alone showed a lowered serum bilirubin level beginning on day four. Infants who received only light therapy showed slight rebound bilirubinemia after the light was discontinued. Though we must be cautious in drawing firm conclusions from these preliminary data, two points seem clear. Phenobarbital and light treatment each modify the course of hyperbilirubinemia in the premature infant. Light reduces the bilirubin level more quickly and more dramatically than phenobarbital treatment since the latter probably depends upon enhancement of hepatic microsomal enzyme activity, which takes several days to occur. At the present time, the data are insufficient to determine if the addition of phenobarbital to light offers an advantage over light alone. One might suspect, however, that the combination might produce a sustained reduction of serum bilirubin level and avoid the rebound phenomenon seen on the fifth day with light alone.

In our study, only one of the infants who received phenobarbital became lethargic, but the lethargy persisted even after the drug was discontinued. No side effects from light therapy were recognized, and a funduscopic examination in the nursery in the majority of children failed to reveal any abnormalities.

Summary

All the observations suggest that phenobarbital and light may be of therapeutic value in controlling neonatal hyperbilirubinemia. In pregnancies in which one might anticipate increased bilirubin formation by the

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<td>Peak total serum bilirubin levels of low birth weight newborn babies during the first 5 days of life</td>
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<tr>
<td>Group</td>
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<td>PB*</td>
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* PB—Phenobarbital

![Figure](image-url)
newborn baby, the use of phenobarbital during pregnancy and in the neonatal period, and exposure of the infant to light may provide methods other than exchange transfusion to reduce the concentration of serum bilirubin in the infant. Clinical trials should proceed cautiously, however, since phenobarbital is known to stimulate the activity of liver microsomal enzymes that metabolize, steroids, hormones, and other normal body substrates. It is not known whether this effect would be harmful if it occurred in the human fetus or neonate. Pediatricians should consider phototherapy in the same cautious manner as they would the use of a new drug available for the treatment of newborn infants. Considering the state of ignorance on this subject, research into the long-term effects of phototherapy is clearly needed.

References


All hemolytic anemias feature shortened red cell survival due to premature hemolysis of the cell. For the purposes of this presentation, we may classify the hemolytic anemias, most broadly, according to the mechanisms leading to hemolysis.

Intrinsic Mechanisms

Hemolytic anemias due to intrinsically defective erythrocytes are essentially of three types. First are those anemias in which the red cells are defective due to lack of an essential factor, eg, pernicious anemia in relapse. The second type includes those in which the red cells have an abnormal shape because of an inherited error in the chemical makeup of the hemoglobin molecules; eg, sickle cells, elliptocytes, spherocytes, and the target cells. Last are those anemias in which the red cells are defective in their basic enzyme systems, as in paroxysmal nocturnal hemoglobinuria (PNH) (bithermal auto-hemolysins) with acetylcholinesterase deficiency, or primaquine sensitivity with glucose-6-phosphate dehydrogenase (G6PD) deficiency.

Such anemias due to intrinsically defective erythrocytes, including those in which the red cells are defective in their basic enzyme systems as in PNH, pose no problem for the Blood Bank. In these cases there is no antibody involved, and compatible group and type specific blood is abundantly available and effective in raising the hemoglobin level of these patients. However, should the reticulocyte count exceed five percent, a direct Coombs test (DCT) may be obtained which complicates matters for the Blood Bank, unless the reticulocytosis is brought to the attention of the Blood Bank staff.

Another complication may occur if the specimen sent for blood group, type, and crossmatch is obtained shortly after hemolysis has occurred. Should the serum contain hemolysins, they might not be detected in the crossmatch unless certain precautions are taken. If missed, incompatible blood may be issued —adding insult to injury.

Extrinsic Mechanisms

Those hemolytic anemias which are due to extrinsic factors may be classified, further, as non-immune or immune. Non-immune mechanisms include a) drugs and chemicals (phenylhydrazine, naphthalene, lead, snake venoms); b) physical agents (heat); c) bacteria and parasites (hemolytic streptococci, Clostridium welchii, Bartonella, plasmodia); and d) acquired sensitivity to penicillin, methyldopa, Keftin®, or fava plant as examples. Some of the agents in this last group serve to lyse the cells, either through direct action or by formation of antibodies.

These hemolytic anemias due to extrinsic factors of the non-immune variety present no transfusion problem for the Blood Bank. However, it must be realized that in those conditions where a positive direct or indirect Coombs test is obtained (as in acquired sensitivity to methyldopa, penicillin, phenacetin) a situation may arise whereby blood may be needed in an emergency; because of this incompatibility the release of blood may be delayed, to the detriment of the patient. Therefore, it is essential that such conditions be recognized within a short period of time. The administration of blood thereafter becomes as easy as the transfusion of any patient in the previous category.

In drug sensitivity cases, however, it must be realized that the hemolytic anemia will cease once the drug administration is stopped, and the DCT usually reverts to negative. Worledge, Carstairs, and Dacie (1966) studied 30 patients who developed hemolytic anemia while taking methyldopa (Aldomet®). Cessation of therapy led to immediate hematological remission. Peculiarly, the auto-antibodies had Rh specificity, instead of being directed against methyldopa or one of its derivatives. In patients on methyldopa, in which the DCT becomes positive, warm auto-antibodies may be detected in the serum so that all donors appear to be incompatible. However, despite the presence of such incompatibility, infused red cells show normal survival. Reversion to a negative Coombs test enables the Blood Bank to obtain and prepare compatible blood for the patient without difficulty or worry. It is essential, therefore,
that the drug causing the hemolytic anemia be withheld.

Sodium cephalothin (Keflin®) is another drug which sometimes causes neutropenia and often a DCT which appears to be causally related to hemolytic anemia. The clinical significance of the DCT in these patients is primarily one of its complicating blood banking—as high as 75 percent of such patients, depending on the dose administered, may give an incompatible minor crossmatch on a non-immunologic basis.

Another antibiotic which complicates blood banking, and may be a cause of hemolytic anemia, is penicillin. In contrast to cephalothin, penicillin produces both a DCT and indirect Coombs test (ICT). This means that the major crossmatch, in addition to the minor crossmatch, is incompatible. In a review of allergic drug-induced hemolysis, Daussset and Contu (1967) found penicillin to be the offending drug in 14/34 cases. Another difference between these two related antibiotics is that an eluate which will react with penicillin as well as cephalothin-coated cells may be prepared. No such eluates may be prepared from the sera of patients receiving cephalothin.

Another drug known to rarely cause hemolytic anemia on an immunological basis is phenacetin (acetophenetidin). This drug also produces a number of side effects: direct dose dependent hemolytic anemia, methemoglobinemia, sulfhemoglobinemia, and (rarely) a severe hemolytic anemia in G-6-PD deficient individuals. Patients who develop sensitivity to the drug produce antphenacetin antibodies which can directly (although weakly) agglutinate red cells, and produce and ICT and an active eluate. The addition of phenacetin to the patient’s serum neutralizes the antibodies in the presence of complement after two hours of incubation. A basic difference between Keflin® and penicillin sensitivity, and phenacetin sensitivity is the failure of normal erythrocytes to absorb phenacetin.

Quinidine, quinine, p-aminosalicylic acid, stibophen, and sulfomethrazine have been cited as rare causes of hemolytic anemia.

Varying mechanisms of action have been postulated for these drugs. The cause-effect relationship of these drugs is dependent on the in vitro demonstration of enhancement of the ICT in the presence of the offending drugs. It seems that the mechanism of immunologic hemolysis induced by some drugs may be different than that produced by others. In 1965, Levin summarized the mechanism leading to drug sensitivity with antibody formation as a drug or a drug degradation product combining with tissue protein to form a complete antigen. These provoke formation of antibodies which possess structural specificity toward either the drug hapten, the aminoaic acid residue binding hapten to protein, or the structural configuration of the protein moiety. If the drug attaches to the red cell membrane proteins first, the formed antibodies sensititize the red cells and lead to their destruction. According to Ackroyd (1952), this is the mechanism of immune hemolysis in penicillin sensitivity. A variation of this scheme has been proposed which suggests that cephalothin causes a positive DCT by binding pre-existing plasma proteins to erythrocytes. Another mechanism of cell destruction has been advanced by Miescher and Cooper (1960) who suggest that drug and antibody combine in the plasma with secondary and non-specific fixation to the cell.

The hemolytic anemias of immune origin may be the result of the presence of a) iso-antibodies or iso-hemolysins, or b) auto-antibodies or autohemolysins. Hemolytic anemias caused by auto-antibodies are acquired. From the standpoint of mechanism they may be classified as primary idiopathic (occurs in patients in whom no primary disease can be demonstrated), or secondary symptomatic (is associated with some other disease; eg, malignant lymphomas, leukemia, virus pneumonia, infectious mononucleosis, or collagen disease). From the standpoint of serology, the auto-antibodies may be classified as warm antibodies, with best reactivity at 37 C; and cold antibodies, with maximum reactivity at lower temperature. In turn, the cold and warm antibodies may be subclassified into normal and pathogenic. The presence of both of these kinds of antibodies is shown best by the DCT, which is the distinguishing feature, and by auto-agglutination. Patients with the pathogenic type of either the cold or warm type are said to have auto-immune hemolytic disease or anemia. Most antibodies eluted from the cells of patients with the warm type of auto-immune hemolytic anemia detect an antigen associated with the Rh agglutinin complex.

Hemolytic anemias due to iso-antibodies and iso-hemolysins are, in the majority of cases, easy to deal with; cells lacking the corresponding antigen are usually relatively easy to find, and compatible blood may therefore be transfused. However, certain iso-antibodies are directed against an antigen that is present in a high percentage of the population; should blood be requested, particularly in large amounts, it would be very difficult to obtain the required number of units of compatible blood. For example, in order to find two units of compatible blood for a patient with anti-k antibody, the blood of 1,000 donors must be crossmatched since the percentage of Cellano negative individuals is only 0.2 percent. Other examples of this type of incompatibility may be cited. It is immensely difficult to transfuse patients with paroxysmal cold hemoglobinuria. These patients show a positive DCT, free plasma hemoglobin, and free antibodies in their serum. These antibodies are of anti-P, or anti-Tj specificity. Since the corresponding
Tj⁺ antigen is present on the red cells of more than 99.9 percent of people, it is obvious that to find a unit of compatible blood more than 1000 randomly selected donors must be crossmatched.

The transfusion of a patient with the warm antibody type of auto-immune hemolytic anemia is also a formidable problem. The cells of such patients give a positive DCT and, infrequently, an ICT. A positive ICT in these patients is associated with a poor prognosis.

Anti-e Antibodies

Anti-e is one of the most disliked antibodies when found in a patient with auto-immune hemolytic anemia who is in need of blood transfusion, for three main reasons:

1. It is a warm antibody; the administration of incompatible blood is certain to be detrimental to the patient who obviously can do without an additional load of plasma hemoglobin.

2. This antibody is directed against a very common antigen. After crossmatching 50 units of blood, one may find one unit of compatible blood if lucky. For each additional unit requested, 50 units must be crossmatched.

3. The adverse effect of the antibodies on the supposedly compatible red cells is often demonstrated clinically by the frequency of hemolytic transfusion reactions in patients having auto-immune hemolytic anemia in general; and in patients having auto-immune hemolytic anemias due to anti-e reacting not only with e-positive cells but also, although much more weakly, with e-negative cells. Consequently, although e-negative cells may have a near normal life span following initial transfusion, subsequent transfusions of these patients result in the appearance of a higher titer of antibodies. This complicates future crossmatching tests. One other result of failure to apply extreme restraint on the use of blood in such cases is the exhaustion of the limited supply of the rare R₂R₂ blood. Hence, when such blood is needed as a life-saving measure, it is often not available. The first case study—auto-immune hemolytic anemia due to anti-e, secondary to chronic lymphocytic leukemia (CLL)—typically illustrates this point.

L.C. a Negro male diagnosed as having CLL in January, 1966, was taking chlorambucil until January, 1968. In October, 1968, he was found to have anemia with a hemoglobin of 4.5 gm percent. A sample of blood sent to the Blood Bank showed the following results:

Blood Group: O Rh₄ (Pos.)
Direct Coomb's Test: Positive (1++)
Most Probable Genotype: R₁ R₂ (CDe/cDE)
Antibody Identification: Anti-e. Detectable only by the Coomb's technique

Blood was released, and three units of R²R² (cDE/cDE) were released and administered.

It is obvious that prior to transfusion the direct and indirect Coombs tests were weak. Although e-negative blood was transfused, both the DCT and ICT became progressively stronger—as evidenced by a rise in antibody titers from one in pre-transfusion to eight in post-transfusion blood sample. As the number of transfusions increased, the patient's serum became increasingly incompatible not only with the blood of e-positive donors, but also with that of e-negative donors. Just before his demise, L.C. received the last two units of R₂R₂ which we were able to obtain from the rare bloods stored in a frozen state at the New York Blood Center.

Anti-I Antibodies

A patient with auto-immune hemolytic anemia of the strictly cold antibody type, which does not agglutinate cells at 37 C, presents a lesser problem. Usually the antibody involved is of anti-I specificity. Dacie reported finding cold auto-antibodies rather than warm ones in about 20 percent of cases of idiopathic acquired hemolytic anemia and in about 30 percent of cases secondary to such conditions as CLL. Further, an upper respiratory tract infection associated with a group of organisms known as pleuro-pneumonia-like organisms was found in all cases in which acquired hemolytic anemia followed an attack of primary atypical pneumonia. Anti-I antibodies are directed against an antigen which is much more prevalent than the e antigen. In order to find a unit of compatible blood, the Blood Bank would have to crossmatch the serum of the patient against the blood of 4,400 randomly selected people. However, as mentioned earlier, such patients are a lesser problem in transfusion mainly because the majority of the patients show an antibody which reacts below body temperature only; therefore, when absolutely essential such patients may be safely transfused, provided transfusion is given conservatively.

The advisability of transfusing these patients, conservatively and only when absolutely essential, is based on the fact that continued transfusion of such patients may eventually result in production of the antibodies which are reactive at body temperature. Blood administered to these patients has a reduced life span and may invoke a hemolytic transfusion reaction which, in turn, may produce warm reacting antibodies. Such patients have an abnormal immune response and the antibodies, although cold, are pathogenic.

A few of the patients with auto-immune hemolytic anemia due to anti-I have antibodies which react initially not only in the cold but also at 37 C. Transfusion of such patients with incompatible blood must be avoided unless it is to alleviate debilitating symp-
TRANSFUSION PROBLEMS

toms or is a life saving procedure. Such patients are susceptible to antibody production when repeatedly challenged with the corresponding antigen.

Therefore, the sooner and more frequently these patients are transfused, the sooner the stage is reached when transfusion of incompatible blood is no longer a life saving measure. One can be more liberal in the use of blood for such patients if the very rare ii genotype blood is available. To our knowledge no individual living in the Richmond, Virginia area belongs to this phenotype. Since these patients are kept relatively comfortable with the judicious use of immunosuppressive therapy and avoidance of exposure to cold, the use of hemotherapy may be avoided for a considerable period of time. The successful blood management of such a patient is represented in the second case study.

D.O. was a 60-year-old white female who had been in good health until ten days prior to hospital admission on October 25, 1965. At that time she noted severe headaches and weakness of her lower extremities with ataxia; she developed nausea, vomiting, and fever. She was diagnosed as having bronchopneumonia and was treated accordingly. She was also found to have severe anemia with a hemoglobin of 5.4 gm percent. History showed no melena, hematuria, or epistaxis and she had noted bruising only during the two weeks prior to admission. She had no history of jaundice. Family history was non-contributor. Later she was referred to William T. Dabney of the Medical College of Virginia, who found her to have a moderately enlarged spleen. Cr51 red cell survival time and studies showed a T 1/2 of 14 days (normal 25-35 days), and a slight increase in the spleen to liver ratio with normal splenic localization index. A blood sample sent to the Blood Bank on December 29, 1966 showed the following:

Blood Group: O Rh0 (Neg.)
Direct Coombs Test: Weakly Positive
Indirect Coombs Test: Positive, Titer—1:4
Antibody Specificity: Anti-I

The anti-i antibodies were found to react not only in the cold but also at 37 C. The Blood Bank accordingly advised withholding transfusing until absolutely essential. This patient was treated and kept on a maintenance dose of prednisone. The transfusion of blood has been avoided purposefully until the present, despite the fact that her hemoglobin has been 6–7 gm percent. Dr. Dabney saw her again in January, 1970 and found her active despite her anemia. The DCT was repeated 15 times between December, 1966 and January, 1970. Reactivity ranged between negative and 1+ strong with the last test, like the first one, being only a weak positive. The failure of the Coombs test to become stronger after four and one-half years may be the result of non-transfusion. This, as suggested previously, indicates the value of withholding blood transfusion in such cases.

Anti-i Antibodies

Another case exemplifying a transfusion problem in hemolytic anemia is that caused by a high titer of anti-i antibodies in patients with IM. Last year we reported the fourth case of IM complicated by severe, but transient, auto-immune hemolytic anemia due to anti-i, as seen in a third case study.

R.P. was a 20-year-old white male who had been in good health until six days prior to admission to the Medical College of Virginia Hospital. On that day, he was involved in an automobile accident. Examination of the patient at the referring hospital emergency room immediately following the accident revealed no serious injury, with negative X-rays, and he was released. Four days later he began to have some vomiting and pain in the right side of the abdomen, which continued until the day of admission. The next day he developed dark urine and a mustard-colored stool. At this time, one day prior to admission, he was found jaundiced with a bilirubin level of 7.6 mg percent, a hemoglobin of 8.0 gm percent, and a hematocrit of 22 percent. There was marked tenderness in the right upper quadrant and a possible epigastric mass. He was referred to the Medical College of Virginia Hospital for further evaluation and admitted on April 20, 1968. The past medical history was not remarkable except for a prior appendectomy. Examination showed a pulse of 120/min and a blood pressure of 130/90. He was jaundiced with some abdominal distress. Eyes showed marked icterus. The abdomen showed tenderness in the right upper quadrant and there was a small, ill-defined, tender epigastric mass. The spleen was barely palpable. Prior to admission the patient had never received blood, blood components, antibiotics, or other drugs. He denied exposure to any chemicals, and there was no history of allergy to drugs or chemicals. The rest of the accessory history was not remarkable. A tentative diagnosis of liver hematoma was made, and surgery to relieve it was entertained. For this purpose eight units of blood were requested. Because of difficulties encountered in blood grouping, typing, and crossmatching, blood was withheld until studies were completed to clarify the discrepancies. The difficulties consisted of a discrepancy between cell grouping which showed him to be group AB and serum grouping which showed him to be group O. The DCT using broad spectrum Coombs serum was strongly positive; his serum agglutinated 60 randomly selected group A bloods and all members of two commercial panels of cells. Using appropriate techniques, the blood group of the patient was found to be A,B. Initial laboratory studies on admission showed a hemoglobin of 5.2 gm percent, and a
hematocrit of 15 percent which steadily fell to 4.5 gm percent and 12 percent respectively over the next eight hours. The white cell count was 13,500/mm³; the differential showed 54 percent polymorphonuclear leukocytes, 31 percent lymphocytes and 14 percent considered monocytes. Nucleated red cells were also present. Blood chemistry studies included amylase-70 (normal 150) Somogyi units, alkaline phosphatase 18 (normal 0.8 to 2.9) Bessay-Lowry units, LDH 570 (normal 20 to 68) International units, and SGOT 225 (normal 0 to 40) Karmen units. Total protein was 6.9 gm percent with 56 percent albumin and A/G of 1.27:1. Plasma hemoglobin was 210 mg percent, and the urine was strongly positive for bile and contained 160 mg of hemoglobin. On the second day of admission, a liver radiotopic scan revealed a moderately increased size and decreased uptake in both lobes, and increased concentration in the spleen which was enlarged. Because of the immunologic reaction which is indicated by the DCT, hemoglobinemia and bilirubinemia, he was started on prednisone. The red cell count on this day was 1.92 mil/cu mm, and the platelet count was 364,000/cu mm. The peripheral blood smear showed nucleated red cells, shift to the left, and 50 percent lymphocytes with one-half appearing as pathological lymphocytes. The bone marrow showed good myeloid, erythroid, and megakaryocytic cellularity with moderate increase in erythroid/myeloid ratio and a slight increase in reticuloendothelial cells. The stain for iron was strongly positive. On this day he received two and one-half units of whole blood without untoward reaction. Permission to release blood was based on compatibility tests with patient's serum after absorption with own cells. Blood was released which showed incompatibility no stronger than a selfcontrol by the saline and albumin methods, and which was negative by the Coombs test. The following day, April 22, the patient showed a clear urine but the abdomen became homoglobinuric almost immediately after the administration of two more units of blood. A heterophile agglutination test was performed, and the unabsorbed serum showed a 1:448 titer which remained unchanged after guinea pig kidney absorption, and became negative after beef cell absorption. Further laboratory studies using rare cells of the phenotype ii and absorption-elution techniques indicated that the antibody causing the incompatibilities was of anti-i specificity. This antibody occurs rather commonly in patients with IM, although in low titer. As such it reacts strongly with cord blood and only weakly, if at all, with adult blood at 4 C only. Therefore, it is usually of no clinical significance and does not present crossmatching difficulties. However, when it does, it is important to identify the antibody within a short period of time. This shows the immunohematologist that it is advisable to release blood for these patients, since transfusion problems which may occur may be outweighed by the complications of withholding blood.

Albumin Autoagglutination Phenomenon

The last case posing a transfusion problem which I present is a rare phenomenon which may coexist in a patient with hemolytic anemia. This phenomenon has been termed the albumin autoagglutination phenomenon. The first example was reported by Weiner (1956).

V.P. was a seven-year-old white female patient. Three weeks prior to admission to the Medical College of Virginia Hospital the patient had followed a truck spraying 30 percent dichloro-diphenyl-trichlorethane. She had also been exposed to dog spray insecticide and had been given chloromycetin in March and April, 1964 for pharyngitis. She was admitted to a hospital on July 6, 1964 with 5 gm/100 ml of hemoglobin and jaundice. She was successfully transfused with 250 ml of whole blood without crossmatching difficulties. On July 10 she was transferred to the Medical College of Virginia Hospital with a hemoglobin level of 6.8 gm/100 ml. She was diagnosed as having acquired hemolytic anemia, possibly on the basis of inhalation of an insecticide. Despite the fact that blood was urgently needed, due to difficulties in the albumin phase of the crossmatch the Blood Bank could not release the blood requested. Further studies showed that this case was an example of the non-immune autoagglutination phenomenon. On this basis, on July 17 the patient was successfully transfused with 125 ml of packed red cells compatible in the Saline and Coombs tests, although very strongly incompatible in the albumin test. Admittedly, this phenomenon is rare. Rarity, however, does not detract from its practical significance, since blood required in an emergency for such a patient may be withheld on the presumption that it contains specific high-protein active antibodies.

Transfusion Criteria

It is worth remembering that transfusion could lead to complications, particularly in patients with autoimmune hemolytic anemia. Therefore, the longer the transfusion is avoided, the longer the complications of blood transfusion are delayed and the better the prognosis. In all hemolytic anemias, the anemia per se is not an indication for transfusion, and transfusions are given only when the symptoms justify. Transfusions are not means of maintaining the hemoglobin levels in the normal range.

Does every severe anemia always demand transfusion? The answer to this vital question is NO. This answer is based on the fact that in patients with extremely marked anemia, transfusion may easily overload the circulation and precipitate cardiac failure. Therefore, it is mandatory that whenever the chance
exists that the patient will respond to some other form of treatment, transfusion of blood should be avoided. An ideal example is the patient with pernicious anemia in relapse. It is commonly agreed that transfusion of such patients should be avoided unless (1) there is hemorrhage associated with thrombocytopenia and (2) the patient has pneumonia or some other infection which may interfere with the response to Vitamin B₁₂. Inhalation of 100 percent oxygen by such patients may effectively raise the O₂ carrying capacity of the blood, to tide them over a short period of extremely severe anemia. A blood with 3 gm Hb percent normally carries about 4 ml oxygen per 100 ml, while the inhalation of 100 percent oxygen increases the oxygen content of the plasma by an increment of 2 ml oxygen per 100 ml. This means that such treatment may effect an increase equivalent to 1.5 gm Hb percent, which in the case of the patient with 3 gm Hb means a rise to a level of 4.5 gm percent. Should these patients be transfused, the transfusion rate should not exceed 0.5 ml/lb of body wt/hr. Sharpey-Schafer (1945) suggested that it would be wise to limit the volume of transfused blood to 500 ml/48 hrs (preferably as PRC) and to take various measures to reduce right auricular pressure, such as keeping the patient propped up in a sitting position. The central venous pressure should be observed and any rise should be an indication for slowing or stopping the transfusion. Other safety measures are (1) warming of the patient and (2) administration of a diuretic.

Summary

In this presentation I have attempted to present some of the transfusion problems that face the Blood Bank and the physician treating the patient. I have briefly discussed methods of recognizing the complicating factors, interpretation of their clinical significance, and the proper hematotherapeutic management of such cases. Finally, I hope that this presentation is a convincing thesis for a better understanding of the Blood Bank and its problems, since the patient's welfare is better served when there is a rapport between the clinicians and the Blood Bank staff.

References


WILLIAMS D: Personal communications

Immune Suppression in Auto-Immune Hemolytic Anemia*

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The acquired auto-immune hemolytic anemias represent a diversity of disease states in which the most constant immunologic finding is a positive direct anti-human globulin test (van Loghem, 1965; Swisher et al, 1965). This is true of the symptomatic variety of acquired hemolytic anemia as well as the idiopathic form. The positive Coomb’s test has been seen in association with primary atypical pneumonias, occasionally in favism, in some bacterial and drug induced hemolytic anemias, in patients with malignancies of the lymphoid tissues, and in collagen-vascular disorders—chiefly SLE.

The original hope that the direct Coomb’s test would be negative in all forms of hereditary hemolytic disease and thus differentiate them from acquired forms of hemolytic anemia has not been borne out.

Acquired auto-immune hemolytic anemias vary widely in the severity of the hemolysis, in the association of additional pathologic states and in the comparative clinical influence of these factors. Those cases of auto-immune hemolytic anemia that are associated with self-limiting viral diseases such as infectious mononucleosis, measles or bacterial infections, usually subside with the primary disease. Forms of Coomb’s positive hemolytic anemias associated with drug therapy can usually be eliminated by withholding the offending drug. In instances where the Coomb’s positive hemolytic anemia is associated with benign or malignant tumors or cysts, surgical correction of the abnormality may result in permanent hematologic and serologic remission. It has also been observed that a positive Coomb’s test may occur in association with pernicious anemia, folic acid deficiency and iron deficiency anemia. In these circumstances, replacement of the specifically deficient material may result in complete remission of the hematologic abnormality. This discussion will concern itself primarily with the idiopathic disease, since immunosuppression is not necessary or desirable in most of the symptomatic varieties.

Since the diagnosis of an auto-immune hemolytic anemia of the idiopathic variety is tied to the exclusion of recognizable causative disease, the frequency with which the diagnosis is made will first depend on the aggressiveness of the attending physician in looking for secondary causes and secondly, the nature of the patient population. Since the disease has a peak incidence in the fourth through seventh decades, a large pediatric population would diminish the incidence. Likewise, since about 60 percent of the patients in reported series are women, a largely male clinic or hospital population would have a similar effect. No racial discrepancies have been noted to date. In Dacie's series (1969) followed over a 20 year period, 111/210 or 52 percent adult patients with a positive direct Coomb’s test were classified as idiopathic, whereas only 18.2 percent of Pirofsky’s 234 cases were idiopathic (1969). A positive direct Coomb’s test is, of course, central to the diagnosis of auto-immune hemolytic anemia although a positive direct Coomb’s may be present for years with hemolysis.

The demonstration that the globulins coating the red cells are “true” antibodies is now generally accepted with the proviso that the coating of the red cells with a protein giving rise to a positive antiglobulin reaction does not necessarily mean that the reacting substance is an erythrocyte antibody. It may even be complement absorbed as a result of antibody activity. By performing a gamma globulin neutralization test one can elucidate whether the coating globulin of the erythrocyte is a gamma globulin, complement or mixed type. Further evaluation of the protein can be done with antiglobulin sera specific for IgG, IgM, or IgA. The great majority of auto-immune hemolytic anemias will have IgG antibody.

The cardinal points which identify the coating globulins as antibodies are their transferability to normal red cells; the frequent presence in serum of similar globulins which can be absorbed by normal cells; and in many instances, their ability to react with specifically identifiable red cell antigens. For instance, in patients who form warm antibodies, it is often possible to demonstrate a clear specificity for one or more Rh
AUTO–IMMUNE HEMOLYTIC ANEMIA

antigens, but the nature of specificity of other antibodies which do not have affinity with Rh antibodies is yet unknown. It appears probable that an antibody reacting indifferently with all human cells may be involved. Although the etiology of the auto-immune hemolytic anemias remains obscure, current theory (Parker and Vavra, 1969) surmises that somatic mutation leads to the development of forbidden clones of antibody forming cells which are not susceptible to, and escape from, the normal homeostatic mechanisms which prevent auto-antibody formation. The association of auto-immune hemolytic anemias with other diseases thought to be of an auto-immune nature, such as disseminated lupus erythematosis and ulcerative colitis, lends unity to this concept. Chronic lymphocytic leukemia and lymphosarcoma may represent malignancies of potential antibody forming cells in which the tumor cells, in some instances, retain their ability to form antibodies, particularly abnormal ones which react with erythrocytes. Another possibility is that the antibodies primarily formed against the abnormal neoplastic lymphoid cells act as antigens; the antibodies which cross-react with red cells. It also seems conceivable that the malignant lymphomas represent an enormous monoclonal proliferation of lymphoid cells which displace the immunologically competent cells and interfere with the delicate balance between the two. This allows the development of a clone of auto-antibody forming cells. Whichever of these mechanisms ultimately proves to be true, the current therapy for auto-immune hemolytic anemias of a sufficient degree of severity to require treatment involves attempts at immunological manipulation.

Current Concept of the Immune Response

The current concept of the immune response (Fig 1) is divided into afferent, recognition, stimulatory and effector phases. All phases of this scheme can be manipulated to produce immunologic tolerance, as we shall see.

(1) In the afferent phase it is believed that the antigen is processed by macrophages into highly immunogenic complexes with RA and other cellular constituents.

(2) In the recognition phase the macrophage transmits messenger RNA to “antigen sensitive cells” which have the morphologic appearance of a small lymphocyte. These cells arise from stem cells in the marrow.
Surgical Ablation of Lymphoid Tissue

where they are incapable of responding to antigen. Upon leaving the marrow they migrate to the thymus where partial maturation takes place and then seed peripheral lymphoid tissue where they acquire immunologic competence. Stimulation of these "antigen sensitive cells" appears to be confined to lymphoid organs. They do not in themselves produce antibody, but they give rise to clones of antibody producing cells as well as to additional antigen sensitive cells.

(3) In the stimulatory phase exposure to antigen initiates an immunologic response after a latent period of four to 24 hours. Mature antibody processing cells appear to arise from a large immunoblast (hemacytoblast) and reproduce rapidly. Serum antibody arises logarithmically and gradually declines. Small lymphocytes are also formed, committed to react specifically with antigen. They seem to be the primary mediators of cellular immunity and probably carry long term immunologic memory.

(4) In the effector phase the small lymphocytes are responsible for such forms of hypersensitivity as allograft rejection, and contact skin sensitivity.

 Responses attributable to serum antibody include local and systemic anaphylaxis, Arthus phenomenon, hemolytic anemia and thrombopenia.

Approaches to Immunosuppression

In clinical immunosuppression the number of options available to the clinician is somewhat restricted by the clinical setting. Since the immune response in auto-immune hemolytic anemia is mediated by humoral antibody, attempts at immunosuppression should be so directed.

Table I shows the theoretical areas where immunosuppression might be possible in immuno hemolytic anemia. Administration of antigen might be considered as analogous to desensitization of patients with allergic asthma. Since the nature of the erythrocyte antigen is unknown, this approach does not seem feasible at present. Administration of specific antibody as a form of immunosuppression has received renewed interest as a result of the successful use of Rh antibody in the prophylaxis of erythroblastosis. It seems dubious that such an approach would prove successful in auto-immune hemolytic anemia for a variety of reasons.

I would like to discuss the types of immunosuppression which have been attempted in auto-immune hemolytic anemia, paying particular attention to those methods which are most commonly used in treatment today (Table II).

Surgical Ablation of Lymphoid Tissue

One of the four major approaches to immunosuppression has been that of surgical ablation. Splenectomy, of course, is the oldest form of immunological manipulation that has been attempted in autoimmune hemolytic anemias. The rational behind its first application in 1911 by Michelle was a result of Banti's work incriminating the spleen as a primary site of blood destruction. The mechanistic concept of its function in auto-immune hemolytic anemia is probably still a prime reason for its removal; but it is included in the list of immunological manipulations because of the evidence showing splenic hyperplasia, particularly an increased production of lymphocytes and plasma cells, in the spleen. This suggests that hemolysis can potentiate the ability of the spleen to engage in antibody production. Jandl (1965) speculated that the enhanced immunologic reactivity of the hyperplastic spleen could take two pathways. In one it could initiate immunologic responses directed against related or coincidental antigens of the erythrocyte; in the other it could convert reactions which were initially nonimmune into immune forms. The latter mechanism could possibly permit metabolically modified antigenic determinants to be recognized as foreign. In this manner, it could supply the background to the auto-immune state described as an enhanced sensitivity of antibody forming tissues. Splenic hyperplasia creating these two antibody producing states could result in an immune relationship which is auto-catalytic and which would appear as an auto-immune hemolytic anemia.

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<th>TABLE I</th>
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<th>TABLE II</th>
<th>Approaches to immunosuppression in idiopathic autoimmune hemolytic anemia</th>
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<td>I. Surgical ablation of lymphoid tissue</td>
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<td>A. Splenectomy</td>
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<td>B. Thymectomy</td>
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<td>C. Thoracic duct drainage</td>
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<td>II. Administration of cytotoxic drugs</td>
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<td>A. Corticosteroids</td>
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<td>1. spleen</td>
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The beneficial effects of splenectomy have been amply documented in several series reported since 1911. Therapeutic response has been noted in about 70 percent of the patients treated with half of the patients apparently being cured of their disease (Allgood and Chaplin, 1967). The mortality rate, however, of splenectomy in patients with auto-immune hemolytic disease is high and averages about 17 percent in the series reported. There is a further increase in mortality if splenectomy is delayed for over a year after the onset of the disease. This high surgical mortality rate has tempered the enthusiasm for splenectomy in most clinics.

In addition to the immediate surgical mortality of splenectomy, the predisposition of patients with auto-immune hemolytic anemia to thrombosis may be enhanced by the thrombocytosis occurring after the spleen is removed. In younger patients, such a thrombocytosis is usually well tolerated, but in the older age groups with co-existing atherosclerosis, it may constitute a major hazard to the patient's survival. The increased frequency of overwhelming infections in infancy following splenectomy seems to be real, but since auto-immune hemolytic anemia occurs very rarely in this age group, it is seldom a consideration. A more serious difficulty following splenectomy is the hyperplasia which sometimes occurs within the reticuloendothelial cells in the liver leading to progressive hepatomegaly, liver dysfunction and death. Responses to splenectomy in patients with auto-immune hemolytic anemias secondary to reticuloendothelial neoplasm seem to be somewhat less frequent than in the idiopathic form of disease. Splenectomy may be of value after patients have failed on corticosteroids, and Crosby's data suggest that the remission rates following splenectomy in patients who are unresponsive to steroids may be as good as those treated de novo with splenectomy. Several authors have emphasized criteria which may enable one to select candidates for splenectomy. Among these are the patients with warm acting, incomplete erythrocyte auto-antibodies and documentation of splenic sequestration. Several methods of determining significant splenic sequestration of Cr\(^{51}\) labeled red cells are in use including those devised by Korst (1955), Jandl (1956), and McCurdy and Roth (1958). All suffer the same defect, ie, they are not entirely reliable in predicting either response to splenectomy or failure of response.

Goldberg and his associates have suggested that a reduced Chromium 51-T \(\frac{1}{2}\) may be of importance in selecting candidates for splenectomy; this seemed to be the case in their series of 13 patients in whom 11 had good results. It is difficult to know what this means since all patients with auto-immune hemolytic anemia have reduced Cr\(^{51}\) red cell survival times and the response rate is what one would expect. Evidence of sequestration of red cells in the liver is a poor prognostic sign and suggests that splenectomy will offer little benefit.

Thymectomy is an attractive approach to immunosuppression in auto-immune hemolytic anemia because of the central role of the thymus in the maturation of immunologically competent cells. It is also technically easier than splenectomy and should carry a lower mortality rate. No data are available on adults treated in this manner, but in infants there have been two remissions induced with this procedure and one failure. Thymectomy appears to be of no value when thymoma is associated with auto-immune hemolytic anemia.

Thoracic duct drainage seems potentially useful because primary antigen sensitive cells and immunologic memory cells can be removed. Unfortunately, immunologic impairment is very short lived.

**Cytotoxic Drugs**

The administration of cytotoxic drugs is a second approach to immunosuppression. The immunosuppressive effects of corticosteroids resemble those of x-irradiation in that they produce lymphocytolysis particular in the germinal centers of lymph nodes and spleen with resultant lymphocytopenia. The mechanism by which the cell damage is effected is unknown. They are also active in suppression of delayed hypersensitivity but their effect on serum antibody synthesis is less impressive.

Because of the high surgical mortality associated with splenectomy in auto-immune hemolytic disease, the observed lympholytic activity of adrenocorticoids was investigated in 1951 by Dameshek in auto-immune hemolytic anemias.

In a series reported in 1956, Dameshek and Komninos claimed that 90 percent of the cases of auto-immune hemolytic anemia would show an initial therapeutic response to corticosteroids. Of these, 65 percent were complete remissions and in an additional 25 percent a definite response was obtained. Two-thirds of the cases, however, relapsed when corticosteroids were discontinued. Subsequent series have shown similar results. Horster reported a 6.8 percent cure rate with corticosteroids, and remissions lasting over a year in about a third of the cases after discontinuation of corticosteroid therapy. About half of the patients could be maintained in remission only while corticosteroids were continued.

Pirofsky's data suggests that the corticosteroids, too, are less effective in the auto-immune hemolytic anemias associated with reticulo-endothelial malignancy. The dosage of corticosteroids necessary to induce remission has been variable in the series reported but in the average adult, 300 mg of cortisone per day or its equivalent of prednisone, triamcinolone, or dexamethasone, appear to be an effective dose level. If using corticosteroids is effective, time of onset of the evidence for reduced
hemolysis is rapid. Response is usually clearly apparent within the first one to two weeks, although a few patients will respond as long as 21 days after therapy is begun. In the patients who respond slowly, the response tends to be less good and it is difficult to remove such patients from steroid coverage.

Hematologic remission may occur while the direct anti-globulin tests remain positive. In idiopathic autoimmune hemolytic anemias, there may be reduction in spleen size, although this effect is not as marked in the symptomatic varieties of the disease. Since the side-effects of steroids are not inconsiderable, reductions from the high dose levels initially used, should be made as soon as possible, and the minimally effective dose of corticosteroid should be used as the maintenance dose. Even with maintenance, a significant number of relapses will occur within the first six months, although the relapse is usually of gradual onset. If the patient is being seen at frequent intervals, there is normally sufficient warning to increase the amount of steroid. In a few patients, prednisone may gradually be discontinued over a period of five or six months and the patients will remain in remission.

Alkylating agents include such compounds as the nitrogen mustards, sulfur mustards, sulfonate esters and ethylenimines. They are chemically highly reactive and are capable of combining irreversibly with DNA, proteins and other essential macromolecules in the cell.

Cyclophosphamide, a latent compound activated in vivo after tissue phosphorylases cleave the cyclic phosphamide moiety to expose the alkylating radicals, has been shown to be a very promising immunosuppressive agent. It appears to be capable of reducing the antigen sensitive cell population, blocking cellular proliferation during the inductive phase and is ever active in reducing antibody synthesis. Hersh found it to be the most effective immunosuppressive agent among the various alkylating agents. I am unaware of any published reports on its effectiveness in auto-immune hemolytic anemias, although there are isolated reports of remission induced by other alkylating agents (Taylor, 1963).

Purine and pyrimidine antagonists are another approach included among the cytotoxic drugs. In 1957, Sturtzle and Holub suggested that 6-Mercaptopurine (6-MP) might interfere with antibody synthesis, but were unable to confirm it in the test system that they were using. This was independently confirmed in extensive studies by Schwartz and Dameshek (Schwartz and Dameshek, 1959; Dameshek and Schwartz, 1960; Schwartz, Eisner and Dameshek, 1959; Schwartz and Dameshek, 1960) who demonstrated that 6-MP could suppress antibody formation in rabbits immunized with Bovine albumin. In addition, 6-MP apparently could induce a state of immunologic unresponsiveness in adult rabbits and could suppress transplantation rejection of skin graft although not completely at tolerated doses. Further studies on the mechanism by which 6-MP was capable of inducing this effect led to the conclusion that the drug had its primary effect on the lymphoid hemocytoblast (immunoblast) and morphologic studies of lymph nodes subsequent to 6-MP after a homograft administration revealed extensive disruption of the lymphoid follicles (Andre et al, 1962). Borel also showed suppression of IgG response with prolonged administration. Studies were extended (Schwartz and Dameshek, 1962) to include cases of human auto-immune hemolytic anemias treated with 6-MP or Thioguanine with a favorable effect in three of six cases. 6-MP and thioguanine have been sporadically reported since (Demis, Brown and Crosby, 1964; Shearn, 1965; Hitzig and Massimo, 1966) to cause remissions in auto-immune hemolytic anemia. The primary limiting factors to the administration of these agents is bone marrow toxicity (Demis, Brown and Crosby, 1964). Azothiaprine (an imidazole substituted 6-MP) is converted to 6-MP in vivo. It has been used in the auto-immune hemolytic anemias alone and in combination with corticosteroids with some success. Its spectrum of immunosuppression is similar to 6-MP but it appears to have a better therapeutic ratio (Frisch and Davis, 1962; Frisch, Davis and Milstein, 1962) and produces less bone marrow suppression. It also produces fewer G.I. symptoms when given orally.

It seems probable that these agents do not show cross resistance with corticosteroids, and failure on the latter does not necessarily militate against induction of remission with the former. Response rates are difficult to access on the basis of the limited number of cases reported, but the drugs are somewhat less effective than corticosteroids. This may merely reflect the fact that the patients are further advanced in their disease.

Fig 2 shows the clinical course of a 79-year old white man, who was first seen here in August 1964 for treatment of progressive anemia of four months' duration. Physical examination was essentially unremarkable. His laboratory data revealed a hemoglobin of 7.8 gm percent, reticulocyte count 29 percent, MCV 96, MCH 32, MCHC 34, and Bilirubin 2.2 mg percent with 0.3 mg percent direct reacting fraction. Coomb's test was weakly positive. Cold agglutinins were positive in a titre of 1:64. Chromium$^{51}$ red cell survival was 7 ½ days, and the spleen to liver ratio was 0.72. This patient was started on prednisone 40 mgs per day with a prompt rise in his hemoglobin concentration, as shown in Fig 2. His hemoglobin was maintained well until steroid toxicity forced the reduction of his dose of prednisone. Three months later his hemoglobin gradually fell to levels of seven to eight grams percent. He required an interim hospitalization in September 1966, at which time his
hemoglobin was 8.5 gm percent, reticulocyte count was 40 percent, cold agglutinins were positive at a titre of 1:1, 280, and Coomb's was strongly positive. He was seen again in consultation and the recommendation was made that he be offered splenectomy. Because of his age and the patient's wishes this was not done, and he was instead started on 6-MP in a dose of 25 mg per day, which was continued uninterruptedly until the time of his terminal hospitalization in August 1969. We have only random blood counts available for this period of almost five years, because he was being followed elsewhere. He was admitted in August 1969 because of massive G.I. bleeding. At that time his hemoglobin concentration was 5.5 gm percent with a reticulocyte count of 16 percent. Prednisone, which had been continued at a low dose level of 5 mg per day was increased because of the uncertainty as to whether the marked fall in his hemoglobin concentration was the result of bleeding or an accelerated rate of hemolysis. His upper G.I. series was normal. His barium enema revealed some diverticula in the colon. His Coomb's test was strongly positive and his cold agglutinin titre was too high to read. Serum protein electrophoretic pattern was essentially normal, except for a questionable blip in the slow gamma region. Immunelectrophoretic assay of his serum revealed an IgM level of 1,168. IgG and IgA levels were normal. He was started on leukeran, 12 mg per day on September 11, 1969, which was continued through October 5, 1969, when it had to be discontinued because of leukopenia of moderate degree. His G.I. bleeding continued throughout his hospital illness, and he was transfused several times when his hemoglobin concentration fell to dangerous levels. It was not apparent, in this particular patient, who had what we would classify as cold agglutinin disease, that his immunologic process was altered either by the long-term administration of small doses of 6-MP or by the relatively aggressive therapy with chlorambucil at the time of his terminal illness.

**Radiation**

The primary effect of total body irradiation appears to be on DNA of lymphoid and other sensitive cells.
Unfortunately, when used alone, high doses are required which are lethal in themselves. At present there seems to be no place for total body irradiation in the clinical context of auto-immune hemolytic anemias.

Local irradiation has proven to be a more effective approach to immunosuppression. Since splenectomy is an efficient form of treatment for autoimmune hemolytic anemia it has seemed natural to try to circumvent the surgical mortality by splenic irradiation. Results have generally been poor. However, some transient responses have been obtained in secondary forms of auto-immune hemolytic anemias, particularly those associated with malignancy of the reticuloendothelial tissues.

As far as the lymph node is concerned, nodal irradiation seems to offer little promise in auto-immune hemolytic anemia but there are scattered reports by Wasserman, Brown, Eisner (Eisner, Ley and Mayer, 1967), and others of a beneficial effect on symptomatic hemolytic anemias from Hodgkin's disease to radiation of local tumor masses.

**Administration of Anti-Lymphocyte Serum**

Anti-lymphocyte serum has been shown to be of value in the treatment of the allograft rejection response but its primary effect seems to be in suppressing cellular immunity rather than humoral. There is some evidence that it may abolish immunologic memory and thus might be of value in auto-immune hemolytic anemia. We are unaware of any attempts to use the material in the clinical setting of auto-immune hemolytic anemia.

**Prognosis**

The outlook in auto-immune hemolytic anemia is unpredictable but a large number of the patients can be controlled by judicious therapy with corticosteroids. Despite the improvement in mortality rates since the advent of steroids (46 percent Dacie's series) the mortality rates are still high—31 percent Daussel and Columbani and 28.2 percent Allgood. If the disease cannot be controlled with low doses of steroid which produce minimal toxicity, splenectomy should be done. The time at which this should be performed will require individualization for each patient but, in general, a failure of corticosteroid therapy at acceptable levels of toxicity will require splenectomy at four to six months.

If splenectomy fails to halt the immune process or cannot be done because of the patient's poor condition, a trial of other immunosuppressive agents may be attempted or thymectomy may be resorted to. Information at present is not firm enough to recommend a specific line of approach. On the basis of the experimental data, drug therapy with either cyclophosphamide or azothiaprine would be my personal choice.

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**References**


Berenbaum MC, Brown IN: Dose-response relationships for agents inhibiting the immune response. Immunology 7: 65, 1964


Solar Retinopathy Following the Eclipse of March 7, 1970: A Follow-Up*

WALTER J. GEERAETS

Department of Ophthalmology, Medical College of Virginia, Richmond 23219.

In the last issue of this journal (Medical College of Virginia Quarterly 6: 3, 1970) Thomas W. Nooney, Joseph R. Svoboda, Florencio Ching and I reported two cases of binocular injury and one case of unilateral retinal burn of the fovea caused by watching the recent solar eclipse in the Richmond, Virginia area. Since reporting our local cases, a survey has been completed through other Virginia ophthalmologists of the occurrence of solar retinopathy throughout the state. Nine additional cases of ocular involvement were revealed in the survey and are reported here in table form. The cases are listed in order of probable severity.

Several persons who observed the eclipse without protection and reported to an ophthalmologist for fear of some eye damage showed no evidence of injury. The relatively low incidence of retinal burns following this event may be attributed to the efforts made by television, press and radio to inform the public of potential hazards and methods of eye-sight protection. Scientific research over several decades, aided by private organizations such as the National Society for the Prevention of Blindness and the Commission for the Visually Handicapped, has greatly increased understanding of and ability to predict eye damage by light.

* I am grateful to the many ophthalmologists throughout the State of Virginia who provided data for this report.
# Summary of Ocular Injuries Resulting from Watching the Recent Solar Eclipse

<table>
<thead>
<tr>
<th>Patients Age / Sex / Race</th>
<th>Location at Time of Eclipse</th>
<th>Approximate Time of Observation (PM)</th>
<th>Approximate Duration of Observation</th>
<th>Ocular Findings</th>
<th>Eye used</th>
<th>Visual Fields</th>
<th>Visual Acuity</th>
<th>Protection used</th>
<th>Other Findings</th>
<th>First Seen by Ophthalmologist</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 / F / W</td>
<td>Richmond</td>
<td>1:00</td>
<td>15 - 30 sec</td>
<td>OS foveal lesion with mild surrounding edema</td>
<td>OS</td>
<td>OS central acotoma (2⁰)</td>
<td>OS 20 / 200 (20 / 70 scan)</td>
<td>OD 20 / 20</td>
<td>OS none OD closed</td>
<td>chroma-topala</td>
</tr>
<tr>
<td>14 / F / W</td>
<td>Waynesboro</td>
<td>1:30 - 1:45</td>
<td>*</td>
<td>OS marked macular edema OD some edema</td>
<td>OU possibly</td>
<td>hazy vision (fields not done)</td>
<td>OS 20 / 100-1 OD 20 / 40</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>20 / M / *</td>
<td>Norfolk</td>
<td>*</td>
<td>3 - 4 times for 20 - 30 sec</td>
<td>macular edema cystic macular changes</td>
<td>OS</td>
<td>*</td>
<td>OS 20 / 80 -20 / 200</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>15 / M / W</td>
<td>Altavista</td>
<td>from start to finish</td>
<td>intermittent total about 1 / 2 hr</td>
<td>yellow burns OU macula</td>
<td>OD</td>
<td>OU central acotoma (relative)</td>
<td>OD 20 / 30 OS 20 / 40</td>
<td>none</td>
<td>chroma-topala (steroid therapy)</td>
<td>3 / 10 / 70</td>
</tr>
<tr>
<td>26 / F / W</td>
<td>Charlotte-stville</td>
<td>*</td>
<td>intermittent</td>
<td>OD foveal edema</td>
<td>OD</td>
<td>OD abacol. acotoma (3 - 4⁰)</td>
<td>OD 20 / 30 OS 20 / 20</td>
<td>looked through pinhole in cardboard</td>
<td>none</td>
<td>3 / 18 / 70</td>
</tr>
<tr>
<td>62 / M / W</td>
<td>Roanoke</td>
<td>1:38</td>
<td>4 - 5 sec</td>
<td>OS foveal lesion 1 / 4 - 1 / 2 disc diameter</td>
<td>OS</td>
<td>OS black spot (4 - 5⁰)</td>
<td>OS 20 / 25 OD 20 / 20</td>
<td>none</td>
<td>chroma-topala</td>
<td>3 / 13 / 70</td>
</tr>
<tr>
<td>24 / F / W</td>
<td>Danville</td>
<td>1:30</td>
<td>sufficient to get light burn of face</td>
<td>OS 'mottling' lesion below macula about 2x size of fovea</td>
<td>OS</td>
<td>hazy vision (fields not done)</td>
<td>OS 20 / 20 OD 20 / 15</td>
<td>none</td>
<td>none</td>
<td>3 / 12 / 70</td>
</tr>
<tr>
<td>22 / M / W</td>
<td>Richmond</td>
<td>1:30</td>
<td>several sec</td>
<td>maybe mild macular edema for one day</td>
<td>OU</td>
<td>hazy vision (no field defects)</td>
<td>OU 20 / 20-</td>
<td>none</td>
<td>(steroid therapy)</td>
<td>3 / 8 / 70</td>
</tr>
<tr>
<td>62 / M / W</td>
<td>Salem</td>
<td>Noon</td>
<td>3 sec</td>
<td>OS mild macular edema</td>
<td>OS</td>
<td>hazy vision</td>
<td>OU 20 / 20</td>
<td>none</td>
<td>none</td>
<td>4 / 3 / 70</td>
</tr>
<tr>
<td>28 / F / W</td>
<td>Marion</td>
<td>2:00</td>
<td>2 min</td>
<td>OU 'burning of eyes'</td>
<td>OU</td>
<td>fields not done</td>
<td>OU 20 / 20</td>
<td>x-ray film</td>
<td>none</td>
<td>*</td>
</tr>
<tr>
<td>9 / M / W</td>
<td>Arlington</td>
<td>1:30</td>
<td>several sec</td>
<td>none</td>
<td>*</td>
<td>fields not done</td>
<td>OU 20 / 20</td>
<td>used telescope with sun filter and 3 layers of exposed film</td>
<td>none</td>
<td>3 / 11 / 70</td>
</tr>
<tr>
<td>*/ M / N</td>
<td>Newport News</td>
<td>*</td>
<td>*</td>
<td>none</td>
<td>*</td>
<td>hazy vision (fields not recorded)</td>
<td>*</td>
<td>none</td>
<td>none</td>
<td>3 / 10 / 70</td>
</tr>
</tbody>
</table>

* Not Reported
Mechanism of Baroreceptor-Induced Changes in Heart Rate*

MARC D. THAMES (M-69)

The mechanism of mediation of baroreceptor-induced changes in heart rate is uncertain. According to the classical view reciprocal changes in parasympathetic and sympathetic efferent activity are involved; newer studies, however, suggest that increases in heart rate in response to systemic hypotension are mediated exclusively by increased sympathetic activity while decreases in heart rate in response to systemic hypertension are due solely to increases in parasympathetic activity. To resolve these differences, we studied dogs unanesthetized, anesthetized with chloralose and urethane, or with chloralose and morphine. Beta-adrenergic receptor blockade with propranolol reduced significantly but did not abolish the tachycardia in response to the hypotension induced by intravenous nitroglycerin. It also reduced significantly the bradycardia in response to hypertension produced by intravenous phenylephrine. Parasympathetic efferent blockade with atropine essentially abolished the tachycardia in response to nitroglycerin and the bradycardia in response to phenylephrine. In a second group of experiments, propranolol reduced but did not abolish the tachycardia in response to bilateral carotid arterial occlusion. In a third group of experiments, parasympathetic blockade with atropine reduced but did not abolish the bradycardia in response to bilateral electrical stimulation of the carotid sinus nerves. These results clearly show that baroreceptor-induced changes in heart rate are mediated by reciprocal alterations in cardiac parasympathetic and sympathetic efferent activity.

Preceptor: HERMES A. KONTOS, Division of Cardiovascular Disease, Medical College of Virginia.

Inhibition of Fibroplasia with Lung Implants in the Peritoneal Cavity of the Swiss White Mouse*

KENNETH D. YOUNER (M-71)

Well documented observation has shown that pneumococcal lobar pneumonia undergoes resolution without the fibroplasia that often follows inflammatory processes. As noticed by Dumont, neutrophiles invade the lung alveoli, however, the usual lymphocyte infiltration does not follow. Using pulmonary homografts in dogs, Barnes et al, have shown that intralveolar edema, inflammation and neutrophilic infiltration occur. However, lymphocytes either do not appear, or do so only in the peripheral vessels. An attempt was made to compare the ability of lung tissue to inhibit fibroplasia using tissue implants into the peritoneal cavity of a Swiss white mouse. The lung of a donor mouse and the kidney of the same mouse were used as the experimental-comparison tissues. Three variables were considered. First the tissue types were chosen due to close anatomical and histological similarity. Each organ has a parenchyma with a basement membrane type structure with a connective tissue capsule surrounding the entire organ. The presence of the capsule, and the possible role it may play in stopping a lymphocyte infiltration was the second factor. Each mouse received the entire left kidney and the entire left lung, each with an intact capsule. Half of the other lung and half of the other kidney was also implanted into the same mouse. The remaining half of each tissue was used as a normal histological comparison. The third factor is time. Barnes noted a maximum tissue response at 5–6 days. Therefore, I used two series. Series of 6 and 12 days were used. The second series was used to see if any effect the lung may have on fibroplasia is time dependent. The whole lung implant gained more weight (used to


* Supported in part by an A. D. Williams Fellowship.
reflect the amount of fibrosis) than the whole kidney. However, once the capsule is opened the half kidney gains more weight than the whole lung. With the 12 day series whatever effect the lung parenchyma had on fibrosis is now gone, for the whole lung and the half lung both gained more weight than the whole or half kidney. These weight findings were corroborated by histological sections comparing the amount of fibrosis in the implanted tissues.

Preceptor: William Regelson, Department of Medical Oncology, Medical College of Virginia.

Roentgen Evaluation of the Hepatic Arterial Bed

Parham R. Fox (M-71)

Angiographic alterations sustained by the hepatic arterial bed in diffuse parenchymal disorders such as cirrhosis, and congestion, have heretofore not been critically evaluated. Tortuosity of intrahepatic arteries, a readily detectable and often striking angiographic feature, has generally been considered as indicative, or even specific for cirrhosis. The influence of aging, liver size, and other factors, have not been objectively considered. We have attempted to correlate the gross and histologic appearance of thirty livers at post mortem with the roentgen appearance of their barium injected hepatic arteries; this report constitutes our preliminary results. Diagnostic criteria of in vivo hepatic arteriography will be subject to correlative evaluation with the data provided by these post mortem studies. Liver weight, gross description, color photographs, and microscopic sections were obtained for pathologic correlation. Pertinent clinical details were culled from the patients' chart. High resolution radiographs were randomized and shown to a panel of three radiologists who assessed and categorized each into the following groups:

1. Normal ............... 14 cases
2. Tortuosity ............. 10 cases
   a. Mild
   b. Moderate
   c. Severe
3. Hypo- and hypervascularity; curvilinear stretching .... 6 cases

Of the 10 cases radiographically demonstrating hepatic arterial tortuosity, none showed gross or histologic evidence of cirrhosis. Of the cases with histologically proven cirrhosis the radiographic arterial pattern was assessed "Normal" in all. It is clear from our data that the specificity of arterial tortuosity as a reflection of cirrhosis, a concept generally accepted, does not withstand objective appraisal. In view of this significant (although negative) observation we are encouraged to continue efforts in seeking a statistically significant correlation with this angiographic finding.

Preceptors: Melvin Vinik, Department of Radiology; I. Nakoneczna, Department of Pathology, Medical College of Virginia.

Effect of Gravity on the Distribution of Blood in the Dog Lung

David H. Bristow (M-71)
Frank Martorano (M-71)
Battina Groome

The distribution of blood was measured in nineteen anesthetized dogs. Ten were placed vertical, head up, and nine were placed head down. The dogs were in the vertical position for at least an hour and a half, then blood samples were drawn, the heart was fibrillated, the thorax entered, the major vessels of the heart and lung were clamped, the heart and lung removed together and frozen in liquid nitrogen. Cubes were cut from the lungs at various levels from apex to base and were measured on each edge, weighed, and ground in a colloid mill. Blood pigments were extracted from the ground cubes and measured to find blood volumes in each cube. The total volume of each cube was known and tissue and gas volumes were calculated with algebraic equations. In the head up dogs, per cent of blood was three times as great in the base as in the apex and tissue increase 1.5 times from the apex to the base. In the head down dogs, there was no gradient from apex to base of blood or tissue. One implication of these results is anatomic difference between the apex and base of the lung.

Preceptor: John L. Patterson, Jr., Division of Cardiopulmonary Laboratories and Research, Medical College of Virginia.
A Study in CPK Iso-Enzymes

JOHN ELWOOD OWENS (M-71)

Heart muscle has three CPK iso-enzymes; CPK$_1$ in smallest amount and nearest the anode on electrophoresis; CPK$_2$ in medium amount and in the middle of electrophoretic pattern; and CPK$_3$ in greatest amount at the cathode end. The four chambers of the heart and the septum of the heart are apparently possessive of slightly different levels of concentration of CPK enzyme, a fact which, upon CPK iso-enzyme differential analyses of the four chambers and septum of heart, may be resolved by proving that the fluctuating total level of CPK in a particular area is a function of a certain specific iso-enzyme of CPK. CPK$_2$ and CPK$_3$ appear to be the enzymes that fluctuate not only among the extracts of cardiac muscle from these five areas of the heart but also among the extracts of cardiac muscle of different pathological states. With respect to tissue from the same heart, there appears to be a decreasing level of CPK$_3$ in going from RA to RV to LA to LV to septum. This is particularly true in cases where death had heart involvement. This pattern does not hold true at present in cases where death had no heart involvement. Results are apparently well-reproducible not only in different electrophoretic chambers but also with long intervals of time intervening (i.e., if tissue and/or extracts are frozen). The prospects of this study include the possibility of diagnosing area of heart involved in myocardial infarction by serum analysis of altered CPK iso-enzyme levels.

Preceptor: FRANKLIN LIM, Division of Clinical Pathology, Medical College of Virginia.

Drug Usage in a Medical Ward

JAMES B. BLITCH (M-70)
JEFFREY BIENER (M-71)

Drugs are an essential part of medical therapy and their use has increased rapidly in recent years. Their benefits are obvious. Nevertheless, the physician must be ever aware of their hazards. A six week drug study under the direction of A. J. Wasserman, M. D. was conducted in order to assess the use of drugs in a general medical ward. Drug sheets containing information about dosage, route, instructions, indications, discontinuation and side effects were kept for every drug order for each patient during the entire six weeks. Data was collected by two medical students who accompanied the house staff on daily rounds and questioned each doctor about his patients' medications. This study included 85 patients with a total of 884 drug sheets. There was a mean of 10.5 ± 8.2 S.D. drug sheets per patient with a range of 1 to 47, and a mean of 6.3 ± 3.7 S.D. drugs per patient with a range of 1 to 22. 13% (11 out of 85) of the patients had side effects (this result is similar to that obtained in other drug studies on side effects). There was no statistically significant correlation between side effects and age, sex, number of drugs per patient or number of days in the hospital. However, a correlation between side effects and number of days in the hospital may have existed although it was not statistically significant in this study. In addition, only 5% of the drugs were discontinued because they were effective and no longer necessary. 15% of the drugs were continued at time of discharge, and 8% were discontinued at time of discharge. These figures cast doubt on the efficacy of drugs being employed. Concerning route of administration, 52% were p.o., 17% I.M., 5% S.C. and 20% I.V. 20% I.V. is a surprisingly high figure. Tabulation of instructions for drug usage revealed 48% standard orders, 10% p.r.n, and 40% stat. That 40% of the orders were stat is again a surprising figure.

Preceptor: ALBERT J. WASSERMAN, Division of Clinical Pharmacology, Medical College of Virginia.

Acquired Absence of Alpha Lipoproteins and Acanthocytosis in Severely Burned Patients*

MARVIN ZELKOWITZ (M-70)

Severely burned patients develop decreased to absent alpha lipoprotein (LP) and concomitant "burr shaped" RBC. In serial observations of all hospitalized burn patients, only one of 13 having more than 30% body burn did not develop decreased alpha LP during the first to the tenth week after the burn, the onset depending on the severity of the burn. The beta LP and other lipid measurements tended to be low, although two patients had increased beta LP. Acanthocytic RBC (30–90%) were found in each patient with absent alpha LP, and the findings were temporally related. Patients with less than 30% total body burn did not develop either abnormality. Neither septicemia, hepatic failure, or renal failure could be incriminated. Preliminary studies of the lipid content of RBC membranes from acanthocytic cells suggest that the phospholipid content is less than that of normal RBC, but the cholesterol content is unchanged, as is the distribution of RBC phosphatides. We suggest the following sequence: alpha LP weep through the damaged skin in amounts exceeding synthetic capacity; the RBC phosphatides which are in equilibrium with the plasma phosphatides are leached from the cells.

* Acquired Absence of Alpha Lipoproteins and Acanthocytosis in Severely Burned Patients.
altering membrane lipids and membrane structure with a resultant change in shape. Thus, an alphasialipoproteinemia developing after a severe burn can be associated with burr-cell formation, perhaps because of membrane lipid changes. These findings contrast with the absence of morphologic RBC changes in hereditary an-alphasialipoproteinemia (Tangier Disease) and provide another facet to the association between LP and acanthocytosis.

Preceptor: WILLIAM R. HARLAN, JR., Clinical Research Center, Medical College of Virginia.


Proteinuria and Glomerular Lesions in Rats Induced by Sera from Human Renal Transplant Recipients

R. C. SMALLRIDGE (M-70)
D. B. WALDMAN (M-72)

Glomerulonephritis appears to be due to a variety of factors, one of which may be circulating antibodies to glomerular basement membrane. Since some kidney transplant recipients develop massive proteinuria, a model was established to see if kidney lesions could be produced in experimental animals by inoculations of serum from patients with recurrent proteinuria. Sera (5-8 cc.) taken after transplant rejection from 5/7 transplants produced no proteinuria or histologic changes in the kidneys of unilaterally nephrectomized rats. One patient's serum produced massive proteinuria of 36.6 mg./24 hrs. and 127 mg./24 hrs. in two rats. (control rats averaged 8 mg./24 hrs.) No significant changes were observed on microscopic examination. The serum and acid eluate from the kidney of another patient produced glomerular lesions with obliteration of Bowman's space, thickening of the glomerular vasculature, increased cellularity, and the deposition of PAS positive material in the region of the glomerular basement membrane. We conclude that transferable serum factors present in some patients with glomerulonephritis are even pathogenic in rats. Thus far, we have seen two different effects: (1) massive proteinuria, and (2) obliteration of glomeruli without significant proteinuria. It is possible that this type of bioassay will help in determining which patients have a high risk of recurrent glomerulonephritis following transplantation, and, more important, that it will distinguish types of glomerulonephritis.

Preceptor: G. MELVILLE WILLIAMS, Department of Surgery, Medical College of Virginia.
Contributors

Ali A. Hossaini (Transfusion Problems in Hemolytic Anemias) is associate professor of clinical pathology and director of the blood bank at the Medical College of Virginia. He completed his undergraduate studies at the American University of Beirut, Lebanon, received his M.A. and M.S. degrees from Texas Christian University, and earned his Ph.D. at Ohio State University. Before coming to MCV, Dr. Hossaini was on the faculty of the department of pathology at West Virginia Medical Center in Morgantown.

Harold M. Maurer (New Concepts in Management of Neonatal Jaundice: Use of Enzyme Induction and Phototherapy) is an assistant professor in the department of pediatrics at the Medical College of Virginia. Before coming to MCV, he was chief of pediatrics at the U.S. Public Health Service Hospital in Norfolk, Virginia, and then on the attending staff of Babies Hospital and Vanderbilt Clinic, Columbia-Presbyterian Medical Center. Dr. Maurer received his M.D. from the State University of New York, interned at Kings County Hospital in Brooklyn, and trained in pediatrics at Babies Hospital.

Daniel N. Mohler (Glucose-6-Phosphate Dehydrogenase Deficiency) is associate dean and professor of internal medicine at the University of Virginia School of Medicine. He attended the University of Kentucky and Ohio State University, then received his medical degree from the University of Virginia. After internship at Massachusetts Memorial Hospitals and residency at U. Va., Dr. Mohler had a research fellowship in hematology at the Washington University School of Medicine, then returned to U. Va. where he has remained on the full-time staff in medicine.

John H. Moon (Immunosuppression in Autoimmune Hemolytic Anemia) is an associate professor of medicine in the department of medicine at the Medical College of Virginia. He attended the University of Richmond, then the Medical College of Virginia where he received his medical degree. Dr. Moon interned at Methodist Hospital, Indianapolis, and returned to do his residency at MCV, where he has remained in the field of hematology.
Robert I. Weed (*New Thoughts on Hereditary Spherocytosis*) is a professor of medicine and of radiation biology and biophysics at the University of Rochester School of Medicine and Dentistry; he is also head of the hematology unit. Dr. Weed received his B.S. and M.D. from Yale University, interned at Strong Memorial Hospital, and was an assistant resident in medicine at the Yale-New Haven Medical Center. He held fellowships in hematology with the American Cancer Society and the U.S. Public Health Service. Dr. Weed is also an associate editor of *Blood*.

Wendell F. Rosse (*Paroxysmal Nocturnal Hemoglobinuria—New Thoughts*) is associate professor of medicine and of immunology at Duke University. A graduate of the University of Omaha, he received his M.S. in physiology from the University of Nebraska and his M.D. from the University of Chicago. After internship and residency at Duke, Dr. Rosse was a clinical associate at the National Cancer Institute, a fellow in hematology at the Postgraduate Medical School, London, and a senior investigator at the National Cancer Institute. Dr. Rosse is recipient of a Public Health Service Research Career Development Award.

M. Robert Cooper (*The New Hemoglobinopathies*) received his M.D. degree from the Bowman-Gray School of Medicine of Wake Forest University. After his internship at the University of Virginia Hospital, Dr. Cooper returned to Bowman-Gray where he completed his residency in medicine, was a fellow in clinical oncology, and is now assistant professor of medicine (hematology and oncology). His primary research interests are in white cell metabolism, platelet survival and sequestration, and viscosity changes in hemoglobinopathies.

Walter J. Geeraets (*Solar Retinopathy Following the Eclipse of March 7, 1970: A Follow Up*), professor of ophthalmology and associate professor of biophysics at the Medical College of Virginia, was born in M. Gladbach, Germany. He obtained a doctor's degree in medicine from the University of Bonn, later serving as a research fellow at the Radiation Institute of that university and as the chief assistant of the surgical clinics at Bochum, Germany. In 1957 he came to MCV with appointments in the departments of ophthalmology and biophysics. Dr. Geeraets is currently director of ophthalmic research and coordinator of the NIH ophthalmic resident training program at MCV.
A new service from A. H. Robins

Late in 1969, the A. H. Robins Company, because of their special interest in gastrointestinal medicine, began distributing to Residents and Interns a series of brochures entitled G.I. SERIES. This publication was designed to provide a quick, yet comprehensive review of basic procedures and practices in G.I. medicine— with particular emphasis on the physical examination as performed in the office or at bedside. Designed primarily for the student physician, the contents have been arranged and programmed for quick reading, rapid comprehension and easy review.

An excerpt from the first of the six-part series on physical examination of the abdomen is shown at right. If you have teaching responsibilities, limited quantities of the already published sections of the series are still available on a “first come, first served” basis. Presently available are Part 1—Inspection, Part 2—Palpation and Part 3—Percussion. Simply write to: The Medical Department, A. H. Robins Company, 1407 Cummings Drive, Richmond, Virginia 23220

the spasm reactors in your practice deserve "the Donnatal® effect"

<table>
<thead>
<tr>
<th></th>
<th>each tablet, capsule or 5 cc. teaspoonful of Elixir (23% alcohol)</th>
<th>each Donnatal No. 2</th>
<th>each Extentab®</th>
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</thead>
<tbody>
<tr>
<td>hyoscyamine sulfate</td>
<td>0.1037 mg.</td>
<td>0.1037 mg.</td>
<td>0.3111 mg.</td>
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<td>phenobarbital (¼ gr.) 16.2 mg.</td>
<td>(½ gr.) 32.4 mg.</td>
<td>(¼ gr.) 48.6 mg.</td>
<td></td>
</tr>
</tbody>
</table>
Excerpt from Part 1, *Inspection*

**Abdominal profiles**

Careful inspection of the profile of the abdomen from the side may give the first clue of abnormality, directing attention to a specific region and prompting a search for further signs. While distinction begins with visual inspection, palpation, percussion and auscultation are required to make an accurate differential diagnosis.

---

**The stress-spasm syndrome and the “Donnatal Effect.”**

Donnatal provides a predictable, inexpensive way to help the patient who responds to stress situations with gastrointestinal or other smooth-muscle spasm. The characteristic over-all effect of Donnatal achieves two essential objectives: calming the patient, pacifying the gut. Outstanding in effectiveness, safety, economy, uniformity of composition and dosage convenience, Donnatal continues to be prescribed by more physicians than any other agent in its class.

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Particularly useful when anxiety and tension accompany, aggravate or account for smooth muscle spasm, Donnatal is indicated for the symptomatic relief of recurring, persistent or chronic visceral spasm, as in gastritis, pylorospasm, esophageal spasm, irritable stomach and colon, nervous indigestion, duodenal or gastric ulcer. Donnatal is also indicated in dysmenorrhea, nausea, motion sickness and nocturnal enuresis. Since Donnatal helps reduce gastric hypersecretion, it is useful in hyperchlorhydria, peptic ulcer and other conditions mentioned above.

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**Brief summary:** Blurring of vision, dry mouth, difficult urination, and flushing or dryness of the skin may occur on higher dosage levels, rarely on usual dosage. Administer with caution to patients with incipient glaucoma or urinary bladder neck obstruction. Contraindicated in acute glaucoma, advanced renal or hepatic disease or a hypersensitivity to any of the ingredients.
Whenever anxiety induces or intensifies clinical symptoms

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(chlordiazepoxide HCl)

Quickly relieves anxiety—Helps improve response in psychophysiologic disorders—Seldom impairs mental acuity or physical coordination, on proper dosage—Has wide margin of safety

Before prescribing, please consult complete product information, a summary of which follows:

Indications: Indicated when anxiety, tension and apprehension are significant components of the clinical profile.

Contraindications: Patients with known hypersensitivity to the drug.

Warnings: Caution patients about possible combined effects with alcohol and other CNS depressants. As with all CNS-acting drugs, caution patients against hazardous occupations requiring complete mental alertness (e.g., operating machinery, driving). Though physical and psychological dependence have rarely been reported on recommended doses, use caution in administering to addiction-prone individuals or those who might increase dosage; withdrawal symptoms (including convulsions), following discontinuation of the drug and similar to those seen with barbiturates, have been reported. Use of any drug in pregnancy, lactation, or in women of childbearing age requires that its potential benefits be weighed against its possible hazards.

Precautions: In the elderly and debilitated, and in children over six, limit to smallest effective dosage (initially 10 mg or less per day) to preclude ataxia or oversedation, increasing gradually as needed and tolerated. Not recommended in children under six. Though generally not recommended, if combination therapy with other psychotropics seems indicated, carefully consider individual pharmacologic effects, particularly in use of potentiating drugs such as MAO inhibitors and phenothiazines. Observe usual precautions in presence of impaired renal or hepatic function. Paradoxical reactions (e.g., excitement, stimulation and acute rage) have been reported in psychiatric patients and hyperactive aggressive children. Employ usual precautions in treatment of anxiety states with evidence of impending depression; suicidal tendencies may be present and protective measures necessary. Variable effects on blood coagulation have been reported very rarely in patients receiving the drug and oral anticoagulants; causal relationship has not been established clinically.

Adverse Reactions: Drowsiness, ataxia and confusion may occur, especially in the elderly and debilitated. These are reversible in most instances by proper dosage adjustment, but are also occasionally observed at the lower dosage ranges. In a few instances syncope has been reported. Also encountered are isolated instances of skin eruptions, edema, minor menstrual irregularities, nausea and constipation, extrapyramidal symptoms, increased and decreased libido—all infrequent and generally controlled with dosage reduction; changes in EEG patterns (low-voltage fast activity) may appear during and after treatment; blood dyscrasias (including agranulocytosis), jaundice and hepatic dysfunction have been reported occasionally, making periodic blood counts and liver function tests advisable during protracted therapy.

Usual Daily Dosage: Individualize for maximum beneficial effects. Oral—Adults: Mild and moderate anxiety and tension, 5 or 10 mg t.i.d. or q.i.d.; severe states, 20 or 25 mg t.i.d. or q.i.d. Geriatric patients: 5 mg b.i.d. to q.i.d. (See Precautions.)

Supplied: Librium® (chlordiazepoxide HCl) Capsules, 5 mg, 10 mg and 25 mg—bottles of 50. Libritabs® (chlordiazepoxide) Tablets, 5 mg, 10 mg and 25 mg—bottles of 100. With respect to clinical activity, capsules and tablets are indistinguishable.

Also available: Libritabs® (chlordiazepoxide) 5-mg, 10-mg, 25-mg tablets

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Also available: Libritabs® (chlordiazepoxide) 5-mg, 10-mg, 25-mg tablets