Studies on Erythrocytes in Cases with Past History of Favism and Drug-Induced Acute Hemolytic Anemia

By A. Szeinberg, C. Sheba, Nina Hirshorn and Eva Bodonyi

Most acquired hemolytic anemias (with the exception of acquired idiopathic hemolytic anemia, incompatible blood transfusions and Rh sensitization) and all cases of thalassemia show in this country a well defined ethnographic distribution. Only Jews originating from oriental and Mediterranean countries who have a darker complexion and are called Sephardic Jews in contrast to the light complexioned Ashkenazic Jews from eastern, central and western Europe are victims of such hemolytic occurrences. Gross blood destruction is encountered in some individuals or families of this population group after ingestion of fava beans or various drugs (e.g., sulpha drugs, plasmoquine, para-aminosalicylic acid) as well as following some infectious diseases (e.g., typhoid).

Such distribution of susceptibility suggested that some common constitutional factor must be connected with the pathogenesis of these disorders and that such a factor could give rise to hemolysis only after exposure to an external agent (for instance sulpha drugs or fava beans) no matter what the age of the victim be at the time of such exposure.

To illustrate this, a case of favism in a 74 year old Persian Jew is reported in brief. This was his first hemolytic crisis and followed the ingestion of fava beans. In his country and district of origin Vicia faba was apparently not grown and as he arrived to this country only three years ago, exposure could have taken place in the suitable season (spring) only since then. He developed an acute hemolytic episode and on admission showed less than one million RBCs. He recovered after blood transfusions.

Another case illustrating the same is that of a 57 year old woman, who immigrated from Iraq; there she never ate fava beans. In this country, during the first (or the second) spring season she noticed extreme fatigue and red tinged urine, which she interpreted as menstrual flow. When in the following spring she came down with severe hemolysis and very red colored urine she again blamed the Holy Land for its known miraculous effect on old women (allusion to Sarah, Wife of Abraham) and it was our task to dispel her misinterpretation of what was actually hemoglobinuria after ingestion of Vicia faba.

It was known already in Roman times that among the Mediterraneans certain families show a tendency to hemolysis after ingestion of fava beans, as the well-known sentence “Pythagorei faba se abstinerunt” possibly implies.

To illustrate this familial occurrence we mention from our material a Yemenite family in which one brother suffered both from favism and from hemoglobinuria after sulphapyridine, a second from favism and a third brother from hemoglobinuria...
binuria after ingestion of some unknown drug. The case of one of the brothers in which some hemolysis developed following both beans and sulphapyridine suggested to us the presence of a “hemolytic constitution.” This assumption seemed to us to be supported by the racial incidence of R. Lederer’s “Baghdad Spring Anemia”, a hemolytic anemia (probably favism) he observed in Baghdad, and almost limited to the Jewish population and not found in the Moslem majority. The Mediterranean origin of the Babylonian Jews, which apparently, judging from the hematologists’ point of view, has been maintained all through 2000 years of exile, is certainly of great interest.

In our endeavor to investigate this particular “hemolytic constitution” of the “non-Ashkenazic Jews” we made use of such well established methods as were introduced for the study of the hemoglobinopathies associated with sickle-cell disease, thalassemia and similar disorders. Although we could confirm that all our cases of thalassemia major (also occurring only among non-Ashkenazic Jews) showed the presence of a significant content of fetal hemoglobin, we failed to prove its pathognomonic presence in any of the cases forming the material for the present publication.

The investigations of Dern, Beutler and associates, who proved the existence of erythrocytes abnormally sensitive to primaquine and a number of other aniline derivatives in about 10 per cent of the American Negroes served as the starting point for our own studies reported in this paper.

In the following we are bringing our investigations on patients who suffered in the past from acute hemolytic anemias, all of them non-Ashkenazic Jews and on representative samples of both Ashkenazic and non-Ashkenazic control cases.

**Material**

The subjects of this investigation were divided into 4 groups:

1. Persons with past history of favism or acute hemolytic anemia due to sulpha drugs or PAS. They were investigated during 6-24 months following the recovery from hemolytic crisis. (This group will be designated throughout this report as “test cases”).

---

* By inference one would have to assume that the Ashkenazic Jews have been less successful in maintaining their racial purity and have lost through the generations their “hemolytic constitution.” It is left to be seen what will be the pattern of Cooley’s anemia in the U. S. A. among Americans of non-Mediterranean origin intermarrying with Mediterranean.

Central and Western Europe have learnt about favism from Mediterranean countries and though America was first to draw attention to Cooley’s anemia, it was stated to occur only among Italians, Greeks and Mediterranean Moslems who live in the U. S. A.

Eduard Moericke sang about favism in 1837, nearly 70 years before Fermi coined the name FAVISM. This song goes as follows:

_Vicia faba minor (1837)_

Fort mit diesem Geruch dem zauberhaften!  
Er mahnt mich an die Haare die mir einst alle Sinne bestrickt.  
Weg mit dieser Blüte der schwarzen und weissen!  
Sie sagt mir, dass die Verfuhrerin ach! schwer mit dem Tode gesehen!  

(For the discovery of this song I am (C. S.) grateful to Dr. Paul Nathan of the Beilinson Hospital).
TABLE 1.—Composition of the Investigation Material

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Time after Recovery</th>
<th>No. of Cases</th>
<th>Country of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Test cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemolysis due to sulphamides, drugs</td>
<td>6-24 months</td>
<td>10</td>
<td>Iraq</td>
</tr>
<tr>
<td>(Relatives of above)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemolysis due to PAS</td>
<td>6 months</td>
<td>1</td>
<td>Yemen</td>
</tr>
<tr>
<td>Favism</td>
<td>12-24 months</td>
<td>10</td>
<td>Israel, Yemen, Iraq, Persia</td>
</tr>
<tr>
<td>(Relatives of above)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Control groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy blood donors and hospital patients</td>
<td></td>
<td></td>
<td>Europe, Orient and Mediterranean</td>
</tr>
</tbody>
</table>

2. Relatives of the above persons.
3. Control group of Sephardic Jews (healthy blood donors or hospital patients with no history of hemolytic reactions).
4. Control group of Ashkenazic Jews (selected as above).

The detailed composition of the various groups is summarized in Table 1.

METHODS

1. Fragility studies. Osmotic fragility was tested by the method described by Wintroub; mechanical fragility by the methods of Crosby.11 Susceptibility to hemolysis by Viola faba was investigated with an extract prepared as follows:

Mince green fava beans (including sheaths) were suspended in 10 volumes of buffered (pH 7.2) saline and shaken mechanically for one hour. The suspension was left overnight in refrigerator and then shaken again for one hour. The supernatant fluid was decanted and filtered several times till a clear fluid was obtained. Twofold dilutions of the extract were prepared in saline, normal sera and sera of the “test cases.” Suspensions of washed erythrocytes from control and test cases were added to the diluted extracts and incubated at 4°C, room temperature and 37°C for 2 hours. The results were read after slight centrifugation. The susceptibility to hemolysis by sulphadiazine was tested by a similar method in saline and sera (control and test) containing 5-20 mg.% sulphadiazine.

The erythrocytes of the case which suffered from hemolytic anemia following PAS administration were similarly tested in saline and serum (normal and own) solutions of 0.5-10% PAS.

2. Fetal hemoglobin. Alkali resistant fetal hemoglobin was estimated by the method of Singer and Chernoff.12

3. Paper electrophoresis of hemoglobin. Blood hemolysates were prepared as for fetal hemoglobin. Paper electrophoresis investigations were carried out with a Kellab A.B. (Stockholm) apparatus, model 5k51 at 200V, 0.7 milliamperes per strip for 5 hours. The mobility in the following buffers was investigated: Barbiturate buffer, Ionic strength 0.05 and 0.1, pH 7.0; 7.25; 7.6; 8.0; 9.0; 9.8.

Phosphate buffer; Ionic strength 0.1, pH 7.3; 7.5; 8.0; 8.6; 9.18.

Acetate buffer: Ionic strength 0.1, pH 4.0, 5.0, 5.5.

4. Amino acid content of hemoglobin hydrolysates was studied qualitatively by paper chromatography.

Hemoglobin hemolysate was prepared as for fetal hemoglobin. An aliquot containing exactly 0.01 Gm. hemoglobin was added to 10 cc. of 8X sulphuric acid and autoclaved at 120°C for 12 hours. Each aliquot was neutralized with hot saturated solution of barium hydroxide using phenolphthalein as indicator till the appearance of red color and then dilute sulphuric acid was added just to the discoloration point. Subsequently the material was centrifuged and filtered through glass wool. The barium sulphate precipitate was well
washed with hot distilled water and filtered again. The combined filtrates were evaporated under reduced pressure in a glycerine bath (120 C.) almost to dryness. The residue was taken up in a 10% isopropanol-water solution, transferred into a graduated test tube and concentrated on a water bath to exactly 1 cc. volume. An aliquot of 0.01 cc. was used for paper chromatography.

As by this method of preparation tryptophane is decomposed, another sample of the hemoglobin solution was hydrolysed by barium hydroxide.13

Two dimensional paper chromatography of the hydrolysates was carried out by the ascending method on Whatman No. 1 paper. The following pair of solvents was used:

1. Isopropanol (3 vol.): 0.5% urea solution in 1% sodium chloride (1 vol.).
2. Phenol (4 vol.): Water (1 vol.) with 0.1% 8 Hydroxyquinoline.

As in this system tyrosine appeared together with alanine, an additional sample was chromatographed one dimensionally in butanol:acetic acid:water (4:1:5), where tyrosine was well separated from the other amino acids and was easy to identify it. Examinations for cystine and methionine were also carried out after oxidation of the samples by hydrogen peroxide and ammonium molybdate.14

The detection of the amino acids on the chromatograms was carried out with ninhydrine reagent at 110 C. Proline was also identified with an isatin reagent.

5. Glycolytic Activity of RBC: The method described by Hollingsworth21 for measurement of aerobic glycolytic activity was employed. No cases with reticuloocytes count above 2% were included in our control groups.

6. Catalase Activity: Catalase was measured by the iodometric titration method as described by Allison.22 Erythrocytes separated from heparinized blood and washed with saline were used for the examination. As the enzyme is quickly inactivated in very dilute solution,23 the dilution of blood to the required concentration (0.4% hemoglobin) was made just before the test. No cases with reticuloocytes count over 2% were examined.

7. Glutathione (GSH) has been estimated by the method of Gruener and Philips,29 as modified by Beutler and assoc.9 The results were calculated as mg % glutathione (GSH) in 100 cc. packed red cells.

Results

No differences in the osmotic and mechanical fragility between the test cases and the 2 control groups could be shown. No hemolytic action of the Vicia faba extract or the drugs was demonstrated. No raised content of fetal hemoglobin was found and no electrophoretically abnormal hemoglobin was detected.

The analysis of the amino acids content of hemoglobin hydrolysates gave identical results for the test and control groups. 15 amino acids were identified, (aspartic, glutamic, lysine, glycine, serine, arginine, histidine, threonine, alanine, valine, phenylalanine, leucine, proline, tyrosine, and tryptophane) and the spots on the chromatograms were approximately of the same strength in both groups. Two amino acids, namely, cystine and methionine found by various investigators in human hemoglobin15-18 were not detected by us and were probably lost during the preparation.

Glycolytic activity of RBC. It has been shown that the intrinsic defect in congenital spherocytosis is connected with abnormalities of carbohydrate metabolism of the erythrocytes.19,20 Studies of normal controls and cases of various blood disorders have shown also that young erythrocytes show increased glycolytic activity. Strikingly high activity (unproportional to the percentage of reticuloocytes) have been found in homogenous hemoglobin C disease.21 We investigated whether some abnormality in glycolytic activity was connected with abnormal sensitivity of RBC in our “test cases.”

The results are presented in figure 1 and are summarized in table 2.
Fig. 1. Glycolytic activity of R.B.C. (Rate of glycolysis expressed in mg. glucose utilized by 100 cc. RBC in 1 hour) Group A. Ashkenazic controls. Group B. Sephardic controls. Group C. Test cases.

Fig. 2. Catalase activity of RBC. (Results expressed as velocity constant (min⁻¹) per 1 cc. of 14.8 Gm. hemoglobin. Group A. Ashkenazic controls. Group B. Sephardic controls. Group C. Test cases.

<table>
<thead>
<tr>
<th>Table 2. Glycolytic Activity of RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>A. Ashkenazic controls</td>
</tr>
<tr>
<td>B. Sephardic controls</td>
</tr>
<tr>
<td>C. Test cases</td>
</tr>
</tbody>
</table>

* Results are expressed as mg. 18 glucose utilized during 1 hour by 100 cc. packed red blood cells.

<table>
<thead>
<tr>
<th>Table 3. Catalase Activity of RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>A. Ashkenazic controls</td>
</tr>
<tr>
<td>B. Sephardic controls</td>
</tr>
<tr>
<td>C. Test cases</td>
</tr>
</tbody>
</table>

* Results are expressed as velocity constant (min⁻¹) per 1 cc. of 14.8 Gm. hemoglobin.

No significant differences were found in the glycolytic activity of the "test group" and the 2 control groups.

Catalase activity. It has been suggested by Ponder that catalase slows down the rate of formation of methemoglobin and choleglobin in erythrocytes, while
sulfonamides accelerate their formation. Thus, low catalase activity of RBC could lead to an increased choleglobin accumulation and destruction of the deficient cells. With this possibility in view we examined the catalase in our control and “test cases” groups. The results are presented in figure 2 and summarized in table 3. No significant differences were found in the catalase activity of the erythrocytes of the “test cases” and the control groups.

Non-Protein Sulfhydryl Compounds—Glutathione (GSH)

Glutathione (GSH), which comprises the major component of the nonprotein sulfhydryl compounds of the erythrocytes, has a widespread biological importance. It is necessary for cell division, shows protective action against certain poisons and x-ray irradiation and participates in several enzymatic reactions.25-27 Beutler and assc. have shown that sensitivity to primaquine and some other aniline derivatives encountered among American Negroes is associated with low glutathione content of erythrocytes.9 Our findings are presented in figure 3 and table 4. The results in the “test cases” group present an average of 2-3 examinations performed at an interval of 1 month at least. No cases with hemolytic disorders, fever, liver impairments or psychotic patients were included in the control group.26

We found that all cases with past history of favism or hemolytic anemia due

![Fig. 3](image1)

**Fig. 3.** Glutathione (GSH) content of blood (mg./100 cc. RBC) Group A. Ashkenazie controls. Group B. Sephardic controls. Group C. Test cases. Group D. Relatives of “test cases.”

![Fig. 4](image2)

**Fig. 4.** RBC glutathione (GSH) concentration in 3 families (figures represent glutathione per 100 cc. RBC). (1) past history of hemolysis due to sulpha drugs. (2) past history of favism. (3) past history of hemolysis due to an unknown drug. (4) past history of favism and hemolysis due to sulphapyridine.
A. Szeinberg, C. Sheba, N. Hirshorn and E. Bodonyi

TABLE 4.—Glutathione (GSH) Content of Erythrocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Cases</th>
<th>Range</th>
<th>Mean</th>
<th>Stand. Deviat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Ashkenazic controls</td>
<td>111</td>
<td>47-111</td>
<td>77</td>
<td>13.5</td>
</tr>
<tr>
<td>B. Sephardic controls</td>
<td>111</td>
<td>32-110</td>
<td>77</td>
<td>15.2</td>
</tr>
<tr>
<td>C. Test cases</td>
<td>21</td>
<td>35-56</td>
<td>47</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* The results are calculated as glutathione (GSH) concentration per 100 cc. packed erythrocytes.

TABLE 5.—Blood Concentrations of Glutathione (GSH) in 7 Families, (mg. % glutathione per 100 cc. RBC)

(1) A case with past history of hemolysis due to sulpha drugs. (2) A case with past history of favism.

<table>
<thead>
<tr>
<th>First Generation</th>
<th>Second Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Family 3</td>
<td>52</td>
</tr>
<tr>
<td>Family 4</td>
<td>63</td>
</tr>
<tr>
<td>Family 5</td>
<td>?</td>
</tr>
<tr>
<td>Family 6</td>
<td>49</td>
</tr>
<tr>
<td>Family 7</td>
<td>?</td>
</tr>
<tr>
<td>Family 8</td>
<td>40</td>
</tr>
<tr>
<td>Family 9</td>
<td>34</td>
</tr>
</tbody>
</table>

Glutathione (GSH) was also estimated in the blood of a small group of relatives (parents and children) of our test cases. The results are presented in figure 3 (group D), fig. 4* and table 5. The results in this group show a very high incidence of low glutathione values. Out of 48 persons studied, 38 (about 80%) had glutathione concentration below 60 mg./100 cc. RBC. This suggests that we are dealing with a real familial anomaly and not with a casual finding. The figures presented convey an impression of the presence of a recessive gene responsible for this occurrence, but the material is still too small for a thorough genetic evaluation.

* (In family No 10 no hemolytic occurrences have been known; a girl in the control group was found to have blood glutathione of 53 mg./100 cc. RBC and her whole family has been subjected to investigation. The findings in this family are reported here as they bear also on the problem of familial incidence of glutathione deficient erythrocytes.)
Glutathione (GSH) Content of R.B.C. During the Acute Stage of Hemolysis

Some of our cases were studied by us also during the acute stage of favism and have shown interesting changes in their RBC glutathione content. When studied a few days after the onset of the disease it was found to be within normal limits, and remained such for at least 6–8 weeks. About three months after the attack the glutathione (GSH) concentration dropped to definitely low values (35–45 mg/100 cc. RBC). Two examples of such cases are presented in Table 6.

These findings could be explained by a hypothesis, that young cells formed in great numbers after the hemolytic crisis are normal in regard to glutathione, but in old erythrocytes a defect appears leading either to its increased destruction or decreased synthesis.

Study of this problem is the subject of further investigation.

Discussion

The prevalent conception of the pathogenesis of favism is that of allergic sensitization.30, 31a, 106 Dacie classifies this condition as “hemolytic anemia, probably of allergic origin.”

This conception is based on a possibility of precipitating an acute hemolytic attack by ingestion of a very small amount of fava beans (even one bean is sometimes sufficient) or by inhalation of its pollen, short incubation period and positive skin tests as reported by several authors. A serologic mechanism underlying this disturbance is suggested by a positive Coombs test and the presence of hemolysins reported by some investigators.35 A similar explanation is suggested for acute hemolysis occurring during sulpha-drugs therapy.106, 21b

The findings reported in this paper suggest that a constitutional anomaly of erythrocytes, namely glutathione deficiency is essential for the pathogenesis of
these conditions. Statistical analysis of the data show that it is highly improbable that the differences between the control and test groups are chance only.

It should be stressed at this point, that it seems certain that the glutathione deficiency is not a result of hemolysis, but exists prior to it. All the cases studied by us were examined a long time after recovery, when the hematologic status returned to normal. We can assume therefore that this deficiency is a permanent feature in these cases. It seems to be hereditary in view of the high proportion of persons with low glutathione concentration among the relatives of affected cases.

A similarity between the findings presented here and the glutathione deficiency in the primaquine sensitive Negroes is evident. Still, it should not be concluded at present that the mechanism of hemolysis is identical in both groups of patients, as there seem to be some points of difference.

The primaquine-caused hemolytic disease is associated with the formation of many Heinz bodies, which have not been found in favism.

The primaquine-caused anemia is self limited. In controlled studies hemolysis appeared 3–5 days after the beginning of administration of the drug and the maximal hemoglobin fall ranged from 22 to 48 per cent. The incubation period of favism is usually very short and the blood destruction may be so severe and rapid as to cause death (in our material no death occurred).

When Beutler and associates administered primaquine to 8 persons with low blood glutathione, all of them developed hemolysis. In our material persons with low glutathione (among the "relatives group") claimed to have eaten fava beans without evident harm. A patient in our hospital with a glutathione value of 52 mg. 100 cc. RBC was administered sulphadiazine and did not develop any hemolysis, but when a few weeks later she again took the drug, a hemolytic reaction followed. A theory could be proposed that favism or hemolysis due to sulpha drugs appear only when 2 simultaneous conditions are fulfilled: (1) glutathione deficiency of erythrocytes, (2) sensitization to noxious agent. We think that our knowledge is still too fragmentary to decide on this.

The changes in glutathione level during the acute stage of favism and following it are similar to those described by Flanagan and assoc. in cases of primaquine induced anemia, with one important exception. We did not find the initial sharp drop in the GSH level occurring in the prehemolytic period during primaquine administration. As all the patients studied by us reached the hospital already in the hemolytic stage, we had no opportunity to study the preceding period. (The in vitro test described by Beutler has not yet been exploited by us.)

Another problem which awaits elucidation is the mechanism causing the glutathione deficiency of erythrocytes. Carson et al. demonstrated in a recent communication an abnormality in the direct oxidation of glucose in the red blood cells of primaquine sensitive subjects. This abnormality affects glucose 6 phosphate dehydrogenase activity and possibly also the mechanism for reducing oxidized glutathione.

We studied the oxidized glutathione content in the erythrocytes of our test group and did not find any increased values. On the contrary, most cases were also deficient in oxidized glutathione as well. These results suggest that no defect in the mechanism for reducing glutathione exists in the subjects under our investigation.
STUDIES ON ERYTHROCYTES

SUMMARY

Studies have been carried out on erythrocytes of cases with past history of acute hemolysis due to Favism, sulpha drugs or PAS.

No indication was found of abnormal osmotic or mechanical fragility, electrophoretic mobility of hemoglobin or its amino acid content. Similarly no abnormal glycogenic or catalase activities were detected.

Glutathione (GSH) deficiency of erythrocytes was discovered in all cases studied. Similarly, glutathione (GSH) deficiency was a frequent finding among the relatives of affected cases.

No such glutathione deficiency could be established in the cases of thalassemia major so far studied, though both patient-groups without any exception belong to the same ethnographic denomination (non-Ashkenazic).

This investigation was aided by a grant from the Israel Research Council.

SUMMARIO IN INTERLINGUA

Studios del erythrocytos ha essite executate in casos con historias de acute hemolyse cause per favismo, drogas sulfa, o acido para-aminosalicylic.

Esseva trovate nulle indication de anormal fragilitate osmotic o mecanic, mobilitate electrophoretic de hemoglobina, o contento de amino-acido. Similmente nulle activitate anormal de glycolyse o catalase esseva detegite.

Carentias de glutathiona in le erythrocytos esseva discoperite in omne le casos studiate e esseva un constatation frequente inter le consanguineos del patientes.

Nulle tal carentia de glutathiona poteva esser establite in le casos de thalassemia major usque nunc studiate, ben que ambe gruppos de patientes representava sin exception le mesme division ethnographie (i.e. judeos non-ashkenazim).

Iste investigation esseva subventionate per le Consilio de Recercas de Israel.

REFERENCES

A. SZEINBERG, C. SHEBA, N. HIRSHORN AND EVA BODONYI 613

10bid, p 576.
31bIbid., p. 393.
36Szeinberg, A. and Aviva Chari-Bitron: Blood glutathione concentration in cases with past history of hemolytic anemia due to Vicia fava or sulphonamides. To be published.
Studies on Erythrocytes in Cases with Past History of Favism and Drug-Induced Acute Hemolytic Anemia

A. SZÉINBERG, C. SHEBA, NINA HIRSHORN and EVA BODONYI