Congenital Nonspherocytic Hemolytic Anemia, Associated with Glutathione Deficiency of the Erythrocytes

Hematologic, Biochemical and Genetic Studies

By H. K. Prins, M. Oort, J. A. Loos, C. Zürcher and T. Beckers

GLUTATHIONE, γ-glutamyl-cysteinyl-glycine, was discovered in 1921 by Hopkins1 as a constituent of yeast cells; since then it has been demonstrated in a large variety of cells, including human erythrocytes, where it is present in high concentration (approx. 0.002 mol/1 packed cells). Many attempts have been made to specify the function of this widely distributed tripeptide,2 but so far only one reaction is known in which glutathione is an essential factor: the glyoxalase reaction.3 As there is no evidence that glyoxalase is of importance for the red cell, there is no direct biochemical indication that glutathione is an indispensable red cell component. There is considerable indirect evidence, however, that it has a biologic function in the cell, as there is an increased susceptibility to hemolysis in erythrocytes with a low or “unstable” level of reduced glutathione—e.g., in glucose-6-phosphate dehydrogenase deficiency,4 glutathione reductase deficiency,5 and certain diseases of the liver.6 In these cases it has not been proved that the decreased viability of the erythrocytes is a direct consequence of the low level of

Abbreviations

GSH—Glutathione, reduced form.
GSSG—Glutathione, oxidized form.
ATP—Adenosine-triphosphate.
NAD (DPN)—Nicotinamide-adenine-dinucleotide.
NADH (DPNH)—Nicotinamide-adenine-dinucleotide, reduced form.
NADP (TPN)—Nicotinamide-adenine-dinucleotide-phosphate.
NADPH (TPNH)—Nicotinamide-adenine-dinucleotide-phosphate, reduced form.
G6PD—Glucose-6-phosphate dehydrogenase.
T½Cr—Apparent half survival time, as obtained with the radiochromium method.
AcPH—Acetylphenylhydrazine.
Hp—Haptoglobin.
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reduced glutathione, and a number of investigators have therefore studied the behavior of erythrocytes in which the glutathione level has been lowered by chemical methods such as incubation with acetylphenylhydrazine or N-ethylmaleimide. Although it is highly likely that other red cell constituents are altered by these chemical methods as well as glutathione, important information has been obtained from these experiments. Jacob and Jandl concluded from their investigations on N-ethylmaleimide-treated erythrocytes that the glutathione content of erythrocytes can be drastically lowered (down to 10 per cent) without changing metabolism in vitro or survival time in vivo.

Survival studies with erythrocytes pretreated with para-mercuri-benzoate (a thiol-blocking agent, which does not penetrate the red cell) showed that the thiol groups on the cell surface appeared to be far more important for the integrity of the cell.

In the present paper we wish to describe extensive investigations on a family in which a hereditary deficiency of red cell glutathione has been found, in the hope that the results will contribute to our knowledge of the function of glutathione in the red cell.

**Materials and Methods**

*Blood sampling:* Blood was obtained by venepuncture. For the estimation of the hematocrit, hemoglobin, haptoglobin, reduced glutathione and the Cr51 binding capacity, the blood was mixed with heparin (approximately 1 mg./10 ml. blood). For the estimation of enzyme activity, ATP, lactate production, and incubation studies, the blood was taken into a citrate-glucose solution. All samples were cooled in ice during storage or transport and were examined within 4 hours of collection.

*Plasma haptoglobin* was determined by Nyman's electrophoretic method, modified so as to use cellulose acetate strips.

*Spectrophotometric assays* were performed in cells of 1 cm. light path in a Unicam SP 500 spectrophotometer.

*Reduced glutathione* was estimated with bis(p-nitrophenyl) disulphide, by the method of Stevenson et al.

*Preparation of hemolysate for enzyme studies:* The red cells were washed three times with 0.9 per cent saline, the layer of white cells being removed after each centrifuging. The packed cells were then mixed with an equal volume of distilled water and the resulting mixture was kept at -20 C. to -15 C. for a minimum of one night and a maximum of 3 days.

*Spectrophotometric determination of the activities of the enzymes glutathione reductase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphoglyceraldehyde dehydrogenase* was performed at a wavelength of 340 mμ.

Results were expressed as μmol NADPH₂ or NADH₂/min./Gm. of hemoglobin at 25 C., using an extinction coefficient of 6.22 cm.²/μmol at 340 mμ for NADPH₂ and NADH₂, and an optical density of 8.58 at 540 mμ of a 1 per cent HbO₂ solution. Temperature correction was performed by means of correction curves obtained experimentally on normal hemolysates.

*Glyoxalase* activities were estimated at 37 C. by the method of Valentine and Tanaka and expressed as μmol methylglyoxal/h/ml. packed cells, using an extinction coefficient of 3.37 cm.²/μmol S-lactoyl-glutathione.

*ATP level and lactate production; preparation of red cell extracts with perchloric acid:* One ml. of washed packed cells was incubated for one hour at 37 C. under aerobic conditions in two ml. of a Ringer-phosphate-glucose solution and similarly in a Ringer-phosphate-inosine solution, the solutions containing 0.0020 M CaCl₂, 0.0005 M MgCl₂, 0.103 M NaCl, 0.0027 M KCl, 0.024 M NaHCO₃, 0.0030 M phosphate buffer pH 7.4, and
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0.0175 M glucose or inosine, respectively. The pH of the final solution was adjusted to 7.4. A 1 ml. sample was taken from each mixture before incubation and after 60 minutes. Each sample was pipetted into 1 ml. of a 6 per cent perchloric acid solution, and shaken vigorously for 10 minutes in a cold room. The precipitate obtained was removed by centrifugation at 2000 g for 10 minutes at 0-4 C. One ml. of the clear supernatant was neutralized with 0.2 ml. of a solution containing 2.6 M KHCO₃ and 1 M tris (hydroxymethyl) aminomethane, and the neutral extracts were kept at 0 C or -20 C. Lactate determinations were performed as described by Hohorst;¹⁹ the lactate production was expressed as μmol lactate/ml packed cells/hr. ATP was estimated as described by Adam,²⁰ and was expressed as μmol ATP/ml. packed cells.

Chromatographic fractionation of red cell extracts on columns of Dowex-1-formate²¹:
Three columns (0.9 X 22 cm.) of Dowex - 1 X 2 (200-400 mesh, FLUKA), previously converted into formate were connected with a single mixing chamber, containing 240 ml. of acidified distilled water. Gradient elution of red cell extracts (5-25 ml.) was performed by successive introduction into the mixing chamber of 470 ml. of 4 M formic acid, 200 ml. of a solution of 4 M formic acid + 0.2 M ammonium formate, 320 ml. of 4 M formic acid + 0.4 M ammonium formate, and 470 ml. of 4 M formic acid + 0.8 M ammonium formate. The effluents were collected in time-operated fraction collectors, provided with plastic cups (diameter, 3.5 cm.; height, 1.5 cm.). The flow rate was 4 ml. in 15 minutes per column and the temperature was approximately 10 C.

The C¹⁴ content of the fractions obtained chromatographically was estimated without further treatment, using an end window counting tube (end window, 1.5-2 mg./cm²; effective diameter, 27.8 mm.), shielded by a guard tube, and mounted on an automatic sample changer attached to a recording unit (Philips). One μc. of C¹⁴ in a total volume of 4 ml., placed in the plastic cup, gives a count of 300 cpm against a background of 5 cpm. Strips obtained from paper chromatography or paper electrophoresis were scanned in an automatic scanning device (Frieske & Hoepfner) provided with 2 Philips end window counting tubes as described above, operated in conjunction with an automatic recording unit.

C¹⁴-labeled preparations (Philips-Duphar): Uniformly labeled L-glutamine, specific activity 18.5 mc./mM; glycine-1-C¹⁴, specific activity 8.2 mc./mM. were used.

Evaluation of apparent half-life with Cr⁵¹ (method of Necheles et al.²²): Na₂Cr₂O₄ (Philips-Duphar), specific activity 10 mc./mg. Cr, was used for the labeling of red cells.

Evaluation of the red cell survival time (Ashby technic²³,²⁴): As all patients were of group A, the ABO system could not be used and the MNS system was therefore chosen. The erythrocytes of one patient (group A, NN) were injected into the circulation of normal recipients (group A, MM). Preliminary experiments showed that the available anti-M serum did not react with NN-cells, but gave complete agglutination of MM-cells.

Uptake of chromate by erythrocytes²⁵: Heparinized blood was centrifuged and separated into packed cells and plasma. Into a counting tube were pipetted 1.3 ml. of packed cells, 1.5 ml. of plasma, and 0.5 ml. of a solution containing 0.9 per cent NaCl, 0.5 μc. of Na₂Cr₂O₄, and carrier chromate in concentrations ranging from 0 to 0.016 M. The (micro) hematocrit was then estimated, the suspension was incubated for one hour at 37 C., and the radioactivity was measured in a well-type scintillation counter with a pulse-height discriminator (Philips). Following this the counting tube was centrifuged for 10 minutes at 1000 g, one ml. of the supernatant was diluted to the original volume of the incubation mixture (3.3 ml.), and the radioactivity was measured. The red cells were washed three times with saline, and the radioactivity measured after addition of saline up to the original volume. The percentage of chromate taken up by the red cells was calculated from the formula

\[
\% \text{ chromate uptake} = \frac{\text{cpm washed cells}}{\text{cpm original mixture}} \times 100
\]

In order to check whether any radioactivity had been lost from the red cells during washing, the binding percentage was also calculated from the formula
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Fig. 1.—Pedigree of family with glutathione-deficiency.

% chromate uptake = \frac{\text{cpm in 1 ml. supernatant} \times (100\text{-hematocrit}) \times 3.3}{\text{cpm of the original incubation mixture}}

Chromatographic fractionation of hemolysate on columns of Amberlite IRC-50

Columns of Amberlite IRC-50 XE 64 (2\frac{1}{4} \times 30–35 cm.) were equilibrated with a citrate buffer (pH 6.1, 0.20 gr. ion Na+/l.) Two-10 ml. of hemolysate (0.3–1.5 Gm. Hb) were fractionated at 2 C. in each run. After elution of the HbA\(_4\) fraction, the main hemoglobin component (HbA\(_1\)) was desorbed with a 0.3 M citrate buffer solution pH 6.5.

RESULTS

Clinical and Hematologic Data

The original patient, Mrs. Z.-K., born 1930 (pedigree V 17, Fig. 1), was referred to our laboratory in 1960 with the following history:

Since childhood the patient had been pale and slightly jaundiced. There had been episodes of marked jaundice, usually lasting for about 2–4 days and not associated with nausea, vomiting or abdominal pain. No abnormalities were noticed by the patient in the appearance of urine or stools. In November 1959 Mrs. Z.-K. underwent a gynecologic operation under general anesthesia. After the operation marked jaundice was noticed, and the patient was examined by Dr. W. H. Borst, specialist of internal medicine, whose findings were in brief:

Physical examination: A pale, jaundiced woman. Heart and lungs clear on percussion and auscultation, no enlargement of lymph nodes. Liver and spleen not palpable.

Laboratory investigations: Hb, 7.5 Gm. per cent, RBC, 2.1 \times 10^6; reticulocytes, 11 per cent. White cell count and differentiation normal. Serum bilirubin, 2 mg. per cent (indirect); total content and electrophoretic pattern
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The patient was given two 500 ml. units of blood, and was followed up in the outpatient department. A diagnosis of chronic hemolytic anemia was made. The Hb ranged from 10-11 Gm. per cent; reticulocytes, 2.5-5.0 per cent; serum bilirubin, 1-2 mg. per cent. Serum-Fe, however, was 30-90γ per cent. The Hb-level did not increase after oral iron medication.

In April 1960 the patient was referred to our laboratory. Physical examination there revealed a pale, slender, slightly jaundiced woman; liver and spleen were not enlarged. Routine laboratory investigations: Hb, 10.0 Gm. per cent; RBC, 3.0 × 10⁶; PCV, 34 per cent; MCV, 110 μ; MCH, 33 γ; MCHC, 31 per cent; reticulocytes, 8.5 per cent. Peripheral blood smear: slight anisocytosis and moderate polychromasia; leukocytes, 5400/mm³ with normal differentiation; thrombocytes, 240,000/mm³; serum bilirubin, 3.0 mg. per cent, 0.7 mg. per cent direct. Additional investigations revealed a lowered haptoglobin level, the absence of any abnormal antibodies against red cells (direct and indirect Coombs tests negative), a normal complement level and a normal immunoelectrophoretic pattern of the serum. A glutathione stability test revealed an extremely low level of erythrocyte glutathione, both before (4 mg. per cent) and after (3 mg. per cent) incubation with acetylphenylhydrazine. This proved to be a specific biochemical defect of the patient's red cells, as will be discussed later in detail. The possibility was considered that the recurrent jaundice might be caused by sensitivity of the patient's erythrocytes to certain drugs. Before 1960 she had been taking analgesics containing aspirin and phenacetin and the possibility of a correlation between drug ingestion and jaundice could not been ruled out. Jaundice or dark urine had never been noticed after eating broad beans.

The patient has now been followed for several years. Apart from being easily tired, there have been no specific complaints. Physical examination in July 1963 revealed slight jaundice and mild hepatosplenomegaly (both organs were palpable 1 finger-width below the respective costal margins). Laboratory data have been summarized in Table 1.

Mr. K., brother of the propositus, born 1916 (pedigree V, 2) has no difficulty in carrying on his work as a farmer. Since childhood there have been recurrent episodes of jaundice (duration 2-3 days), in the patient's opinion mainly after fatty meals, and never after eating broad beans. The patient takes no drugs except occasional aspirin; no jaundice has been noted after this medication. Physical examination (1962, 1963) revealed a powerfully built man, with slight scleral icterus and moderate hepatosplenomegaly. Hematologic data pointed to a mild hemolytic anemia (Table 1). The erythrocyte GSH level is extremely low (3 mg. per cent). No irregular antibodies have been found against red cells (direct and indirect Coombs tests negative). Liver function tests: SGOT 11 U, SGPT 11 U, alkaline phosphatase 1, 7 U (Bodansky). A plasma bromosulfalein disappearance curve after injection of 4 mg. of the dye per Kg. body weight was normal. Biochemical studies of the erythrocytes and the results of survival studies will be discussed later in detail.
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<th>Date of Invest.</th>
<th>Hb Gm. %</th>
<th>Hematocrit %</th>
<th>Ret. %</th>
<th>Serum Fe γ%</th>
<th>Total Fe Binding Cap. γ%</th>
<th>Serum Hemoglobin mg. %</th>
<th>Serum Bilirubin mg. %</th>
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**Table 1.** Hematologic Data of Glutathione-Deficient Patients
Mrs. W.-K., sister of the propositus, born 1918 (pedigree V, 5) tires easily when doing her chores as a housewife. She has had short periods of jaundice, not related to drug ingestion. She never eats broad beans. In 1952 a cholecystectomy was performed because of cholelithiasis and cholecystitis. The postoperative course was complicated by the occurrence of a pseudocyst of the pancreas, which was treated by marsupialization. Physical examination revealed no abnormalities (no jaundice, no hepatosplenomegaly). Hematologic findings, apart from a normal serum bilirubin, pointed to slight hyperhemolysis (Table 1). The erythrocyte GSH was 2 mg. per cent. Liver function tests (serum transaminase, alkaline phosphatase, cephalin flocculation test, bromosulfalein disappearance curve and prothrombin time) were all within normal limits.

Mrs. M.-K., sister of the propositus, born 1931 (pedigree V, 19) has had at least 4 brief periods of jaundice, not related to the ingestion of drugs or broad beans, but possibly following fatty meals. On physical examination, no abnormalities were detected apart from slight jaundice. Laboratory data pointed to a hemolytic anemia (Table 1). The erythrocyte GSH was 4 mg. per cent. The serum protein pattern, alkaline phosphatase, and thymol turbidity test were normal, but after intravenous injection of bromosulfalein (4 mg. per Kg. body weight), 10 per cent of the dye was still in circulation 30 minutes after the injection, 5 per cent being the upper level of the normal range.

Patient VI, 7 (nephew of the propositus), a 9-year-old boy, had a prolonged period of neonatal jaundice, but thereafter no jaundice has ever been noticed. Apart from aspirin, there is no ingestion of drugs. Consumption of broad beans produces no ill effects. On physical examination no abnormalities were found, but hematological investigation revealed signs of slight hyperhemolysis (Table 1). As in the other patients, erythrocyte GSH is low (8 mg. per cent).

Family Studies

A family study revealed, that besides the original patient there were 4 other siblings and relatives with glutathione deficiency associated with signs of mild to moderately severe hemolytic anemia, as has been described above. The pedigree of the family is presented in Figure 1. The defect was observed in both sexes, but only in the offspring of consanguinous marriages. In the close relatives of the patients with glutathione deficiency the red cell glutathione values are normal. There are no obvious signs of hyperhemolysis; slight hyperhemolysis, however, cannot be excluded with certainty in all cases (e.g., slight hyperbilirubinemia in IV, 3; lowered haptoglobin levels in V, 15 and VI, 3). One of them (VI, 3) was studied in detail, and the history, physical examination and repeated laboratory studies (including reticulocyte

*Note added in proof: The patient has never eaten broad beans, except once (July, 1964). Shortly afterwards she passed "cola-colored" urine and developed severe jaundice, which disappeared spontaneously. No hematologic data are available, since the patient has not consulted a physician.
counts, serum bilirubin and haptoglobin levels) all failed to disclose any signs of hemolytic anemia.

**Erythrocyte Morphology; Heinz Body Formation**

No striking irregularities have been observed in blood smears from the glutathione-deficient patients; there is a slight anisocytosis and slight hypochromia of the erythrocytes. The osmotic fragility is normal (Table 1). In the Heinz body formation test (Beutler) the glutathione-deficient erythrocytes behave abnormally: they differ from both normal and G6PD-deficient erythrocytes by the formation of a much greater number of comparatively small Heinz bodies. In a “blank” experiment (omission of acetylphenylhydrazine) no Heinz body formation was observed. We have not yet had an opportunity to carry out tests on the glutathione-deficient patients during a period of jaundice, and we are therefore unable to say whether Heinz bodies occur in vivo during hemolytic crises.

**Red Cell Survival Studies; the Effect of Primaquine**

In patients V, 2 and V, 17 Cr51 survival studies showed a very short apparent half-life of the red cells (11 and 2 days, respectively); moreover, the survival curves indicated the existence of two cell populations in each patient. In view of the clinical and hematologic findings, however, we felt that on this occasion the radiochromium method was giving unreliable results, and we therefore continued survival studies by comparing the radiochromium method with the Ashby method.

In Figure 2a the result is shown of an experiment in which the red cells from 150 ml. of glutathione-deficient blood (patient V, 5; blood group A, NN), washed 6 times, were given to a normal recipient (F. P., blood group A, MM). A small portion of the blood (20 ml.) had previously been labeled with 20 μc. of Cr51. There is a striking difference between the Ashby curve (50 per cent survival at approximately 14 days), and the Cr51 curve, which indicates a loss of 50 per cent of the radioactivity within the first two days. After the second day the disappearance of the Cr51 from the circulation is much slower.

From the accumulation of radioactivity in the spleen and the absence of appreciable amounts of Cr51 in the urine, it was concluded that the disappearance of Cr51 from the blood stream was not caused by elution of chromium from intact erythrocytes. It seemed more likely that the viability of some of the Cr51-labeled glutathione-deficient erythrocytes had been reduced by the labeling procedure, possibly as a result of a toxic effect of chromate. In the next experiment (Fig. 2b) the total volume (150 ml.) of glutathione-deficient blood (from the same patient (V, 5)) was therefore incubated with 20 μc. of Cr51, so that the same amount of the possibly toxic chromate was distributed over 7½ times as many red cells. The washed erythrocytes were then given to a second normal recipient (J. L., blood groups A, MM). There was now greater similarity between the curves obtained with the Ashby and the Cr51 methods and the initial loss of radioactivity was markedly smaller than in the previous experiment. The results of the two experiments
are consistent with the assumption that the chromate induced loss of viability of some of the glutathione-deficient red cells. This will probably have been the older cells: from the flat section of the Cr51 curve in Figure 2a it may be assumed that it was mainly the younger cells which remained in the circulation.

In Figure 2b the effect of daily oral administration of 30 mg. primaquine (base) on the survival of the donor erythrocytes is recorded. After a sharp drop in the number of circulating donor erythrocytes between the second
and the fourth day of primaquine administration, both the Cr$^{51}$ and the Ashby survival curves flatten out, despite prolonged drug ingestion. However, during a second period of primaquine administration, 9 days later, nearly all remaining donor erythrocytes were eliminated. Every fall in the survival curve was accompanied by an increase of radioactivity over the spleen.

The experiment was complicated by the fact that the second recipient had a meal of fava beans, two days after the first dose of primaquine (Fig. 2b). At that time a considerable drop of the radioactivity had already taken place.
but a contribution of the fava beans to the further decrease of circulating glutathione-deficient erythrocytes cannot be ruled out. The possibility is enhanced by the result of an experiment with the first normal recipient (F. P., Fig. 2a). Following a meal of fava beans on the 18th day of the survival test the majority of the remaining donor cells disappeared from the circulation within three days.

No irregular antibodies were demonstrable in the serum of the recipients, either before and after the cross-transfusion experiments.

**Chromate Binding**

In Figure 3 the results are shown of experiments on the uptake of chromate by glutathione-deficient erythrocytes, compared with normal and G6PD-deficient erythrocytes. Even when trace amount of chromate are used, uptake by glutathione-deficient erythrocytes is significantly lower than by normal and G6PD-deficient cells. The percentage of chromate taken up by normal red cells is almost constant up to $8 \times 10^{-4}$ mol chromate/1 supernatant (60 μg. Cr/ml. cells), whereas in glutathione-deficient erythrocytes this percentage falls rapidly with increasing chromate concentrations. In these experiments G6PD-deficient erythrocytes behave like normal cells at the lower chromate concentrations. At the higher chromate concentrations there was some divergence of the two curves, that of the G6PD-deficient erythrocytes being the lower. In conjunction with the fall in the chromium binding ratio (Fig. 3) a fall occurs in the level of reduced glutathione in the normal and G6PD-deficient erythrocytes.

No indications were obtained of any difference in the attachment of chromium to the cell constituents of normal and glutathione-deficient erythrocytes. On incubation of normal and deficient red cells in saline and in plasma for 48 hours, no abnormally high elution of chromate from the deficient cells was observed. By Amberlite chromatography of hemolysates of washed cells, previously incubated with chromate (0.0 and 5 μmol chromate/ml. packed cells incubated for one hour at 37 C.), no significant differences could be detected between normal and glutathione-deficient red cells with regard to the distribution of the chromium over the nonabsorbed portion of the hemolysate, the HbA₃-like fraction, and the main hemoglobin fraction.

**Enzyme Activities, ATP Content, and Lactate Production**

From Table 2 it appears that in general the enzyme activities of the deficient erythrocytes, as well as lactate production, are within the upper limit of normal, probably as a reflection of the low mean cell age; ATP values are completely normal. The most striking finding is the reduced glyoxalase activity. It was demonstrated that this deficiency is caused by the lack of glutathione, which is an indispensable cofactor of the enzyme. When the incubation with methylglyoxal was carried out using hemolysate instead of packed cells, addition of reduced glutathione (2.5 mg. per ml. of the mixture to be incubated) induced glyoxalase activity (Table 3). From this table it can be seen that even in normal hemolysate glyoxalase activity is low (due to the
Fig. 3.—The binding of chromium by glutathione-deficient, G6PD-deficient and normal erythrocytes. Both the amount of GSH and the chromate uptake in normal red cells, on incubation with tracer amounts of chromate, have been arbitrarily taken as 100 per cent.

dilution of GSH in the reaction mixture), and must be restored by the addition of extra glutathione (see also Valentine and Tanaka\(^9\)). The normal glyceraldehyde phosphate dehydrogenase activities in GSH-deficient erythrocytes are consistent with the recent finding of Harris et al.\(^{31}\) that glutathione is not a necessary cofactor or constituent of this enzyme.

A series of comparative experiments was carried out to investigate the effect of acetylphenylhydrazine (AcPH) on the glycolysis and the ATP level of normal, glutathione-deficient, and G6PD-deficient erythrocytes. Cells from three glutathione-deficient patients (V, 5, V, 17 and VI, 7), three male Caucasian G6PD-deficients, and three normal controls were washed, packed, and suspended in Ringer-phosphate-glucose solution (AcPH content: 0 and 7.5 mg.AcPH/ml., respectively); the ATP and lactate content of the incubation mixtures was estimated at various intervals. During the first 2 hours of incubation with AcPH there was no change in the ATP level of the three cell types. Lactate production was increased in the presence of AcPH in the case of normal and the glutathione-deficient erythrocytes (with 24 and 29 per cent, respectively, probably as a result of the stimulation of the pentose-monophosphate shunt. The lactate production of the G6PD-deficient cells was not stimulated.
HEMOLYTIC ANEMIA AND GLUTATHIONE DEFICIENCY

Table 2.—Enzyme Activities, ATP Content, and Lactate Production of Glutathione-Deficient Erythrocytes

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>V. 2</th>
<th>V. 6</th>
<th>V. 17</th>
<th>V. 19</th>
<th>VI, 7</th>
<th>Normal Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>5.8</td>
<td>5.9</td>
<td>6.4</td>
<td>6.0</td>
<td>6.5</td>
<td>5.7 ± 1.08</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>2.3</td>
<td>2.3</td>
<td>3.1</td>
<td>2.3</td>
<td>1.7</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>3.1</td>
<td>3.1</td>
<td>2.2</td>
<td>—</td>
<td>—</td>
<td>2.3 ± 0.42</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase</td>
<td>3.1</td>
<td>3.1</td>
<td>2.2</td>
<td>—</td>
<td>—</td>
<td>2.3 ± 0.42</td>
</tr>
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<td>—</td>
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</tr>
<tr>
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<td>3.1</td>
<td>2.2</td>
<td>—</td>
<td>—</td>
<td>2.3 ± 0.42</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase</td>
<td>3.1</td>
<td>3.1</td>
<td>2.2</td>
<td>—</td>
<td>—</td>
<td>2.3 ± 0.42</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Glyoxalase</td>
<td>184</td>
<td>88</td>
<td>22</td>
<td>79</td>
<td>68</td>
<td>769 ± 145</td>
</tr>
<tr>
<td>ATP</td>
<td>—</td>
<td>1.11</td>
<td>1.24</td>
<td>—</td>
<td>1.13</td>
<td>1.13 ± 0.13</td>
</tr>
<tr>
<td>Lactate production</td>
<td>—</td>
<td>2.5</td>
<td>2.4</td>
<td>—</td>
<td>2.3</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>3.2</td>
<td>2.8</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.—Generation of Glyoxalase Activity in Hemolysates of Glutathione-Deficient Erythrocytes by the Addition of Glutathione

<table>
<thead>
<tr>
<th>Glyoxalase activity</th>
<th>V. 2</th>
<th>V. 5</th>
<th>V. 17</th>
<th>V. 19</th>
<th>VI, 7</th>
<th>Normal Hemolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>before addition</td>
<td>39</td>
<td>65</td>
<td>79</td>
<td>0</td>
<td>0</td>
<td>22, 0</td>
</tr>
<tr>
<td>after addition</td>
<td>349</td>
<td>821</td>
<td>642</td>
<td>478</td>
<td>565</td>
<td>867, 715</td>
</tr>
</tbody>
</table>

The Primary Defect

Attempts were made to localize the primary defect in the glutathione-deficient erythrocytes. From the enzyme estimations it was deduced that the glutathione-reducing system in the abnormal erythrocytes is intact. This conclusion was confirmed by the results of the following experiment: One volume of washed, packed, glutathione-deficient erythrocytes was mixed with one volume of distilled water. Five ml. of the resulting hemolysate was incubated for 2 hours at 37 C. at pH 7.4 with 4 ml. of a solution, containing 0.005 M glucose-6-phosphate, 0.005 M MgCl2, 0.0003 M NADP (TPN), and 0.005 M oxidized glutathione (GSSG). Two similar mixtures were incubated omitting the NADP and GSSG, respectively. Hemoglobin and reduced glutathione estimations were performed at intervals. The results are shown in Figure 4. It was demonstrated that hemolysate of glutathione-deficient erythrocytes is able to reduce added oxidized glutathione in the presence of glucose-6-phosphate and NADP, and even in the presence of glucose-6-phosphate alone. Addition of glucose-6-phosphate and NADP, but omission of oxidized glutathione, however, did not result in the formation of reduced glutathione, apparently due to the absence of oxidized glutathione in the glutathione-deficient red cells.

This was confirmed by the fact that in a normal hemolysate in which all the glutathione had been oxidized by treatment with H2O2 (0.15 Gm.
H₂O₂/100 ml. hemolysate, 0 C.), nearly 60 per cent of the original amount of reduced glutathione was found within 15 minutes of incubation with glucose-6-phosphate and NADP.

**Incubation with glycine-C¹⁴** Four ml. of washed, packed, erythrocytes of a glutathione-deficient patient (V, 5), her son (VI, 3), and a normal healthy subject were suspended in 6 ml. of Ringer-phosphate-glucose solution, also containing 0.010 M L-cysteine/1 and 0.010 M L-glutamic acid/1. To each of the suspensions 10 μC. (1.22 μmol) of glycine-1-C¹⁴ was added, and during the subsequent aerobic incubation at 25 C. the radioactivity of the erythrocytes (0.3 ml. washed, packed cells) and of the supernatant (0.3 ml.) was estimated at fixed intervals. After 46 hours of incubation approximately 45 per cent of the total amount of C¹⁴ had been taken up by the erythrocytes in all three suspensions. The mixtures were centrifuged, the cells were washed and hemolysed, and the hemolysates were submitted to chromatography on columns of Amberlite IRC-50 XE-64.

From the hemolysates of the normal subject and the patient's son two radioactivity peaks were eluted prior to the hemoglobin peaks, in which little radioactive material was demonstrated (about 10 per cent of the total amount of C¹⁴). The first radioactive fraction was investigated by paper electrophoresis (pH 8.6 in barbital buffer, low pH in N acetic acid) and by paper chromatography (phenol-water and butanol-acetic-acid-water). In all cases the radioactivity migrated with oxidized glutathione. After hydrolysis with 6 N hydrochloric acid (16 h, 105 C.) a radioactive substance was
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obtained which had the chromatographic and electrophoretic properties of glycine. The second radioactive fraction from the Amberlite column was glycine-C\textsuperscript{14} as was concluded from paper electrophoresis and paper chromatography.

By Amberlite column chromatography of the hemolysate of the glutathione-deficient erythrocytes only the glycine-C\textsuperscript{14} peak was detected.

In a second experiment with normal and glutathione-deficient erythrocytes (patient V, 19), 5 ml. of washed packed cells were incubated with 10 ml. of the same incubation mixture (containing 10 \textmu c. glycine-C\textsuperscript{14}) for 8 hours at 37 \textdegree C. The erythrocytes took up approximately 35 per cent of the total radioactivity. After washing and hemolysis the low molecular components were separated from the bulk of the hemoglobin by filtration through 1\frac{1}{2} \times 45 cm. columns of (coarse) Sephadex G50; the last traces of protein were completely removed by drop-wise addition of 60 per cent perchloric acid until flocculation occurred, followed by centrifuging. The extracts (containing about 90 per cent of the C\textsuperscript{14} taken up by the erythrocytes) were neutralized with KOH and submitted to chromatography on Dowex-1-formate columns. The chromatogram of the normal extracts contains two radioactive components (Fig. 5), with the paper chromatographic properties of glutathione and glycine, respectively, whereas in the extract of the glutathione-deficient erythrocytes only a negligible part of the total radioactive material had the chromatographic properties of glutathione. An identical result was obtained with the erythrocytes from the glutathione-deficient patient V, 2.
From the high yield of C\textsuperscript{14} obtained in the low molecular portion of the Sephadex-G50 filtrate, it appears that very little radioactive material is attached to protein. We obtained much lower yields (60-70 per cent) by the perchloric acid extraction normally used; this extraction must therefore be incomplete.

\textit{Incubation with glutamine-C\textsuperscript{14}}: Preliminary studies with glutamic acid-C\textsuperscript{14} revealed that glutamic acid was not taken up by human erythrocytes; the incubations were therefore performed with uniformly labeled L-glutamine-C\textsuperscript{14}. In an experiment with erythrocytes from a normal person and from a glutathione-deficient patient (V, 2), 10 ml. of washed, packed cells were incubated for 8 hours at 37 C. with 20 ml. of a Ringer-phosphate-glucose solution, containing 0.01 mol cysteine/1, 0.01 mol glycine/1, and 10 \mu c. (=0.54 \mu mol) glutamine-C\textsuperscript{14}. An equal uptake of C\textsuperscript{14} by the two categories of erythrocytes was observed (24 per cent of the total radioactivity in 8 hours, or 0.0017 \mu mol glutamine/ml. packed cells per hour; this is a low rate of uptake, as compared with the glycine uptake of 0.011 \mu mol glycine/ml. packed cells/h.). After incubation the erythrocytes were washed, hemolysed with an equal volume of water and deproteinized by the addition of 6 per cent perchloric acid. The resulting extracts were submitted to chromatography on columns of Dowex-1-formate. From the extract of the normal cells four radioactive fractions were obtained (Fig. 6), identified by paper chromatography as glutamine, glutamic acid, reduced glutathione, and oxidized glutathione. In the extract of the glutathione-deficient cells only glutamine and glutamic acid were detected. Two minor radioactive fractions (X\textsubscript{1} and X\textsubscript{2}) of the Dowex-chromatograms could not be identified with glutathione.

\textit{Incubation of hemolysate with glutathione-C\textsuperscript{14}}: Ten ml. of washed, packed
erythrocytes from a normal healthy subject and from a glutathione-deficient patient (V, 2) were mixed with 10 ml. of distilled water; 7 mg. of reduced glutathione was added to the glutathione-deficient hemolysate. The hemolysates were then incubated for 6 hours at 37 C. with 20 ml. of a Ringer-phosphate-glucose solution pH 7.4, to which approximately one μc. of C\(^{14}\)-labeled oxidized glutathione had been added. (This was obtained from the incubation studies on normal erythrocytes with glycine-\(^{14}\)C.) After incubation extracts were prepared with perchloric acid as previously described and submitted to chromatography on columns of Dowex-1-formate.

In both cases part of the C\(^{14}\)-labeled substance was not absorbed by the columns. By paper chromatography this portion appeared to be very heterogeneous, and could be identified neither with glycine nor with glutathione. In each case most of the labeled substance was absorbed on the column and later eluted as a single peak; this was shown by paper chromatography to consist of glutathione.

In this experiment 75 per cent of the labeled glutathione remained unaltered in the normal hemolysate, and 78 per cent in that from the glutathione-deficient patient.

**Discussion**

In this paper the results are given of an investigation on two male and three female patients, all relatives, with congenital nonspherocytic hemolytic anemia. The very low level of reduced glutathione in the erythrocytes appeared to be a characteristic abnormality in these patients. Little or no signs of hyperhemolysis have been observed in the relatives with a normal glutathione level, so that there appears to be a correlation between the glutathione deficiency and the hyperhemolysis.

The absence of the abnormality in the parents and the offspring of the patients, and the consanguinity of the parents of all the patients, are consistent with an autosomal recessive pattern of inheritance.

The life span of the glutathione-deficient erythrocytes, as measured by the Ashby method, is markedly shortened (approximately 30 days). That this shortened survival is not due to any incompatibility can be deduced from the form of the survival curves and from the absence of irregular antibodies in the serum of the recipients both before and after cross-transfusion experiments.

Comparison of the curves obtained with the Ashby method and the Cr\(^{51}\) method leads to the conclusion that the abnormal erythrocytes are easily damaged by the chromate labeling procedure, possibly by a toxic effect of chromate, even at a concentration below 0.2 μg. Cr/ml. red cells. The shape of the Cr\(^{51}\) curves is compatible with the assumption that the older erythrocytes in particular are irreversibly damaged and removed from the circulation. Donohue et al.\(^{34}\) measured the effects of increasing amounts of chromate (0.6, 2, 5, 10, 50, and 100 μg. metallic chromium/ml. normal human red cells) on the disappearance rate of Cr\(^{51}\) in circulation; there was a definite shortening of the T\(_{1/2}\)Cr when the cells were treated with 100 μg. Cr/ml., and
a possible effect with 50 μg. It is interesting to note that the decrease in viability of normal red cells occurs at the same range of chromium concentrations at which the chromium binding percentage begins to decline (approximately $8 \times 10^{-4}$ mol chromate/1 supernatant; approximately 60 μg Cr/ml red cells). It appears therefore that above a certain “saturation level” chromate is toxic for erythrocytes. The T\(^{1/2}\)Cr and the chromium binding capacity of glutathione-deficient red cells are reduced even at a chromium concentration below 0.2 μg. Cr/ml red cells; this may mean that the “saturation” is already exceeded even at this low concentration.

Although the binding of chromate by the abnormal erythrocytes is lower than in normal erythrocytes, we did not detect any differences in the distribution of chromium in hemolysates of glutathione-deficient cells. Glutathione is obviously not essential for the binding of chromium, but it possibly inhibits damaging side reactions caused by the chromate.

The survival curve of Figure 2b, reflecting the effect of primaquine administration on survival time, closely resembles the survival curves obtained by Dern, Beutler and Alving\(^3\) in experiments with G6PD-deficient patients. In our experiments it seems, as in theirs, that the older cells in particular are removed after drug ingestion. It may be, however, that the damaging action of primaquine was enhanced by a meal of fava beans, and that such a meal can itself result in damage to glutathione-deficient cells, as is suggested in Figure 2a.

It is not justifiable to draw general conclusions from these experiments, but the results of the survival studies strongly suggest that glutathione-deficient erythrocytes are susceptible to the effect of oxidant drugs or their metabolites. Moreover, one patient with glutathione reductase deficiency studied by Carson et al.\(^5\) was reported to be primaquine-sensitive. Apparently the three biochemical defects, all associated with the maintenance of reduced glutathione in the red cell, are characterized by increased red cell destruction following the ingestion of primaquine (and probably also other drugs). We have advised therefore the glutathione-deficient patients not to use drugs which we know to be harmful in cases of glucose-6-phosphate dehydrogenase deficiency.

On incubation of glutathione-deficient erythrocytes with acetylphenylhydrazine rapid formation of small Heinz bodies was observed, indicating rapid denaturation of hemoglobin. This is consistent with the finding of Allen and Jandl\(^36\) that the presence of reduced glutathione reduces the speed of hemoglobin denaturation by acetyiphenyihydrazine.

Jacob and Jandl\(^7\) incubated normal erythrocytes with N-ethylmaleimide and with paramcuribenzoate. It was found that N-ethylmaleimide, which enters in cells and reacts with glutathione, has no shortening effect on the life span of cells in vivo when 90 per cent or less of the glutathione has been bound. When ethylmaleimide was added in sufficient amounts to bind more than 90 per cent of the intracellular glutathione, the survival time was markedly decreased, and a high uptake was observed in the spleen. p-Mercuribenzoate can also react with -SH-groups, but does not enter the cell. It was found that very small amounts of it markedly decreased the viability of the erythrocytes, despite the maintenance of a normal intraerythrocytic level of reduced glutathione,
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and a high uptake was again observed in the spleen. It was concluded that
-SH-groups on the membrane are very important for the viability of the
erthrocyte, and that intracellular glutathione is of secondary importance. This
view is in general supported by the clinical findings in glutathione-deficient
patients and by the results of our in vivo experiments, but there remains the
difference that we observed marked primaquine-sensitivity of glutathione-
deficient erythrocytes, whereas Jacob and Jandl observed no increased destruc-
tion of artificially glutathione-deficient cells (90 per cent of the GSH inhibited)
with a daily dose of 60 mg. primaquine. It may be that 10 per cent of the
normal glutathione level is still above the critical value. A second possibility
is that the erythrocyte membranes of our glutathione-deficient patients contain
an abnormally low amount of -SH-groups, whether or not as a result of the
low intracellular glutathione level.

The biochemical properties of glutathione-deficient red cells which we
investigated did not differ markedly from those of normal erythrocytes. Lactate
production from glucose is slightly increased, probably as a result of the
lower mean cell age, and it is stimulated by the addition of acetylphenyl-
hydrazine.

The ATP level does not depart from the normal. It can be concluded that
the oxidation-inducing agent acetylphenylhydrazine has no great immediate
effect on glycolysis of red cells, whether they contain “stable,” “unstable” or
no reduced glutathione, but our experiments give no information as to its long
term effect.

It has been found by Löhrl and Waller7 that in G6PD-deficient erythrocytes
a significant decrease of the ATP content took place after 4 hours incubation
with Ringer-bicarbonate-glucose solution, containing approximately 10⁻³
mol primaquine/1, whereas in normal erythrocytes no change was observed.
Similar results were obtained by Mohler and Williams38 after 6-8 hours
incubation of normal and G6PD-deficient cells with phenylhydrazine. In view
of these experiments it seems necessary to continue our experiments on
glutathione-deficient erythrocytes with long-term incubation studies. So far,
biochemical investigations have produced no unequivocal indications, either
as to the cause of the hyperhemolysis in glutathione-deficient patients or as to
the function of glutathione in the erythrocyte.

On incubation with glycine-C¹⁴ and glutamine-C¹⁴ no formation of labeled
 glutathione could be demonstrated in glutathione-deficient erythrocytes; this
can mean either a low rate of glutathione synthesis or an increased rate of
turnover. On incubation of hemolysate from glutathione-deficient erythrocytes
with oxidized glutathione and glucose-6-phosphate (with or without NADP)
no abnormally high rate of destruction was observed. Moreover, after 6 hours
incubation of normal hemolysate from glutathione-deficient erythrocytes with
equal concentrations of labeled glutathione 75 and 78 per cent, respectively,
of the labeled substance remained unchanged in the hemolysates. The rest of
the radioactive material was in both cases not identical with glycine. At least
in hemolysate therefore, no glycine is formed during the breakdown of
 glutathione.

After incubation of the abnormal cells with glycine-C¹⁴, however, the labeled
material was found to consist almost entirely of unchanged glycine-C\textsuperscript{14}, and after incubation with glutamine-C\textsuperscript{14} most of the radioactivity was recovered with either unchanged glutamine or glutamic acid. The experimental data so far are therefore more in agreement with a low rate of glutathione synthesis in the abnormal cells than with an increased rate of turnover.

Accepting the point of view that incorporation of glycine-C\textsuperscript{14} and of glutamine-C\textsuperscript{14} into the glutathione of normal red cells must be attributed mainly to the de novo synthesis of glutathione,\textsuperscript{33} we consider it probable that the primary defect in the glutathione-deficient erythrocyte is an anomaly in the glutathione-synthetising system.

**Summary**

1. A new biochemical defect of erythrocytes is described: glutathione deficiency (reduced glutathione less than 10 per cent of the amount of reduced glutathione in normal erythrocytes).

2. The defect is associated with a clinical picture of congenital nonspherocytic hemolytic anemia which is fairly well compensated.

3. The results of a family study are consistent with an autosomal recessive pattern of inheritance.

4. Labeling with Na\textsubscript{2}Cr\textsuperscript{51}O\textsubscript{4} has a damaging effect on glutathione-deficient erythrocytes. The erythrocyte life span, as estimated by a serological method (Ashby), was markedly shortened (30 days instead of 100-120 days).

5. Red cell destruction could be increased by the administration of primaquine.

6. Secondary to the glutathione deficiency, low glyoxalase activity was observed. The glutathione-reducing capacity, glycolytic activity, and the ATP level of the abnormal red cells were found to be within the normal range.

7. On incubation of the glutathione-deficient erythrocytes in vitro with glycine-C\textsuperscript{14} and glutamine-C\textsuperscript{14}, no formation of labeled glutathione could be demonstrated.
basse activitate de glyoxalase. Le capacitate de reducer glutathiona, le activitate glycolytic e le nivello de triphosphato de adenosina in le erythrocytos anormal se trovava intra limites normal.

7. Post incubation de erythrocytos a carentia de glutathiona con cyclina-C\textsuperscript{14} e glutamina-C\textsuperscript{14} in vitro, nulle formation de glutathiona a C\textsuperscript{14} poteva esser demonstrate.

REFERENCES


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Congenital Nonspherocytic Hemolytic Anemia, Associated with Glutathione Deficiency of the Erythrocytes: Hematologic, Biochemical and Genetic Studies

H. K. PRINS, M. OORT, J. A. LOOS, C. ZÜRCHER and T. BECKERS