A Series of New Screening Procedures for Pyruvate Kinase Deficiency, Glucose-6-Phosphate Dehydrogenase Deficiency, and Glutathione Reductase Deficiency

By ERNEST BEUTLER

(With the technical assistance of Miss Agnes Halasz)

IT HAS BECOME increasingly apparent that many forms of hemolytic disease are due to hereditary enzyme deficiencies affecting the erythrocytes. The drug-induced hemolytic anemias may be due to glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, glutathione reductase (GSSG-R) deficiency, or to a deficiency in reduced glutathione (GSH). Nonspherocytic congenital hemolytic anemia may be due to pyruvate kinase (PK) deficiency, G-6-PD deficiency or, much more rarely, to GSH deficiency, GSSG-R deficiency, diphosphoglyceromutase deficiency, triosephosphate isomerase deficiency, or to ATPase deficiency. Precise biochemical diagnosis of these disorders is of value, both from the viewpoint of genetic counseling and from that of therapy. Yet, assaying of all of these enzymes is costly, time-consuming, and requires specialized laboratory facilities. For these reasons, there is a need for rapid, simple procedures which will differentiate some of the more common hereditary enzyme deficiencies from one another. It is the purpose of this communication to report a new type of screening procedure which has been readily adapted for the simple detection of deficiency of several enzymes. It is based upon a principle which has not heretofore been utilized in screening procedures.

PRINCIPLE AND GENERAL PROCEDURES

In these procedures advantage is taken of the fact that even relatively minute quantities of reduced pyridine nucleotides fluoresce intensely when activated with long-wave ultraviolet light. The tests are based upon the reduction of oxidized pyridine nucleotide, in the case of the test for G-6-PD deficiency, or on the oxidation of a reduced pyridine nucleotide, in the case of the screening test for PK deficiency or GSSG-R deficiency. Although some quenching of fluorescence occurs in the presence of hemoglobin, this is minimized by two factors. First of all, the exciting wavelength is below the maximum absorption band of

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hemoglobin, the Soret region. Moreover, the emission maximum, 415 millimicrons, is near an absorption minimum of hemoglobin. Secondly, spotting the test solution on ordinary filter paper appears to cause some chromatographic separation of hemoglobin from the pyridine nucleotides, and therefore results in considerable intensification of fluorescence.

The same general procedure is used for all three screening tests. One part of blood or red cell suspension (generally 0.020 ml.) is added to 10 parts of reaction mixture (generally 0.200 ml.). Immediately after addition of the sample to the reaction mixture, a control baseline spot may be made on the filter paper. Additional spots are made after specified periods of incubation. After the spots have dried, inspection of the filter paper in a darkened room under a convenient source of long-wave UV light will establish a provisional biochemical diagnosis.

Unless otherwise indicated, biochemical reagents used were obtained from Sigma or Calbiochem.

**Specific Procedures**

I. Glucose-6-Phosphate Dehydrogenase (G-6-PD)

A. Reaction Sequences

When G-6-PD is present in the hemolysate, glucose-6-phosphate is oxidized to 6-phosphogluconate and triphosphopyridine nucleotide (TPN) is reduced to reduced triphosphopyridine nucleotide (TPNH). The hemolysate also contains 6-phosphogluconic dehydrogenase, which oxidizes the 6-phosphogluconate, reducing more TPN. When activated with UV light, the TPNH fluoresces.

B. Reaction Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
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<tbody>
<tr>
<td>Glucose-6-phosphate, .01 M</td>
<td></td>
<td>0.10 ml</td>
</tr>
<tr>
<td>TPN, .0075 M</td>
<td></td>
<td>0.10 ml</td>
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<tr>
<td>Digitonin, saturated solution, or</td>
<td></td>
<td>0.20 ml</td>
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<tr>
<td>Saponin (Mann), 1%</td>
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<tr>
<td>Potassium phosphate buffer, pH 7.4</td>
<td></td>
<td>0.30 ml</td>
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<tr>
<td>H2O</td>
<td></td>
<td>0.30 ml</td>
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</tbody>
</table>

C. Preparation of Blood Sample

Blood collected in ACD, heparin, or EDTA is suitable. Even several week old samples stored in ACD at 4 C. are suitable for testing.

D. Test Procedure

One volume of whole blood (usually 0.02 ml.) is added to 10 volumes of reaction mixture (usually 0.20 ml.) and the sample is incubated either at room temperature or at 37 C. When saponin is used as the lytic agent, a spot is made on Whatman #1 filter paper after 3 minutes incubation at 37 C. or 5 minutes incubation at room temperature. If the lytic agent used is digitonin, the spot is made after 5 to 10 minutes incubation at 37 C., or after 10 to 15 minutes incubation at room temperature. If normal G-6-PD activity is present, the spot will fluoresce brightly. No appreciable fluorescence appears when G-6-PD deficient blood is tested (Fig. 1).

E. Stability of the Reagent Mixture

The reagent mixture is stable for at least 3 months when stored at -20 C.

F. Comments

Because of the rapidity of the reaction, some fluorescence is usually observed even in a baseline spot if one is made before incubation. Because heterozygotes for G-6-PD deficiency have varying proportions of two-cell populations, detection pre-
Fig. 1.—The results of the test for glucose-6-phosphate dehydrogenase deficiency carried out on two normal blood samples, a blood sample from a patient with sickle cell disease (Anemic-1), and on two Negro males hemizygous for G-6-PD deficiency. Digitonin was used as the lytic agent. Left: Illuminated with visible light. Right: Illuminated with longwave UV light.

sents special problems. These are common to all detection procedures which, as this one, use cell lysates. The efficacy of this procedure in detecting heterozygotes has not been evaluated, but it can be presumed that a certain proportion of them, possibly about 60 per cent, could be identified by making spots at frequent intervals. A special feature of the technic is that it is, to a large extent, self-correcting for anemia. The decreased enzyme activity which may be found in anemic whole blood samples is counterbalanced by the decreasing quenching of fluorescence, brought about by its lowered hemoglobin content (Fig. 2). The reaction occurs sufficiently rapidly, even at 0 C., that the test should be initiated within 5 or 10 minutes of the time that the blood is added to the reaction mixture. If, as in a field screening program, an incubator is not immediately available, the reaction may be allowed to proceed at room temperature. In this procedure, as well as the others described below, it is essential that the spots are dry before they are evaluated under UV light. The intensity of fluorescence is markedly diminished when the spots are moist. Once the spot is dry, the biochemical reaction comes to a halt and fluorescence is easily detectable for several days.

II. Pyruvate Kinase

A. Reaction Sequences

When pyruvate kinase is present in the hemolysate, a phosphate group from phospho(enol)pyruvate is transferred to ADP, forming pyruvate and ATP. Lactate dehydrogenase in the hemolysate catalyzes the reduction of pyruvate to lactate with the oxidation of the reduced diphosphopyridine nucleotide (DPNH) in the reaction mixture to diphosphopyridine nucleotide (DPN). Since DPNH fluoresces and DPN does not, there is gradual loss of fluorescence.
**Fig. 2—The effect of anemia on results of the screening test for G-6-PD deficiency.**

Blood from one normal and one G-6-PD deficient (male Negro) subject was studied by artificially adjusting hematocrit levels by addition of plasma to packed cells. Saponin was used as the lytic agent. The spots were all made after 3 minutes incubation at 37°C. It is apparent that the test is self-compensating for anemia: in anemic samples the decreased enzyme activity is counterbalanced by the decreased quenching effect of the hemoglobin. Top: Illuminated with visible light. Bottom: Illuminated with long-wave UV light.

**B. Reaction Mixture**

- Phosphoenolpyruvate (PEP) (tricyclohexyl ammonium salt) .15 M (neutralized)\(^*\) \[0.03 \text{ ml.}\]
- ADP .03 M (neutralized)\(^*\) \[0.10 \text{ ml.}\]
- DPNH .015 M (neutralized)\(^*\) \[0.10 \text{ ml.}\]
- MgSO\(_4\) .08 M \[0.10 \text{ ml.}\]
- Potassium phosphate buffer.
  - pH 7.4 .25 M .05 ml.
  - H\(_2\)O .05 ml.
  - H\(_2\)O .62 ml.

**C. Preparation of Blood Sample**

A heparinized, EDTA, or ACD anticoagulated blood sample is centrifuged and the plasma and buffy coat are carefully aspirated. A 20 per cent suspension of red cells is prepared by adding 4 volumes of physiologic saline solution.

**D. Test Procedure**

One volume of cell suspension (.020 ml.) is added to 10 volumes of reaction mixture (0.20 ml.). The mixture is incubated at 37°C for 30 minutes. As indicated in Figure 2, the fluorescence has disappeared from normal samples by this time, while fluorescence persisted in the two pyruvate kinase deficient samples tested.

\(^*\)Neutralized to pH 7–8 with pH paper using approximately 0.2 N NaOH.
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E. Stability of the Reaction Mixture

The reaction mixture is stable for at least 4 days but less than 10 days at -20 C. It is stable to lyophilization,* and the lyophilized material, under N₂, is stable for at least 3 months at 4 C. or -20 C.

F. Comments

PK deficient subject #1 had received 1000 ml. of blood approximately 1 month previously. The gradual loss of fluorescence observed presumably represents the enzyme activity of transfused cells. The specificity of the method is further confirmed by the fact that omission of either PEP or ADP from the reaction mixture results in a total failure of defluorescence. Because of the instability of DPNH, it is our practice to standardize solutions spectrophotometrically at 340 nm. A 1:1000 dilution of .015 M solution in phosphate buffer should have an O.D. of approximately .093. Freshly prepared solutions should be satisfactory for screening purposes without prior standardization. White cell pyruvate kinase activity is normal in subjects with hereditary pyruvate kinase deficiency. For this reason, it is desirable to remove the buffy coat in performing a screening test for pyruvate kinase deficiency. In addition, hypotonic lysis is employed to release enzyme from erythrocytes, rather than digitonin or saponin as employed in the other two screening procedures. It has been found that little enzyme is released from white cells using this reaction mixture, while release of enzyme from white cells by digitonin or saponin is equivalent to that obtained with freezing and thawing.

III. Glutathione Reductase

A. Reaction Sequence

When glutathione reductase is present in the hemolysate, oxidized glutathione (GSSG) is reduced to GSH and TPNH is oxidized to TPN. Since TPNH fluoresces when activated with UV light and TPN does not, there is gradual loss of fluorescence as the reaction proceeds.

B. Reaction Mixture

GSSG, .003 M 0.1 ml.
TPNH, .015 M 0.1 ml.
Potassium phosphate buffer.
    pH 7.4, .25 M 0.6 ml.
Digitonin, saturated solution.
    or Saponin (Mann), 1% 0.2 ml.

C. Preparation of Blood Sample

Heparinized, ACD, or EDTA blood may be employed. Samples stored for at least 3 weeks may be used.

D. Test Procedure

One volume of blood (usually .02 ml.) is added to 10 parts of reaction mixture (usually .20 ml.). Spots are made on filter paper every 15 minutes and the disappearance of fluorescence is compared with a control sample (Fig. 4).

E. Stability of the Reaction Mixture

The reaction mixture is stable for at least 10 days when stored at -20 C. It is stable to lyophilization, and the lyophilized material, under N₂, is stable for at least three months at 4 C.

*Lyophilization was carried out through the courtesy of Dr. Allan Louderback, Hyland Laboratories, Los Angeles, California.
Fig. 3.—The results of the examination of 7 blood samples for PK activity. A 20 per cent suspension of red cells in saline was prepared from each sample after centrifugation and aspiration of the buffy coat. The sample designated “Anemic-1” came from a patient with sickle cell disease; sample “Anemic-2” came from a patient with autoimmune hemolytic disease. PK deficient subject #1 had received 1000 ml. of blood approximately 1 month previously. The last two tests represent the addition of a normal sample to reaction mixtures from which phospho(enol)pyruvate or adenosine diphosphate had been omitted. Left: Illuminated with visible light. Right: Illuminated with long-wave UV light.
Fig. 4.—The results of tests for GSSG-R deficiency carried out on three blood samples from clinically normal subjects. The enzyme activity of the red cells of each subject was assayed quantitatively by measuring the rate of GSSG reduction in 0.05 M potassium phosphate buffer, pH 7.0, at 37°C. The units given represent micromoles of GSH formed/gram hemoglobin/minute. It is apparent that the rate of disappearance of fluorescence is proportional to the GSSG-R activity. The specificity of the method is further confirmed by the fact that fluorescence does not disappear when GSSG is omitted from the reaction mixture. Saponin was used as the lytic agent. Top: Illuminated with visible light. Bottom: Illuminated with long-wave UV light.

F. Comments

Because of its instability, TPNH solutions were standardized in the same way as DPNH solutions (see above). However, this should not be required for screening purposes. Glutathione reductase deficiency appears to be quite rare; only one case has been reported from the United States.7 Numerous cases have been collected in Germany, however.8 Because of the rarity of this disorder we have not yet been able to study a case of clinically significant glutathione reductase deficiency utilizing...
this technic. However, there is marked individual variation in the activity of this enzyme in clinically normal subjects. As shown in Figure 4, the disappearance of fluorescence closely parallels the enzyme activity as assayed quantitatively. The specificity of the procedure is confirmed by the failure of fluorescence to disappear from a control mixture without the specific substrate, GSSG.

**DISCUSSION**

Although deficiencies of many red cell enzymes have been described, relatively simple screening procedures have been developed previously only for the detection of glucose-6-phosphate dehydrogenase deficiency. In these procedures reduction of TPN has been linked to a visible receptor, or to endogenously formed methemoglobin through methylene blue. Many of the procedures described are quite satisfactory, but each has some disadvantages. Thus, the dye decolorization technics involving brilliant cresyl blue or methylene blue as receptors require anaerobic conditions. The spot test procedure employing the tetrazolium dyes as receptor requires separation of hemoglobin from enzyme on DEAE paper, and the methemoglobin reduction test requires fresh blood samples and the estimation of methemoglobin levels. The procedure described here is extremely simple, does not require anaerobic conditions and can be carried out at room temperature or at 37°C, requires only a minute quantity of blood, can be carried out on stored blood, and is very easy to interpret. The only apparent disadvantage is the requirement for a source of long-wave UV light, a condition which can be met readily in most laboratories at very little cost. The procedure has not been evaluated for heterozygote detection. None of the screening procedures for G-6-PD deficiency are highly efficient for heterozygote detection, since varying proportions of normal and deficient red cells coexist in heterozygotes. Only specially designed procedures, taking this into account, are suitable.

The screening procedure for pyruvate kinase deficiency should also prove to be a considerable convenience. One other method has been described by Brunetti and Nenci. It depends upon a change in color of pH sensitive dye.

No method for screening for glutathione reductase deficiency has been described. It is possible that this disorder is more common than has heretofore been suspected. Indeed, Waller et al. have reported finding numerous cases in Germany, some with neurologic complications. Screening for this disorder among patients with hemolytic disease, and perhaps even among nonanemic patients with neurologic disorders, may give a truer estimate of its prevalence in other populations.

The principle employed in these screening procedures is readily adaptable to the screening for other enzymatic abnormalities. Thus, merely substituting 6-phosphogluconic acid for glucose-6-phosphate in the screening procedure for glucose-6-phosphate dehydrogenase, produces a screening procedure for phosphogluconic dehydrogenase deficiency. We have used the same method to devise an extremely satisfactory screening method for galactosemia, which has been described in detail elsewhere. The method may also be applicable to screening for triosephosphate isomerase deficiency.
As presented here, the methods have been used to detect fully developed defects. By examining spots made at appropriate intervals, it is also possible to use the same approach for heterozygote detection.

In a preliminary study using our procedure on coded samples, it has been possible to detect all three homozygotes and four of five heterozygotes for PK deficiency using this procedure. The reaction of the fifth heterozygote was considered to be equivocal.

**Summary**

A new type of screening procedure for the detection of enzymatic defects of the red cell has been described. The blood or red cell sample is added to the reaction mixture. After a suitable period of incubation a drop of the mixture is spotted on filter paper, permitted to dry, and examined for fluorescence under UV light. In this way the oxidation of reduction of pyridine nucleotides is readily evaluated. Reaction mixtures for the detection of glucose-6-phosphate dehydrogenase deficiency, pyruvate kinase deficiency, and glutathione reductase deficiency are described. The same general procedure should be readily adaptable to the detection of other enzymatic deficiencies of red cells, such as phosphogluconate dehydrogenase deficiency or triosephosphate isomerase deficiency.

**Summary in Interlingua**

Es describite un nove typo de technica pro le detection de defectos enzymatic in le erythrocytos. Le specimen de sanguine o de erythrocytos es addite al mixtura de reaction. Post un appropriate periodo de incubation, un gutta del mixtura es deponite super papiro-filtro e, post su desiccation, examine pro fluorescentia sub lumine ultraviolette. In iste maniera, le oxydation o reduction de nucleotidos de pyridina es facilemente evalutate. Es describite le mixturas de reaction pro le detection de carentia de dehydrogenase de glucosa-6-phosphato, de carentia de kinase de pyruvato, e de carentia de reductase glutathionic. Il pare pauc dubitabile que le mesme technica general es prestemente adaptabile al detection de altere carentias enzymatic in le erythrocytos. Tales includerea carentia de dehydrogenase de phosphogluconato o carentia de isomerase de triosephosphato.

**Acknowledgments**

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