A Simple Method for Detection of Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency (G-6-PD Spot Test)

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During the past few years it has been clearly demonstrated\(^2\)\(^-\)\(^4\) that deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) is the basis for certain drug-induced hemolytic anemias. This condition is genetically determined\(^5\) and has been found throughout the Mediterranean littoral, in Southeast Asia, and in approximately 11 per cent of American negroes.\(^3\)\(^-\)\(^4\) Substances capable of inducing hemolysis in affected individuals include fava beans, antimalarial drugs such as primaquine, sulfonamides, and a number of other drugs, many of which are in common use.\(^3\)\(^-\)\(^4\) A type of congenital nonspherocytic hemolytic anemia is also associated with this enzymatic defect.\(^6\)\(^-\)\(^9\)

In view of its high incidence among American Negroes, G-6-PD deficiency represents one of the commonest potential causes of hemolytic anemia in the United States. A similar situation exists in many other areas of the world. For this reason, there has been considerable demand for a simple, inexpensive, and rapid method for detection of this condition. The procedure outlined below has been developed to meet this need. It will be seen that the major advantages of this method are its simplicity and the stability and reliability of reagents.

Materials* 

a. The dipotassium salt of glucose-6-phosphate and the monosodium salt of triphosphopyridine nucleotide were obtained from Sigma Chemical Co. of St. Louis, Mo., and from California Corp. for Biochemical Research.

b. Phenazine methosulfate was obtained from Sigma Chemical Co. It is a light brown powder, lemon-yellow in aqueous solution. It is very unstable to light. Stability is enhanced by acidification.

c. MTT [3(4,5 dimethylthiazoly 1-2)2,5 diphenyltetrazolium bromide] is produced by Nutritional Biochemical Co. of Cleveland, Ohio; by Gurr, Ltd. of London, England; and by Dr. H. Harms of Leverkusen, German Federal Republic. Samples have been tested from each of these sources and are fully reactive in the system described below. In the oxidized state, MTT is a light yellow powder which is slightly soluble in water. In the reduced (formazan) state, it is purple, water-insoluble, heat-stable, and relatively light-stable.

*Since, in most hospitals, it may be inconvenient to prepare these reagents except for large-scale screening and research purposes, a reagent kit will be commercially available through California Corporation for Biochemical Research (CalBiochem), 3625 Medford Street, Los Angeles 63, Calif.

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Glucose-6-Phosphate 1.2 mg.
Potassium Phosphate Buffer 0.004 mMoles pH 7.0

Phenazine Methosulfate 0.002 mg.
TPN 0.1 mg.

Fig. 1.—Diagram of reagent tube. Reagents are applied and dried on the inner surface of the tube at the sites and in the amounts indicated. They are subsequently redissolved and mixed in 0.1 cc. of distilled water.

d. Whatman DE-20 paper is produced by W. R. Balston, Ltd., London, England. In the United States, it is distributed by Reeve-Angel & Co., 52 Duane Street, New York 7, N. Y., and on the west coast of the United States by Braun Chemical Co. It is a diethylaminoethyl cellulose paper which strongly binds glucose-6-phosphate dehydrogenase.

The reagents listed under a, b, and c are unstable if mixed together in solution. However, they may be applied separately to the inner surface of a tube at different sites, dried, and subsequently mixed in solution prior to use. Such a tube is illustrated in figure 1. The indicated amounts of reagents are sufficient for dilution in 0.1 ml. of solution.

To prepare a spot test reagent tube, 0.02 ml. of a 10 mg./ml. aqueous solution of MTT is first applied to the inner surface of the tube and dried at 90 C. for approximately 90 minutes. Two mg. of phenazine methosulfate is dissolved in 20 ml. of distilled water, and this is acidified to pH 3.8 with approximately 0.2 ml. of 0.01 M hydrochloric acid. Five mg. of TPN is dissolved in 1.0 ml. of this solution. Two-hundredths ml. of the TPN-acid-phenazine methosulfate mixture is applied to the bottom of the tube and dried at 90 C. for approximately 90 minutes. Finally, 120 mg. of glucose-6-phosphate is dissolved in 1.0 ml. of 0.2 M potassium phosphate buffer, pH 7.0. One-hundredth ml. of the glucose-6-phosphate-phosphate buffer solution is applied to the side of the tube and dried overnight at 40 C. Thus prepared, the reagent tube may be exposed to room light and left at room temperature for at least 6 months with only slight loss of activity. Also, exposure to a constant temperature of 120 F. for 1 week has been found not to alter the potency of the reagents. Once the reagents have been dissolved, they should be used within a few hours, although they are stable for several hours if kept in the dark. Each tube contains sufficient reagents for at least ten spot tests. The current cost of reagents is approximately $0.025 per tube.

Since it reacts nonspecifically with MTT, hemoglobin must be separated from the glucose-6-phosphate dehydrogenase. This is accomplished by the use of DE-20 paper, pretreated so that hemoglobin may be easily eluted, while the paper binds G-6-PD avidly. As pretreatment, DE-20 paper is saturated with 0.02 ml. of 0.26 M, pH 5.0, potassium phosphate buffer per square cm. and dried. DE-20 paper so treated is probably stable indefinitely.
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METHODS

a. Spot Test

1. Procedure: For these studies, 5.0 ml. of blood is mixed with 1.0 ml. of ACD solution. Heparin, balanced oxalate, and potassium ethylene diamine tetraacetate (Versene, Sequestrene) are also satisfactory as anticoaguants. Heparinized capillary tubes may be used for this purpose. To correct for variations in hematocrit, blood is sedimented overnight at 5 C. This simple maneuver obviates the need to determine hematocrit, and the correction for anemia which has been used in other screening methods. Centrifugation gives equally satisfactory results. The packed or sedimented erythrocytes are taken from the bottom of the tube with a Pasteur pipette. Excess blood and plasma are wiped from the surface of the pipette. Erythrocytes are applied to both sides of the treated DE-20 paper over a circular area 5 mm. in diameter. The paper is saturated in this manner, and excess blood is removed by inverting the pipette while the tip is in contact with the paper. Approximately 0.008 ml. of packed cells is applied by this method. The paper is placed in distilled water at room temperature for approximately 20 minutes. It is then carefully removed from the distilled water, and blotted between sheets of filter paper. It is again placed in distilled water for approximately 20 minutes and again blotted with filter paper.* Hemoglobin is thus eluted from the paper, leaving only a faint yellow to tan spot. It is sometimes necessary to immerse the paper briefly a third time to remove traces of hemoglobin. Prior to testing with the spot test reagents, as much water as possible is blotted from the DE-20 paper. The substances in a spot test reagent tube are then redissolved with 0.1 ml. of distilled water. It is essential that all components be brought into solution by vigorous stroking with a glass stirring rod, taking special care to cover the almost invisible, glassy deposit of glucose-6-phosphate and phosphate buffer. The spot test reagent solution is applied to the spots on the DE-20 paper. A strong purple color develops in 2 minutes with normal erythrocytes; little or no color develops with G-6-PD deficient erythrocytes. Interpretation of the test is, therefore, made at 2 minutes. Further intensification of color occurs with the passage of more time, and the reaction may be stopped at any point by flooding the paper with distilled water at 60-100 C. This often results in intensification of color, and for this reason the test is interpreted prior to treatment with hot water. If a semipermanent record is desired, the paper may be treated in this fashion to terminate the reaction, dried, and thereafter kept in a dark envelope. Figure 2 demonstrates the relationship of color intensity to time from the application of spot test reagent.

2. Precautions: The G-6-PD spot test method has been devised to allow maximum simplicity and ease of performance. To achieve this, some degree of sensitivity has been sacrificed. Thus, only one quantitative measurement is required, the pipetting of 0.1 ml. of distilled water into the reagent tube. Furthermore, since none of the substrates is rate-limiting, even this measurement may be made approximate. It might be questioned whether the method of applying the red cells to DE-20 paper is sufficiently accurate. This problem has been studied by measuring the amount of hemoglobin eluted from 21 such spots prepared in the usual manner from single blood samples. The hemoglobin content of the spots averaged 2.1 mg., and ranged from 1.8 to 3.5 mg. This represents a range of 0.006 to 0.013 ml. of packed erythrocytes, with a mean of 0.008 ml. Except in one instance, the volume of packed red cells applied did not vary more than 18 per cent from the mean. It should be pointed out that the enzyme activity in erythrocytes of primaquine-sensitive male subjects is rarely higher than 20 per cent of normal. Therefore, in practice, there seems to be no problem of visual differentiation between the reactions of red cells

*These time intervals were selected for convenience, and need not be rigidly adhered to. Repeated blotting of the spots after brief immersions is acceptable, and may be time-saving.
derived from normal subjects vs. those of sensitive males, and false positive or false negative results have not been observed. For this reason, it seems unnecessary to use micropipettes for measuring the volume of red cells. Furthermore, application of an excessive volume of packed cells does not appear to give rise to increased color intensity. Possibly, excess erythrocytes are washed from the paper immediately upon immersion. It is important, of course, that each spot be completely saturated. It is advised that any excess be pipetted off the paper.

Once the packed erythrocytes have been applied to the DE-20 paper, the latter should be immersed in distilled water without undue delay. It has been found that a delay of 30 minutes or longer results in fixation of erythrocyte pigmented substances, presumably hemoglobin degradation products, to the paper. These interfere with interpretation of the spot test. If large numbers of blood samples are to be tested, it is convenient to do these in groups of 10 or 20, so that this problem may be avoided.

Glucose-6-phosphate dehydrogenase is relatively stable in the intact cell. Its activity decays rapidly following hemolysis, and the rate of decay is a function of temperature. Therefore, manipulations of hemolysates prior to assay for G-6-PD activity are usually done at near-freezing temperature. All steps of this procedure have been standardized at room temperature, with cognizance of the fact that enzyme activity is lost gradually during the procedure. It has been found that appreciable activity persists if the DE-20 paper stands in distilled water at room temperature as long as 2 hours before application of reagent. After 3 hours, sufficient G-6-PD activity is lost so that only a faint purple color develops with normal erythrocytes. It is advised, therefore, that the entire procedure be completed within an hour in order to obtain strong contrast between normal and G-6-PD deficient cells.

The test reagents, prepared as outlined above, should be used within an hour after they are redissolved, if kept in the light. However, if shielded from light, they are stable for several hours. It is preferable to perform 10 or more tests simultaneously, and to discard any excess reagents. Two or three normal controls should be included in each series of tests. (Non-anemic subjects of northern European ancestry may be assumed to have normal G-6-PD activity.)

b. Quantitation of Formazan Generation in Spot Test

In order to allow quantitative comparison of the simple spot test procedure with standard methods, a measure was made of the amount of formazan generated. After the addition of the spot test reagent, the spots were allowed to develop color for about 10 minutes. At this time, the color was quite intense, and the DE-20 paper was immersed in hot water. The paper was then dried, and the formazan pigment was eluted from each spot with a 10:1 ether-acetone solution. The amount of formazan so extracted was determined by measuring light absorption spectrophotometrically at 550 nm, the absorption maximum for this substance.

c. GSH Stability Test

This was performed as described elsewhere by one of the authors.10

d. Spectrophotometric Assay of TPNH Generation

A modification of the method of Kornberg and Horecker11 was used for this assay. Blood was collected in ACD solution, and was centrifuged at 550 g in an International #1 centrifuge at 4 C. for 10 minutes. Plasma and buffy coat layer were removed and the erythrocytes were washed twice with cold isotonic sodium chloride solution. Two-tenths ml. of the packed erythrocytes was hemolyzed by the addition of 1.8 ml. of cold distilled water. The hemolysate was then centrifuged at 5,000 g for 30 minutes at 4 C. to remove stroma. The assay system contained 0.5 ml. of 0.05 M glucose-6-phosphate, 0.5 ml. of 0.25 M glycyl-glycine buffer at pH 7.6, 0.17 ml. of 0.3 M magnesium chloride, 0.2 ml. of 0.001 M triphosphopyridine nucleotide, and 1.08 ml. of distilled water. Five-
Fig. 2.—Appearance of DE-20 paper strip at various stages of test for G-6-PD.
Top row in each panel represents males, of which the fourth and eighth spots are derived from G-6-PD-deficient erythrocytes. Bottom rows represent females, of which fourth, sixth, eighth, and tenth spots are derived from subjects heterozygous for G-6-PD deficiency.

a. After application of sedimented RBC’s.
b. After elution of hemoglobin in distilled water.
c. One minute after application of reagent.
d. Two minutes after application of reagent.
e. Four minutes after application of reagent.
hundredths ml. of hemolysate was added to this solution. Absorption was measured in a Zeiss PMQ II spectrophotometer at a wavelength of 340 mμ with a light path of 1 cm. Optical density readings were made over a period of 5 minutes. The rate of increase in optical density was linear in all but a few instances, in which there was a lag in the first few minutes. A linear segment of the reaction curve was selected and the mean increment in optical density/minute was determined from this. The hemoglobin content of the hemolysate was determined by standard spectrophotometric assay of cyanmethemoglobin. Enzyme activity was calculated as units of optical density change per minute per gram of hemoglobin. By this method, mean normal value was 9.1 U./min./Gm. hemoglobin in 58 subjects. The sample standard deviation was 2.0 U./min./Gm. hemoglobin.

RESULTS

In view of the many procedures now available for the detection of G-6-PD deficiency, any new method must be shown to have advantages not only in terms of simplicity, convenience, time required, and cost, but must also demonstrate a degree of reliability approximating that of accepted methods. To illustrate such correlation, samples were obtained from 10 males and 10 females, including two primaquine-sensitive males and four females heterozygous for G-6-PD deficiency. These samples constituted the sources of the spots shown in figure 2, the top row of each panel representing males, and the lower row representing females. After approximately 10 minutes the reaction was terminated with hot water, and formazan was eluted from each spot and measured spectrophotometrically. The data thus obtained were compared with the post-incubation GSH values, and with the units of enzyme activity. Correlation with enzyme activity is presented in figure 3, and that with the post-incubation GSH values in figure 4. It is apparent that good correlation was found with each of these methods, and the relationship with the direct enzyme assay appeared to be almost linear. This relationship makes valid the application of the MTT dye method as a semiquantitative enzyme assay.

DISCUSSION

All present methods for detection of erythrocyte G-6-PD deficiency depend ultimately upon the reduction of triphosphopyridine nucleotide by glucose-6-phosphate, as indicated in reaction I:

Reaction I:
G-6-PD
Glucose-6-phosphate + TPN → 6 phosphogluconate + TPNH

The conventional spectrophotometric enzyme assay measures the rate of increase in absorption of ultra-violet light by TPNH. Other methods of assay link TPNH generation of reaction I to the reduction of other compounds, e.g., glutathione, dichloroindophenol (DCIP), brilliant cresyl blue, and methemoglobin. Of the several methods currently in use, some are moderately elaborate spectrophotometric assays not readily adaptable for use in the routine clinical laboratory. There are, however, four non-spectrophotometric methods which have been reported as suitable for use in the field. These are the Heinz body test, the brilliant cresyl blue dye test, the
Fig. 3.—Correlation between GSH stability test postincubation values, and formation of formazan pigment following application of test reagent to samples represented in Fig. 2. Arrows indicate specimens abnormal by GSH stability test which were normal by spot test. Spectrophotometric enzyme assay by TPNH generation confirmed these samples as normal.

methemoglobin reduction test, and the DCIP decolorization test. The Heinz body test may give rise to false positives, especially if no correction is made for anemia. It has not been widely adopted. The brilliant cresyl blue dye test depends upon decolorization of the test mixture from blue to violet. The influence of anemia on the results of this test has not been well defined, but anemia probably produces false positive results in this test as well. A major problem with this test has been the failure of most lots of brilliant cresyl blue dye to decolorize at a uniform rate. Indeed, some lots do not decolorize at all. A third screening method is based upon differential reduction of methemoglobin. However, this method employs seven reagents, some of which are unstable, and several quantitative measurements are required. Finally, a screening method which depends upon the decolorization of DCIP (dichloroindophenol) is commercially available through Sigma Chemical Co. This method is very similar to the brilliant cresyl blue test, except for the substitution of DCIP for the former dye. Again, in this process, the in-
fluence of anemia upon test results has not been determined. The hydrogen carrier, phenazine methosulfate, used in this procedure is unstable unless kept dark and dry. Multiple quantitative measurements and the use of a 37°C water bath with prolonged (100 minutes and six hours) incubation seem to be further disadvantages. While it may be that this procedure will be found adequate, no studies of the method have been reported by independent investigators.*

It would seem that the problems inherent in the previously described procedures detract from their usefulness for the clinical laboratory or for the practicing hematologist.

Tetrazolium dyes have been used for some time in the histochemical staining of dehydrogenases, and it appeared feasible to employ such dyes as indicators of G-6-PD activity. A large number of these dyes have been studied, and it was found that only those with higher redox potentials (nitro-BT, MTT, and INT) were suitable. The relative insolubility of nitro-

*Since this paper was accepted for publication, there has been a report23 of a clinical study using a modification of DCIP decolorization test.
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BT made this dye undesirable for our purposes. INT (2p iodophenyl 3p nitro-phenyl-5-phenyltetrazolium chloride) produced a rose-colored spot of moderate intensity. Although results with this dye in the system described above gave fairly satisfactory results, it was felt that the similarity of the color of the formazan pigment to that of hemoglobin would make this dye less desirable than those which produce a blue or purple color. Neotetrazolium has also been studied in this system. This substance has been employed by Marks21 and others for electrophoretic studies of G-6-PD. However, in the system employed for the G-6-PD spot test, this dye produces a faint lavender color only after prolonged incubation, and is entirely unsuitable. MTT was found to be the most practical tetrazolium dye for this procedure. It was also found necessary to employ the hydrogen carrier, phenazine methosulfate, in the system, as indicated in reaction II:

Reaction II:

\[
\text{MTT} + \text{TPNH} \rightarrow \text{TPN} + \text{MTT formazan}
\]

Since it was found that phenazine methosulfate could be stabilized by acidifying it to pH 3.8, followed by drying in the presence of TPN, it is unnecessary to take special precautions to keep it dark and dry as required in another method.16 Reaction II results in the precipitation of the strongly colored formazan, and, as has been shown, the amount of formazan formed is proportional to the amount of TPNH generated in reaction I above.

In addition to the subjects represented in figure 2, the authors have tested a large group of normal subjects, as well as Caucasian and Negro subjects deficient in G-6-PD, and a large family with congenital nonspherocytic hemolytic anemia associated with erythrocyte G-6-PD deficiency. In all instances, the results of the spot test have correlated satisfactorily with the spectrophotometric enzyme assay, and no inconsistencies have been observed. The method has also been employed in several other laboratories, where satisfactory results have also been observed. Although many heterozygous females have been identified by this method, it should be pointed out that such an individual cannot be reliably recognized by the spot test technic unless, as is sometimes the case, her enzyme activity is moderately to markedly depressed. On the other hand, it is doubtful that a heterozygote can be recognized with more than 80 per cent accuracy by any currently available method for screening or quantitative assay of G-6-PD. This problem has been studied by the authors and is the subject of another report.22 It is also clear that heterozygotes with normal or slightly reduced G-6-PD activity are not likely to suffer from clinically significant hemolysis. Thus, it is felt that this simple method will detect those individuals with significant hemolytic proclivity with nearly 100 per cent accuracy.

SUMMARY

A method is described for detection of erythrocyte glucose-6-phosphate dehydrogenase deficiency, based upon the reduction of the tetrazolium dye, MTT, by reduced triphosphopyridine nucleotide. The method is highly re-
liable and correlates well with established methods of enzyme assay. The test is sufficiently simple to perform that it may be done in any routine clinical laboratory or physician’s office. The reagents may be prepared in mailable, stable form at minimal expense.

**SUMMARIO IN INTERLINGUA**

Es describite un methodo pro le detection de un carentia erythrocytic in dishydrogenas de glucosa-6-phosphato. Le principio del methodo es le reduction del colorante MTT (derivato de tetrazolium) per reducite nucleotida triphosphopyridinic. Le methodo es altemente fidel e monstra un bon correlation con le establite methodos de essayage enzymatic. Le test es suficientemente simple a executar de manera que illo pote esser tractate routinarimente in non importa qual laboratorio clinic o mesmo in le laboratorio private del medico individual. Le reagentes pote esser preparate in forma stabile e capace a esser inviate per posta. Lor costo es basse.

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**REFERENCES**

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