Automated Dithionite Test for Rapid, Inexpensive Detection of Hemoglobin S and Non-S Sickling Hemoglobinopathies

Robert M. Nalbandian, Bruce M. Nichols, Frank R. Camp, Jr., Jeanne M. Lusher, Nicholas F. Conte, Raymond L. Henry, and Paul L. Wolf

Automated adaptations of dithionite and urea–dithionite tube tests are accurate, reliable, inexpensive methods for detecting hemoglobin S. More than 3,000 individuals have been screened (120 determinations per hour; reagent cost, 2 to 4 cents per test). The dithionite reagent consists of potassium phosphate, sodium dithionite, and saponin. When S hemoglobin contacts this reagent, the red cells lyse, and the hemoglobin deoxygenates and sickles, forming a hydrophobic-bond-dependent nematic liquid crystal system that is manifested as turbidity. The resulting AutoAnalyzer curve is strikingly and diagnostically different from that produced by hemoglobin A in the same reagent. Specificity of the automated dithionite test may be enhanced by use of the automated urea–dithionite test, which consists of a specimen set of two aliquots: one traverses a dithionite line, the other a urea–dithionite line. A comparison of transmittance in the two lines yields typical diagnostic curves because the urea disperses the sickling, with a consequently increased transmittance over that of the dithionite aliquot. Methods are discussed for recognizing non-S sickling hemoglobins and a few other rare hemoglobinopathies.

Additional Keyphrases sickle cell disease • sickle cell hemoglobin • automated urea–dithionite test • nematic liquid crystal system • hydrophobic bonds • AutoAnalyzer

In this paper we present the automation of the dithionite tube test and the urea–dithionite tube test discussed in the immediately preceding paper (1). With the automated dithionite test, it is possible to screen 120 hemoglobin specimens per hour at a reagent cost of approximately 2 to 4 cents each. With the automated urea dithionite test, the results are more specific but the rate is half that of the automated dithionite test. Thus, about 60 determinations per hour are performed at about 4 to 8 cents per set. Both of these automated methods have proven to be highly reliable in extended field trials in both military and civilian populations.

These automated tests have a molecular basis, reviewed in detail in the preceding paper (1). The reagents consist of potassium phosphate, sodium dithionite, and saponin. When sickling red cells are introduced into such a solution, they lyse immediately, the hemoglobin deoxygenates, the beta globin chains of each hemoglobin tetramer are displaced laterally, complementarity of steric fit between interacting hemoglobin tetramers is achieved in accordance with the Murayama hypothesis for the molecular mechanism of sickling, a nematic liquid system is formed, and in the presence of hemoglobin S or non-S sickling hemoglobins the system becomes turbid. On addition of urea, those nematic liquid systems dependent on hydrophobic bonds are dispersed, whereas others will persist. Thus, on theoretical

From Blodgett Memorial Hospital, Grand Rapids, Mich. 49506 (R.M.N., B.M.N.); U.S. Army Medical Research Laboratory, Fort Knox, Ky. 40121 (F.R.C., N.F.C.); School of Medicine, Wayne State University, Detroit, Mich. 48207 (R.M.N., J.M.L., R.L.H.); and Stanford University Medical Center, Stanford, Calif. 94305 (P.L.W.).
Address reprint requests to R.M.N.
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1 Editor’s Note: “Related to or being in a mesomorphic state, which is the first stage in forming a solid from a liquid melt, and in which the orientation of the molecule...is in parallel, but not layers” (Webster).
grounds this simple test, coupled with molecular insights and electrophoresis of all positive specimens, promises to yield an impressive quantity of information on hemoglobinopathies that, at present, are overlooked.

**Methods**

1. Blood specimens were obtained locally from patients at Blodgett Memorial Hospital and were processed at that facility. Blood specimens were also obtained from Children's Hospital, Wayne State University School of Medicine and mailed to Blodgett Memorial Hospital, where they were processed. Also, another group of patients consisted of recruits at the U.S. Army Medical Research Laboratory at Fort Knox, Kentucky, and specimens so obtained were processed at that facility. The study was so designed to show that two laboratories could reproduce results from the same methods and also that specimens appropriately collected could be mailed at ambient temperatures without adversely affecting the results.

2. The 2% sodium metabisulfite test (2).

3. Hemoglobin electrophoresis at pH 8.4.¹

4. Improved Murayama test (3).

5. Automated dithionite test.


**Materials**

Components shown in Figure 1 are standard AutoAnalyzer modules (Technicon Instruments Corp., Tarrytown, N.Y. 10591).

**Stock buffer.** (1.18M KH₂PO₄ and 1.615M K₂HPO₄). Place in a 1000-ml volumetric flask about 800 ml of distilled water. Add 160.48 g of KH₂PO₄ and 281.88 g of K₂HPO₄, dissolve and dilute to volume.

**Dithionite working solution.** To a 1-liter volumetric flask add 800 ml of the stock buffer. Add 20.0 g of Na₂S₂O₄ and mix until dissolved. Add 60 ml of saponin solution “Zaponin” (Coulter Diagnostics, Inc., Hialeah, Fla. 33010) and sufficient distilled water to make one liter.

**Procedure—Automated dithionite test**

1. Assemble the manifold as shown in Figure 1.

2. Place reagent lines 4 and 6 in dithionite working solution. Place the saline lines into the saline. Start pump and allow 10 min to fill the system with reagents.

3. Adjust the baselines on both recorders to 95% transmittance.

4. Fill one or two cups with 0.5 to 2.0 ml of normal blood (collected in EDTA). Fill 20 cups with the unknowns. After the 20th cup place one cup of saline, one or two cups of normal blood, and 20 cups of unknowns. This loading pattern should be maintained throughout; it is easier to label the peaks if they are in sets of 20.

5. Start mixers and samplers.

6. After all specimens have passed through the system, remove the charts and label the peaks. The “positive” specimens (Figure 2) will have a 15 to 20% (or more) lower transmittance than those specimens that are normal by this procedure. The presence of hemoglobins other than hemoglobin-S in the “positive” specimens should be determined by an electrophoretic pattern.

7. When all specimens have been screened, rinse the system with 1N NaOH (plus 20 ml of “Tergitol NPX” per liter) for 15 min, then with distilled water for 15 to 20 min.

**Procedure—Automated urea–dithionite test**

1. Assemble the manifold and the other Technicon modules as shown in Figure 3.

2. The dithionite working solution is the same as described above.

3. Take one-half of the reagent from step 2 (dithionite working solution) and add sufficient urea to make a 2 mol/liter solution.

4. Place all reagent lines in appropriate reagents. Start pump. Allow 10 min for the system to fill with reagents.

5. Adjust the baseline of the dithionite line (without urea) to 90% transmittance on the chart paper. Adjust the urea-dithionite line to 70% transmittance.

6. Fill three or four cups with 0.5 to 2.0 ml of well-mixed normal blood (collected in EDTA). Fill 20 cups with the unknowns. After the unknowns,

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¹ Method of Helena Laboratories, P. O. Box 752, 1530 Lindbergh Drive, Beaumont, Texas 77704.
mittance on the dithionite line and 30% transmittance on the urea-dithionite line. This pattern of "positive" specimens will only occur with hemoglobin-S and hemoglobin-C (Harlem) (a structural variant of hemoglobin-S). The peaks of both lines will have a lower transmittance if the specimen is a non-S sickling hemoglobin.

9. After the run is completed, rinse the system with 1x NaOH (plus 20 ml of Tergitol NPX per liter) for 15 min, then rinse the system with distilled water for 15 to 20 min.

Results

The historical and rational chemical basis for the use of dithionite and urea-dithionite in the detection of both hemoglobin S and non-S sickling hemoglobins has been discussed and documented in detail in a companion paper (1). The chemistry and principles are exactly the same for the automated methods as in the tube methods previously discussed. A manifold diagram for the AutoAnalyzer for the automated dithionite test is shown in Figure 1. Typical curves produced by the technique are shown in Figure 2.

The AutoAnalyzer manifold diagram for the urea-dithionite test is shown in Figure 3. Typical curves resulting from the technique of diagnostic importance are shown in Figure 4. Data are presented in Table 1.

Discussion

From previous studies by Henry et al. (4, 5) it was shown that when a specimen of hemoglobin S is divided and moves in phase along two channels, one with an appropriate reducing reagent and the other with urea in addition to the same quantities of reducing agent, the transmittance of the liquid in the line carrying the urea will be significantly greater than that of the contents of the other line. Extensive explanations for this

\[ \text{Transmittance} = \frac{I_0}{I} \]

where \( I_0 \) is the intensity of light transmitted through the sample and \( I \) is the intensity of light transmitted through the reference.

\[ \text{Absorbance} = \log \frac{I_0}{I} \]

place a cup of saline, one or two cups of a normal blood, and 20 cups of unknowns. Maintain this loading pattern throughout.

7. Start the mixer and the sampler. Adjust colorimeters so that the peaks in each set of 2 curves of the first 4 specimens are of equal percent transmittance.

8. After all the specimens have passed through the system, remove the chart and label the peaks. The "positive" specimens in the dithionite line (without urea) will have peaks that are lower by 15 to 20% (or more) transmittance than the peak of the corresponding urea-dithionite line (Figure 4). For example, a positive may read 10% trans-

Fig. 2. Typical distinctive curves obtained for hemoglobin AA and heterozygous or homozygous hemoglobin S produced by the automated dithionite test

The reagent (see Methods) produces turbidity in the presence of hemoglobin S, reflected in the high peaks. Hemoglobin A specimens produce the lower curves.
control the hemoglobin

Urea-dithionite

Fig. 0

These, (3)

Typical, specimens

AA have produced

Beta crystal

systems.

Automated automated urea-dithionite test

Dithionite (D) tube test

Urea-dithionite tube test (F)

Improved Murayama test (S)

2% Na2S2O5 Sickling test

Hemoglobin electrophoresis, pH 8.4

Table 1. Data from a Comparative Study with 14 Homozygous S Specimens, 50 Heterozygous S Specimens, and 3235 Control Specimens

<table>
<thead>
<tr>
<th>Hemoglobin S Specimens</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automated dithionite test</td>
<td>14 pos 50 pos 3235 neg</td>
</tr>
<tr>
<td>Automated urea-dithionite test</td>
<td>14 pos 50 pos 3235 neg</td>
</tr>
<tr>
<td>Dithionite (D) tube test</td>
<td>14 pos 50 pos 46 neg</td>
</tr>
<tr>
<td>Urea-dithionite tube test (F)</td>
<td>14 pos 50 pos 46 neg</td>
</tr>
<tr>
<td>Improved Murayama test (S)</td>
<td>14 pos 50 pos 13 neg</td>
</tr>
<tr>
<td>2% Na2S2O5 Sickling test</td>
<td>14 pos 44 pos 6 neg</td>
</tr>
<tr>
<td>Hemoglobin electrophoresis, pH 8.4</td>
<td>14 same 50 same 50 same as SS as AS AA</td>
</tr>
</tbody>
</table>

* Of these, 3 homozygous S, 15 heterozygous S, and 135 control specimens were sent to Blodgett Memorial Hospital in Grand Rapids from Children's Hospital at Wayne State University School of Medicine in Detroit, Michigan, for testing.

dithionite-reagent line is dispersed in the urea-dithionite-reagent line by the urea, which breaks the hydrophobic bonds essential to sickling; hence the clearing and the gain in transmittance when the specimen is hemoglobin S in the urea-dithionite line.

Predictions. This simple observation can be put to good use in the diagnosis not only of hemoglobin S but also rare non-S sickling hemoglobins and, perhaps, other hemoglobinopathies as specified below and discussed elsewhere (6). Other workers who may have access to the rare hemoglobins listed below may care to compare their experimental results with our predictions.

Hemoglobin C (Harlem). This sickling hemoglobin (6-8) is a structural variant of hemoglobin S because it is produced by a homologous crossing over of hemoglobins S and Korle-Bu (9). Hence, the submolecular lesion in hemoglobin C (Harlem) is identical in part with hemoglobin S. Thus, sickling is mediated by hydrophobic bonding, and this hemoglobin should give a positive dithionite test and become clear in a urea-dithionite system.

Hemoglobin C (Georgetown). This sickling hemoglobin (10, 11) forms a nematic liquid crystal system mediated by electrovalent bonding, according to Murayama et al. (12). Hence, this hemoglobin should form a nematic liquid crystal system that is stable in dithionite as well as in urea-dithionite systems.

Hemoglobin Bart's. This sickling hemoglobin (13, 14), found most often at birth in association with alpha thalassemia, is a homogeneous tetramer of gamma globin chains. It has already been shown to sickle under conditions similar to the dithionite test [Lehmann, H., personal communication cited to R.M.N. by W. A. Ascar, M. D.]. We have not had a specimen to work with, but we expect on the basis of theory to see a positive dithionite test.

Hemoglobin I. This sickling hemoglobin (15-17) is an alpha globin chain abnormality, and sickles by a molecular mechanism distinctly different from that of hemoglobin S. The dithionite test was negative on the one specimen tested.

Hemoglobin H. This Heinz-body forming hemoglobinopathy (18), under conditions similar to the Itano solubility test (19, 20), has been reported to give a positive reaction (21). Hence, in the dithionite test it may also give a positive test, possibly along with other Heinz-body forming hemoglobinopathies.

Hemoglobin King's County and Stanleyville II. In both (22-24) of these rare nonsickling hemoglobinopathies, the hemoglobins are known to have low solubilities, approaching that of hemoglobin S. The reactions of these hemoglobins in the dithionite test systems would be of interest.

Hemoglobin Alexandra. This rare sickling hemoglobinopathy (25) has not been sufficiently char-
acterized to make possible a prediction about its reaction in the dithionite test system.

*Hemoglobin Memphis/S*. This sickling hemoglobinopathy is composed of two genetic errors (26, 27), one involving the beta globin chain (S) and the other involving the alpha globin chain (Memphis). Hence, Memphis hemoglobinopathies will give a negative dithionite test but when presenting with beta S globin chains, as in Memphis/S, they may be expected to give a positive dithionite test.

To obtain the greatest accuracy in the diagnosis not only of hemoglobin S, but with little effort in diagnosis of other rare hemoglobinopathies—including non-S sickling ones—all hemoglobin specimens producing a positive dithionite or urea–dithionite tests should be studied additionally by electrophoresis.

Although the automated urea–dithionite greatly increases the specificity of the automated dithionite test, reagent cost per specimen increases to about 4 to 8 cents per set and the rate of determinations is slowed to 60 per hour. Even the positive specimens by the automated urea–dithionite test must be studied by electrophoresis to differentiate specimens of hemoglobin S from its rare structural variant, hemoglobin C (Harlem), a “non-S” sickling hemoglobin.

When the chemistry of the dithionite test is coupled with the ever-expanding fund of molecular information on hemoglobinopathies, specificity and accuracy of detection of both hemoglobin S and non-S sickling hemoglobins will be forthcoming.

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References