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REPORT NO. 898

AN AUTOMATED SCREENING METHOD FOR THE
SPECIFIC DETECTION OF HOMOZYGOUS AND HETEROZYGOUS S HEMOGLOBIN

(Progress Report)

by

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17 September 1970

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Evaluation of Blood Bank Methodology
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Combat Surgery
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ABSTRACT

AN AUTOMATED SCREENING METHOD FOR THE SPECIFIC DETECTION OF HOMOZYGOUS AND HETEROZYGOUS S HEMOGLOBIN

OBJECTIVE

To develop an inexpensive, specific method for the detection of S hemoglobin by an automated method useful for mass surveys of large human populations.

METHODS

The equipment used in this study was a 2-channel AutoAnalyzer. Reagents used were: Sickledex solutions and urea solutions. All data in this report were obtained from the automated, continuous flow analysis of hemoglobin specimens processed at the rate of 60 per hour. The identification of all hemoglobin specimens was achieved by use of electrophoresis, sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) sickling test, the Murayama test, and the Sickledex test.

RESULTS

The Murayama test (USAMRL Reports Nos. 893 and 894), although a specific test for the molecular lesion of S hemoglobin, is not convenient for use in the detection of S hemoglobin in large human populations. In USAMRL Report No. 897, we reported our modification of the Sickledex tube test so that the procedure, previously used only for screening, was shown to be specific for S hemoglobin. The principles of that modification have been adapted to the automated technique described in this report. When S hemoglobin is introduced into the Sickledex working solution (phosphate buffer, sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), and saponin), the S hemoglobin "sickles," constituting a nematic liquid crystal system. In that physical state of hemoglobin, this system acquires several remarkable properties including a marked gain in turbidity. From previous studies (USAMRL Reports Nos. 893-897) it was known that urea will selectively attack specified hydrophobic bonds necessary to the sickling event in S hemoglobin. Accordingly, it was found that when a specimen of S hemoglobin is divided and traversed in phase along two channels (one with the Sickledex solution alone and the other with a urea-Sickledex solution), a comparison of the percent transmittance of the two aliquots will show that the specimen in the urea-Sickledex line gains in percent transmittance as a quantitative function of the amount of S hemoglobin present. The method is sensitive enough to detect both homozygous and heterozygous S hemoglobin. An inexpensive, automated technique for the specific detection of S hemoglobin suitable for the mass survey of large human populations has been developed and is now available.

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AN AUTOMATED SCREENING METHOD FOR THE
SPECIFIC DETECTION OF HOMOZYGOUS AND HETEROZYGOUS S HEMOGLOBIN*

INTRODUCTION

An automated method for the detection of homozygous and heterozygous S hemoglobin is presented. The method is regarded by us as specific, rapid, inexpensive, and ideally appropriate for mass surveys of large populations for the detection of S hemoglobin. Furthermore, the technique may be adjusted so that S hemoglobin and non-S sickling hemoglobins may be detected.

MATERIALS

All Technicon components are listed on attached flow diagram (Fig. 1).

Sickledex test reagent, bulk (Ortho).

Urea.

1 N NaOH.

Tergitol NPX.

PROCEDURE

1. Assemble manifold and other Technicon modules as shown on flow diagram (Fig. 1).
2. Mix Sickledex reagent in accordance with manufacturer's instructions and add 0.5 ml of Brij - 35 per liter of reagent.
3. Take one-half of the reagent from step #2 and add sufficient urea to make a 2 M solution with respect to urea.
4. Place all reagent lines in appropriate reagents and start pump. Allow 5 to 10 minutes to fill the system with reagents.
5. Adjust the base line of the Sickledex line without urea to 94% T on the chart paper and adjust the urea-Sickledex line to 90% T. Run a normal specimen; if the peaks are not at the same percent T, adjust the base lines so the peaks are at equal levels.
6. Fill one or two cups with 1/2 to 2 ml of well mixed normal blood (collected in EDTA). Place these cups on sample tray at regular positions

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followed by 20 unknown specimens in each set. After the twentieth specimen, place a cup with saline in it. Follow the saline cup with one or two normal specimens, followed by 20 unknown specimens. Maintain this loading pattern throughout. It is easier to label the peaks if specimens are gathered in sets of 20.

7. Start mixer and sampler.

8. After all specimens have passed through the system, label the peaks. "Positive" specimens will have peaks in the Sickledex line (without urea) which are lower by 3% T or less than the peak of the corresponding sample aliquot in the urea-Sickledex line. For example, a "positive" heterozygote S hemoglobin may read 50% T on the Sickledex line and 53% on the urea-Sickledex line. The gain in percent transmittance will be even greater with homozygous S hemoglobin.

9. After the run is completed, rinse the system with 1 N NaOH with 20 ml of Tergitol NPX added per liter of reagent for 15 minutes. Then rinse with distilled water for 15 to 20 minutes.

RESULTS

The data are presented in Table 1.

DISCUSSION

We have adapted a dual channel manifold AutoAnalyzer apparatus in such a manner that one channel transports the blood specimen in a Sickledex working solution, while the other channel in phase transports an aliquot of the blood specimen in a urea-Sickledex solution (final concentration of urea equals 2 M). Typical distinctive patterns are plotted on the dual record as shown in Figures 2 and 3. It will be noted that percent transmittance of light is less in the absence of urea than the presence of urea for a given S hemoglobin specimen. The gain in percent transmittance with S hemoglobin specimens in the urea-Sickledex line is quantitatively related to the S hemoglobin concentration. This observation that urea enhances the transmittance of light of S hemoglobin in the Sickledex test solution constitutes the basis for this screening procedure. The reasons for the specificity of this system for the detection of S hemoglobin are given below.

Principle of Sickledex Test. Although the Sickledex test had been discussed a number of times in the literature (1-4), the principle of the Sickledex test has not been disclosed. On the basis of unpublished data, Nalbandian and Kessler originally observed (5) that the Sickledex test was either identical with or derived from the Itano solubility test (6,7). This test was developed by Itano to distinguish between hemoglobins S and D. Guided by studies by Perutz (8,9), Itano observed that deoxygenated hemoglobin S had a uniquely low level of solubility in 2.28 M phosphate

buffer system. Additional unpublished studies by Nalbandian and Henry disclosed that the working Sickledex solution contained phosphate in approximately 2.3 M concentrations, probably a quantity of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$), and a hemolyzing agent considered to be saponin. When hemoglobin S in intact red cells is introduced into such a solution, the erythrocytes will undergo lysis; the S hemoglobin will undergo deoxygenation; microfilament and microcable formation will occur; and the hemoglobin S will be "sickled," constituting a nematic liquid crystal system. Among the remarkable properties acquired for such a solution in that physical state are: (1) birefringence; (2) interference with the transmission of light (hence the positive Sickledex test); (3) circular dichroism; and (4) paramagnetic susceptibility. There are, however, hemoglobins other than S which are known to sickle (5). These include hemoglobin C (Harlem) (10-12), C (Georgetown) (13,14), I (15-18), Bart's (19,20), and Alexandra (21). Since the nonspecificity of the Sickledex test has been discussed elsewhere (5), manipulation of the Sickledex solution is required to achieve specificity.

Urea and Sickle Cell Hemoglobin. We have accepted as correct the recently modified Murayama hypothesis for the molecular mechanism of sickling (22-25). His concept clearly implicates the pathological role of unique hydrophobic bonds formed between interacting tetramers of hemoglobin S. Physical chemists have recognized that hydrophobic bonds in aqueous protein systems are of predominant importance in maintaining the tertiary structure of protein molecules (22-25). Recent discoveries by physical chemists indicate that urea, in addition to its well-known capacity to break hydrogen bonds, also can destroy hydrophobic bonds (22-25). Ponder and Ponder (26) have shown that urea will reverse sickling, but will cause severe hemolysis. Nalbandian and co-workers have provided optical and electron microscopic evidence that urea in invert sugar (inhibits hemolysis) in fact does reverse efficiently sickling in S hemoglobin erythrocytes. The pertinent literature on these aspects has been reviewed and recently published by us elsewhere (27-33).

By the automated technique described above, the hemolysate of hemoglobin S was sickled (constituted a nematic liquid crystal system) in the Sickledex line. When the percent transmittance of the solution of that line was compared with the aliquot of the same blood specimen in the urea-Sickledex line, the solution in the latter channel had gained in percent transmission of light as a quantitative function of the amount of S hemoglobin desickled by the urea. This relative gain in percent transmittance in the urea-Sickledex line for a given S hemoglobin specimen obtains because the nematic liquid crystal system of sickled S hemoglobin is disbursed by urea. This typical characteristic gain in transmittance in the presence of urea under the conditions of this automated method can only occur with hemoglobin S or with its structural variant, hemoglobin C (Harlem) (10-12). Both of these hemoglobins are known to sickle as a result of the formation of identical hydrophobic bonds (22-25). Non-S sickling hemoglobins which have other molecular mechanisms

than the formation of pathologic hydrophobic bonds for sickling will not give such a reaction.

This test system is operated exactly at 37°C for a very important and specific reason. The importance of the formation of specified hydrophobic bonds to "sickling" or the aggregation of S hemoglobin tetramers is obvious from the previous discussion. Not at all well-known is the influence of temperature on the formation of hydrophobic bonds. Scheraga and associates (34) have clearly shown in rigorous, analytical studies that the thermal stability of the tertiary structure of proteins in aqueous media is dependent on counterpoised gain or loss in strength of hydrophobic bonds and hydrogen bonds. In such systems, hydrogen bonds GAIN and hydrophobic bonds LOSE strength with a decrease in temperature. Furthermore, they have shown that for any given protein-aqueous system there is a particular maximum temperature (T_m) beyond which any increase or decrease in temperature adversely affects the integrity of the tertiary structure of the protein molecule. Nalbandian and associates (22-25) have shown in the development of the Murayama test that 37°C is the OPTIMAL temperature for hemoglobin S systems at which hydrophobic bonds are a maximum strength. Thus, for purposes of the automated technique discussed in this report, when the test system is operated at 37°C, this will assure the maximum degree of turbidity in the Sickledex line, and therefore the maximum quantitative gain in percent transmission when compared with the urea-Sickledex line where the formation of hydrophobic bonds has been prevented by the presence of urea.

On theoretical considerations, this system can be easily adjusted to discriminate for S hemoglobin on the one hand, and the non-S sickling hemoglobins alluded to above on the other, since as far as is known these non-S sickling hemoglobins do not sickle on the basis of hydrophobic bonding. Hence, when the transmittance in Sickledex solutions with and without urea are compared, our theories predict that the transmittances should be identical since the hemolysates will be sickled in both lines (i.e., the nematic liquid crystal systems will persist in the presence of urea). A simulated tracing is shown in Figure 4 which indicates our notion of recorded patterns which we anticipate on theory will be produced by non-S sickling hemoglobins tested in this system. Our group welcomes samples of these rare, non-S sickling hemoglobins (Bart's, Alexandra, C (Harlem), C (Georgetown), and Memphis/S) so that we may confirm or correct our theory in this regard.

This report is based on principles and findings reported by Henry et al in USAMRL Report No. 897 (35).

A comprehensive review of our entire body of work on sickle cell hemoglobin ranging from molecular structural aspects, therapeutic theories, diagnostic tests, optical and electron microscopy, and clinical resumes of cases of acute sickle cell crisis including clinical protocols has been presented by us (36).

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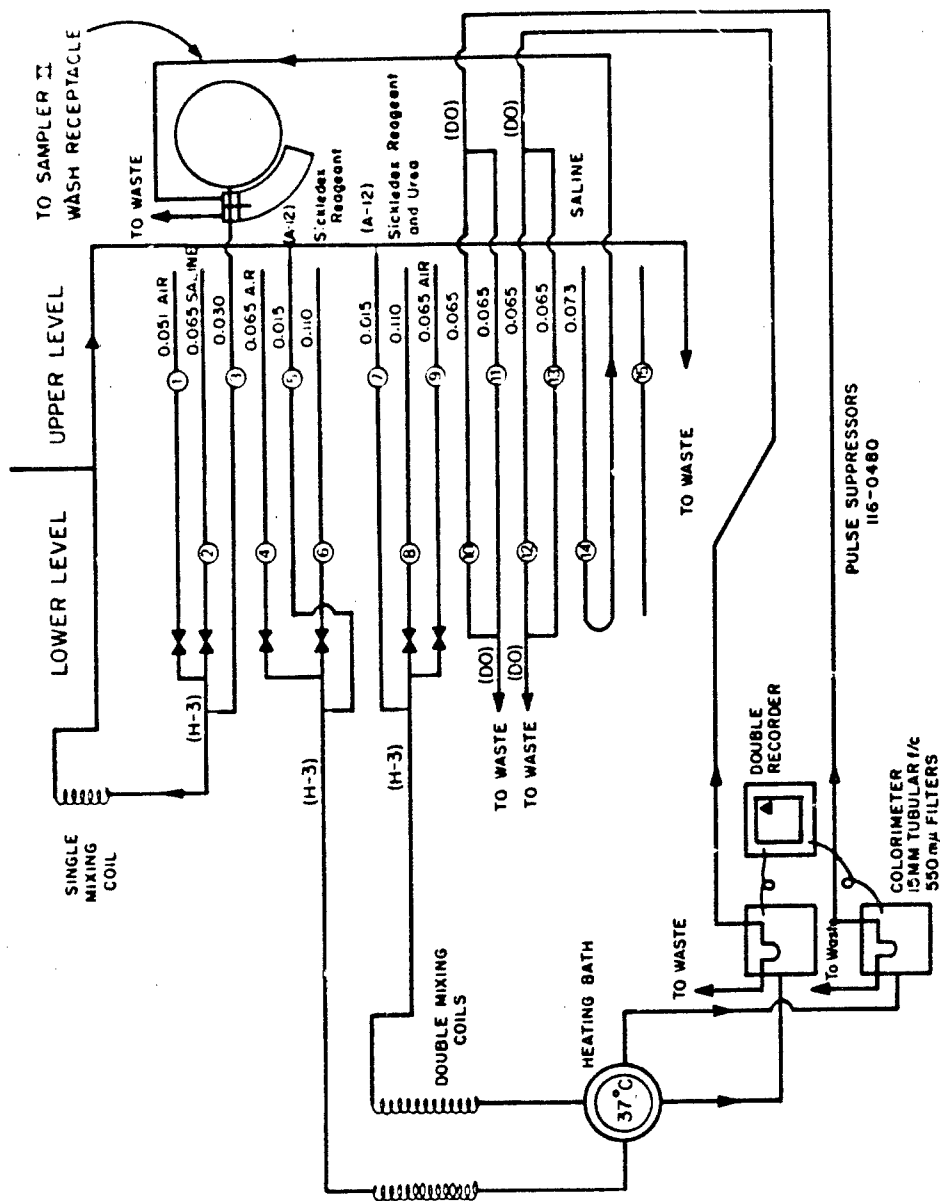


Fig. 1. This figure represents the flow scheme for a dual channel AutoAnalyzer set up to operate as an automated modified Sickledex test system. (See text for details.)

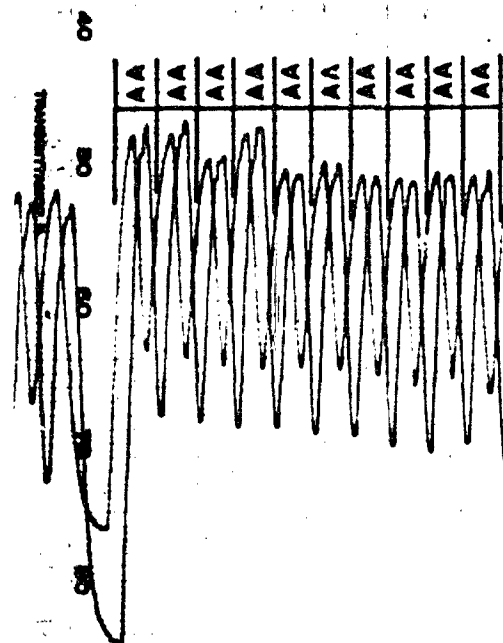


Fig. 2. In this figure and in Figure 3, we have represented typical distinctive patterns plotted on the dual recorder produced in the automated, modified Sickledex test. In the evaluation of each specimen of hemoglobin the identification of the hemoglobin as homozygous S or heterozygous S depends upon the quantitative extent of the GAIN in percent transmittance recorded in sets of two. In each set of recordings, the first tracing represents the plotted percentage of transmittance through a system consisting of the hemoglobin specimen and a urea-Sickledex solution. The second tracing consists of an exact amount of the identical hemoglobin specimen for that set in a Sickledex solution (no urea). It will be seen that after the initial adjustment of the base line, there is no gain in percent transmittance for any set of hemoglobin A specimens.

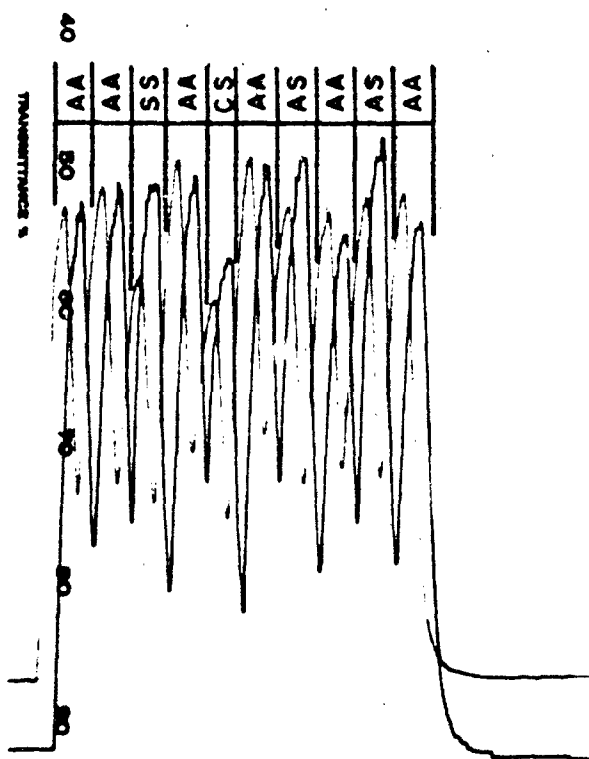


Fig. 3. The conditions producing the illustrated tracing obtained by the automated modified Sickledex test are identical with those described in the legend in Figure 2. Note that in each set of tracings constituting one hemoglobin specimen, when hemoglobin S is present, there is a quantitative gain in the percent of transmittance when the value of the urea-Sickledex line (the first tracing of each set) is compared to the value recorded for the Sickledex line (the second tracing in each set). Furthermore, it will be appreciated that there is a quantitative relationship between the amount of hemoglobin S present and the extent of percent gain in transmittance. Thus, the second tracing of each set represents the percent transmittance of the solution when hemoglobin S is present as a nematic liquid crystal system and the first line of each set represents the percent transmittance of the solution when the nematic liquid crystal system has been dispersed by urea. The quantitative relationship of this percent gain in transmittance is related to the quantity of hemoglobin S present and, therefore, to zygotity. (See text for details of interpretation.)

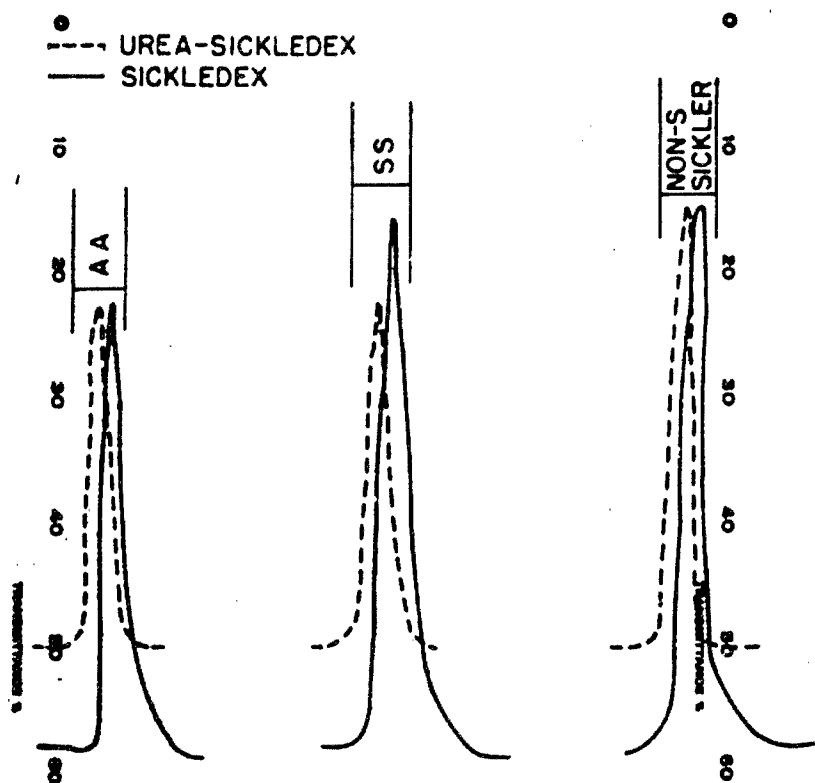


Fig. 4. Figures 2 and 3 should be reviewed in connection with this illustration. THIS IS A SIMULATED REPRESENTATION OF THE EXPECTED TRACINGS UNDER CONDITIONS OF THE AUTOMATED MODIFIED SICKLEDEX TEST WHEN A NON-S SICKLING HEMOGLOBIN IS ENCOUNTERED. The dashed curve represents a urea-Sickledex solution and the solid curve represents a Sickledex solution. Equal aliquots of the unknown hemoglobin specimen are present in both lines in each set. The presence of a non-S sickling hemoglobin is indicated by the fact that a set of peaks is equal and higher relatively than the equal peaks encountered with the hemoglobin A specimens in the test run. The significance of such a pattern in contrast with that typical pattern for hemoglobin S shown in the middle of the three tracings pictured turns on the fact that since the non-S sickling hemoglobins form nematic liquid crystal systems without the formation of hydrophobic bonds, the nematic liquid crystal system persists in the presence of urea. Thus, the percent transmittance of the system in both the urea-Sickledex line and the Sickledex line will be low and at equivalent levels which under conditions of the recording are presented as unusually high dual peaks, as indicated in the right-hand set of tracings.

TABLE 1
Tests of Hemoglobin Identification

Test	Hemoglobin Type					
	SS	AS	CS	AC	AD	AA
Modified Sickledex	8 Pos	22 Pos	3 Pos	6 Neg	1 Neg	264 Neg
Automated Sickledex	8 Pos	22 Pos	3 Pos	6 Neg	1 Neg	264 Neg
Na ₂ S ₂ O ₅ Sickling	8 Pos	22 Pos	3 Pos	6 Neg	1 Neg	264 Neg
Murayama	6* Pos	8** Pos	1* Pos			
Electrophoretic mobility pH 8.4	8 same as S	22 same as A&S	3 same as C&S	6 same as A&C	1 same as S	264 same as A

* Not done in 2 cases (insufficient quantity of specimen).

** Not done in 14 cases (insufficient quantity of specimen).

A total of 304 different human blood specimens was tested. Forty of the specimens represented hemoglobinopathies of S or C; 264 of the specimens were of A hemoglobin type and represent the controls. Confirmation of the hemoglobin type was established by the methods listed. Where quantities of blood were insufficient, the Murayama test was not done. The results are summarized and are in support of the automated method proposed in this report. Aliquots of these identical specimens were used in the development of data for the modified Sickledex tube test reported by Henry and associates (35) in USAMRL Report No. 897.

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13. ABSTRACT The Murayama test (USAMRL Report Nos. 893 and 894), although a specific test for the molecular lesion of S hemoglobin, is not convenient for use in the detection of S hemoglobin in large human populations. In USAMRL Report No. 897, we reported our modification of the Sickledex tube test so that the procedure, previously used only for screening, was shown to be specific for S hemoglobin. The principles of that modification have been adapted to the automated technique described in this report. When S hemoglobin is introduced into the Sickledex working solution (phosphate buffer, sodium hydrosulfite (Na ₂ S ₂ O ₄), and saponin), the S hemoglobin "sickles," constituting a nematic liquid crystal system. In that physical state of hemoglobin, this system acquires several remarkable properties including a marked gain in turbidity. From previous studies (USAMRL Report Nos. 893-897) it was known that urea will selectively attack specified hydrophobic bonds necessary to the sickling event in S hemoglobin. Accordingly, it was found that when a specimen of S hemoglobin is divided and traversed in phase along two channels (one with the Sickledex solution alone and the other with a urea-Sickledex solution), a comparison of the percent transmittance of the two aliquots will show that the specimen in the urea-Sickledex line gains in percent transmittance as a quantitative function of the amount of S hemoglobin present. The method is sensitive enough to detect both homozygous and heterozygous S hemoglobin. An inexpensive, automated technique for the specific detection of S hemoglobin suitable for the mass survey of large human populations has been developed and is now available.			

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