Simple Electrophoretic System for Presumptive Identification of Abnormal Hemoglobins

By the International Committee for Standardization in Hematology

In order to determine if mutant hemoglobins can be identified by relatively simple methods, a Working Group of the ICSH Expert Panel on Abnormal Hemoglobins and Thalassemia analyzed 17 hemolysates containing 14 different mutant hemoglobins by four electrophoretic methods: (1) cellulose acetate in alkaline buffers, (2) citrate agar pH 6.0, (3) urea 2-mercaptoethanol buffer pH 8.9, and (4) urea 2-mercaptoethanol buffer pH 6.0. The examined mutants included several of great numerical and clinical importance as well as some rare ones, namely, HbS, C, D Los Angeles (Punjab), E, G Philadelphia, N Baltimore, and O Arab; also Hb Ft. Worth, Montgomery, Winnipeg, Rush, Q India, Bethesda, and Lepore. Comparative mobilities of these hemoglobins in all of the methods are presented here. The combined data permit their presumptive identification, often with a high degree of specificity. The system has been applied in Iran, where the four prevalent mutants can be differentiated by these methods, at considerable saving of time and resources previously expended on structural analyses. It is proposed as a basis for an ICSH Tentative Standard. There is little doubt that this presumptive identification of hemoglobin variants by simple electrophoresis will be improved or complemented by the introduction of newer techniques, such as immunologic analysis. However, for the present and for some time to come, the system outlined here should be found valuable. The present report does not concern itself with the numerous auxiliary techniques involved in the identification of abnormal hemoglobins—sickle-cell test, solubility tests, lability test—and no claim is made that the simple system described here eliminates these other techniques from the diagnostic armamentarium of the laboratory.

Millions of people throughout the world carry abnormal hemoglobins in various genetic combinations, with consequences ranging from trivial to lethal. Their identification—a public health problem of major importance—includes recognition of the relatively innocuous mutants that must be differentiated from the harmful ones. Most abnormal hemoglobins...
were originally detected by an alteration in electrophoretic charge; although simple electrophoresis is still the most satisfactory method of detecting them, it would not be expected to discriminate between different mutants that, by chance, carry the same electrophoretic charge, such as the several dozen that resemble HbS. An entirely unrelated property—insolubility in the reduced state—forms the basis of the sickling and solubility tests that can detect the β6 Glu → Val substitution of HbS. Identification of most of the other known mutants depends largely on structural analysis, an expensive, lengthy process requiring sophisticated equipment, highly trained personnel, and large amounts of sample. While essential for characterizing new hemoglobins and those of particular importance, structural analysis is clearly not possible for the numerous mutants constantly being found in screening programs and in medical practice. A simple system of identifying them, even on a presumptive basis, would be extremely useful.

One possibility for achieving such a system lies in the recognition that each molecular substitution may produce a unique complex of altered physical (and often functional) properties. Several types of zone electrophoresis, both of hemoglobin and of globin chains, can reveal various aspects of this complex, which involves alterations in electrical charge, molecular configuration, and both intra- and intermolecular contacts. The combined data might provide highly specific identifications.

In order to evaluate simple procedures for identifying hemoglobins, the members of the Working Group undertook to apply them in their own laboratories to several hemoglobin samples sent to them as unknowns. Dr. Winston Moo-Penn of the Center for Disease Control sent each member a standard solution containing HbJ, A, F, S, and C and about 0.5 ml of each of 17 other hemolysates, labeled only by code. They contained 14 different mutants, including several of considerable importance in various parts of the world, namely, HbS, C, D Los Angeles (Punjab), O Arab, E, N Baltimore, and G Philadelphia. Other, rarer hemoglobins were included largely because they were available. Three mutants—HbD Los Angeles, G Philadelphia, and E, were sent twice. The seventeen samples were distributed in four different mailings.

MATERIALS AND METHODS

Four methods of zone electrophoresis were considered likely to supply the most useful identifying data for the least expenditure of effort and time. Brief discussions of the methods are presented here; detailed directions are found in the references and in manufacturers’ instructions. Original references to the mutant hemoglobins are found in the compilations in ref. 1 and general discussions of methods in refs. 2-6.

**Method 1.** Zone electrophoresis in alkaline buffers is a simple and versatile method of initial screening. Filter paper is the cheapest type of support, but separations on it are slow and hampered by considerable adsorption. Nevertheless, it may provide superior discrimination between several mutants, notably HbD and G, or E and C. It has been largely supplanted by cellulose acetate, which provides excellent, rapid separations of HbA, F, S, C, and numerous other mutants, primarily on the basis of net charge. Some laboratories prefer the method of starch gel electrophoresis, which is more complex and time consuming but permits application of larger samples and costs less for materials.

**Method 2.** Citrate agar electrophoresis, pH 6.0-6.2, separates hemoglobins on the basis of many factors other than net charge, such as the molecular location of the substituted residue. This highly empirical method is useful for confirming the identifications of HbA, F, S, C, and many other mutants. It was originally proposed for distinguishing HbS from HbD, but recent improvements have greatly increased its sensitivity and scope. Many hemoglobins
that are electrophoretically altered in alkaline buffers move with HbA on electrophoresis on citrate agar; thus this method cannot serve as a primary method of detection. However, other hemoglobins show a distinctive, or even unique, mobility that is very useful in the identifying process.

**Method 3.** Electrophoresis of globin chains in 6 M urea 2-mercaptoethanol buffer pH 8.9. This method can indicate in which globin chain the abnormality lies. Analyses in both acidic and alkaline buffers are easily performed, and they may reveal highly characteristic electrophoretic differences. As expected, such differences are found in substitutions involving histidine, since its charge changes from positive to neutral in the pH range 4-8. Other differences may also be shown by electrophoresis of globins at both acidic and alkaline pH. They are not demonstrable by zone electrophoresis of hemoglobins, since this procedure is usually not successful in acidic buffers. Although the 6 M urea buffers would be expected to obliterate secondary and tertiary structures of the globin, the process may be incomplete, and variations in exposure of altered residues, with consequent alterations in molecular contacts, may contribute to the electrophoretic differences.

**RESULTS**

The electrophoretic data on all of the examined mutants are presented in Figs. 1 and 2 (α and β chain abnormalities, respectively), and some of the patterns are illustrated in Fig. 3.

By chance, all of the α chain mutants listed in Fig. 1 resemble HbS in electrophoresis at alkaline pH, except that one, Hb Ft. Worth, moves distinctly more anodically. This difference in mobility is perhaps accentuated by the fact that Hb Ft. Worth occupies a characteristically low proportion of the total hemoglobin, about 5%. Hb Montgomery moves between Hb Ft. Worth and HbS in alkaline electrophoresis, and it is clearly differentiated from the other mutants by its distinctive mobility on citrate agar electrophoresis, between HbA and S. This is also the case with HbQ India, which in citrate agar electrophoresis resolves into a tight, sharp band, slightly anodic to HbA. The rare Hb Winnipeg and Russ behave similarly in all of the methods except globin elec-

![Table]

<table>
<thead>
<tr>
<th>Hemoglobin and Structure</th>
<th>Hemoglobin Chains</th>
<th>Cellulose Acetate pH 8.9</th>
<th>Citrate Agar pH 6.0</th>
<th>Mutant Globin Chains</th>
<th>pH 8.9</th>
<th>pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ft. Worth</td>
<td></td>
<td>A</td>
<td>S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α27 Glu→Gly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montgomery</td>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α48 Leu→Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winnipeg</td>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α75 Asp→1,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Russ</td>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α51 Gly→Arg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q India</td>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α64 Asp→His</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q Philadelphia</td>
<td></td>
<td>A</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α58 Asn→Lys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fig. 1. Electrophoretic mobilities, in four methods, of examined α chain mutants, hemoglobin and globin chains.*
Fig. 2. Electrophoretic mobilities, in four methods, of examined \( \beta \) chain mutants, hemoglobin and globin chains.

trophoresis at pH 6.0, in which the \( \alpha \) globin chain of Hb Winnipeg separates more easily from \( \alpha \)-A globin than does that of \( \alpha \) Russ. These two mutants may never be seen by most screening laboratories. Perhaps the chief value of including them in the current exercise is to demonstrate that they, like several other similarly charged \( \alpha \) chain mutants, can be differentiated from the numerically more important HbG Philadelphia, the third most frequent hemoglobin observed in Africans.

The \( \alpha \) globin chain of HbG Philadelphia differs from the others by being
well separated from that of HbA under electrophoretic conditions in which many other α chains are separated very little or not at all. However, the conditions for showing this must be carefully controlled, because some similarly charged α chains, such as α Winnipeg, separate from α-A at slightly higher pH values and on more prolonged electrophoresis. (Four Asp → His substituted α chains—those of HbQ India, Q Iran, Mahidol, and Hasharon—may separate from α-A as well or better than α-G Philadelphia, but they are readily differentiated from it by citrate agar electrophoresis.)

Each of the β chain mutants listed in Fig. 2 differs from the others in at least one, and usually several, methods. HbS, C, and O Arab, all clinically important, are easily identified by a combination of electrophoresis in alkaline buffers and citrate agar. The rare Hb Bethesda moves with HbA in alkaline electrophoresis, but it is readily identified because it separates distinctively—between HbA and S—on citrate agar electrophoresis. This mutant would doubtless be overlooked in any screening that depends on alkaline electrophoresis as the initial method of detection. (Its behavior illustrates the principle that if patients have an hematologic abnormality, their hemolysates, even if apparently normal in alkaline electrophoresis, should be investigated by other methods.)

Hb Lepore resembles HbS in every method except citrate agar electrophoresis, in which, like many other mutants, it moves with HbA. Its quantitative resolution—about 10% of the total hemoglobin—is a distinguishing feature, as are the hematologic and genetic data of the patient. HbN Baltimore is the “fastest” (most anodic) of the examined mutants in alkaline electrophoresis, and the resolution of its β globin chain in urea mercaptoethanol buffers is highly distinctive. HbD Los Angeles is differentiated from HbS by citrate agar electrophoresis and from other, similarly charged mutants by the behavior of its β globin chain in acidic urea 2-mercaptoethanol buffers containing EDTA, in which it moves distinctively close to the β globin chain of HbA. HbE is readily differentiated from HbC and O in citrate agar electrophoresis; its β globin chain also shows a distinctive electrophoretic mobility, differing slightly at acidic, compared with alkaline, pH.

**DISCUSSION**

The methods described are useful not only for screening purposes but also for diagnosing hemoglobinopathies. If, in the initial analysis in alkaline electrophoresis, the hemolysate resolves into HbA without visible increase in the minor normal components HbF and A₂, it is reported as “no hemoglobin abnormality found.” If, however, the patient has an unexplained hematologic abnormality (as is the case with carriers of Hb Bethesda), the hemolysate should be studied by additional methods, including citrate agar electrophoresis and quantitative estimations of HbF and A₂, even if these are not increased on visual inspection of the pattern. Functional studies, including tests for Heinz body formation, heat stability, O₂ affinity, etc. may be valuable. Hematologic and genetic data and the ethnic origin of the patient should be considered in the evaluation.

All hemolysates with an abnormality in alkaline electrophoresis should be analyzed by citrate agar electrophoresis. If HbS is suspected, its presence can
be confirmed by sickling or solubility tests instead of citrate agar electrophoresis; if these are positive, the correct genotype—HbAs, Sβ-thal, or SS—may be deduced on the basis of the proportion of the total hemoglobin occupied by each fraction. This presence in the hemolysate of more HbA than S establishes the HbAS genotype of sickle cell trait, while more HbS than HbA (and a variable amount of HbF) is pathognomonic for HbS β*-thalassemia. Such patterns can, of course, also be seen in patients with sickle cell anemia after transfusions with normal blood or in the case of heterozygosity for the genes for HbS and hereditary persistence of HbF (HPFH). Accurate quantitative estimations are usually unnecessary, since the essential quantitative relationships of the major hemoglobins are readily detectable on simple visual inspection of the pattern. The complete absence of HbA in the presence of a large amount of HbS is usually associated with sickle cell anemia, but the pattern of HbS βα-thalassemia is indistinguishable from it, and complete absence of HbA in the presence of HbS and HbF also occurs in HbS hereditary persistence of fetal Hb (HPFH) or HbS/ββ-thalassemia. Diagnosis of these conditions depends on clinical and genetic findings as well as on laboratory data. Citrate agar electrophoresis is recommended over solubility or sickling tests for confirming the identity of HbS because it also reveals the identity of other mutants, such as Hb Montgomery, Titusville, Hasharon, etc. and because it facilitates recognition of small amounts of either HbF or HbA in the presence of large amounts of the other. Such recognition may be essential for correct diagnosis of hemoglobinopathies.

Many other genetic combinations of hemoglobins and various thalassemic syndromes can also be recognized electrophoretically. The identification of the “fast” hemoglobins, H and Bart, indications of α-thalassemia, are confirmed by electrophoresis in phosphate buffer pH 7.0, while elevated HbA₂ (often, also, HbF) values are characteristic of heterozygotes for β-thalassemia. Details of these identifications are provided in the references.

An illustration of the usefulness of the system was described by Rahbar in Iran; the four prevalent mutants there—HbD Los Angeles, D Iran, Hamadan, and Q Iran—are electrophoretically similar to HbS. Rahbar found (Fig. 4) that he could differentiate them by simple electrophoretic methods at considerable saving of time and resources previously expended in structural analyses. He compared the structural and presumptive identifications of about 80 samples and found no discrepancies. Many other mutant hemoglobins can be similarly identified. In a recent survey of about 250,000 blacks, Schneider et al.12 characterized and presumptively identified 20 different mutants in addition to HbS and C. Sixty samples, each containing 1 of the 20 mutants, were analyzed structurally, with no discrepancies found between definitive and presumptive identifications.

Doubtless not all of the almost 300 known mutant hemoglobins can be identified by this system, and as more data become available a number of limitations will become apparent. Nevertheless, the present exercise shows that the system is both useful and easily learned. Some participating laboratories were previously quite unfamiliar with some of the methods, and some samples arrived in a denatured state that made them difficult to identify. Nevertheless, the four laboratories that applied all of the methods to all of the samples usually made correct identifications, except for those hemoglobins they had not previously
Fig. 4. Differentiation of four mutant hemoglobins prevalent in Iran. Comparative electrophoretic mobilities of hemoglobins and globin chains.

seen. The clinically important mutants were particularly easily recognized, probably because they were more familiar than the others.

At present HbS and C are generally identified solely on the basis of readily detectable properties—sickling, solubility, or electrophoretic mobility in several different systems. Analyzing them structurally would be considered a waste of time and resources; other hemoglobins can also be recognized by simple methods, some of them perhaps as specifically as HbS and C.

REFERENCES

11. Schneider RG: Differentiation of electrophoretically similar hemoglobins such as S, D, G and P or A2, C, E and O by electrophoresis of the globin chains. Clin Chem 20:1111-1115, 1974
Simple electrophoretic system for presumptive identification of abnormal hemoglobins. By the International Committee for Standardization in Hematology

Updated information and services can be found at:
http://www.bloodjournal.org/content/52/5/1058.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml