Isoelectric Focusing of Human Hemoglobin: Its Application to Screening, to the Characterization of 70 Variants, and to the Study of Modified Fractions of Normal Hemoglobins

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Isoelectric focusing on slabs of acrylamide gel was adapted for the screening of abnormal hemoglobins, the characterization of 70 human variants, and the study of minor fractions of normal hemoglobin. The screening method was as fast and inexpensive as conventional techniques, allowed the simultaneous analysis of some 50 samples of whole blood, and yielded resolution superior to that obtained by other methods with hemolysates. Among the 70 variants, 31 mutants could not be separated from HbS by cellulose acetate electrophoresis. The characterization technique of electofocusing allowed us to distinguish between most variants. Only one mutant, Hb Galveston, could be confused with HbS. Hb Köln, the most frequent unstable mutant, exhibited a special pattern. HbA\text{1c} was separated from HbA. Preliminary results indicate that quantitation of HbA\text{1c} by gel scanning is feasible.

The usual method employed for the screening of abnormal hemoglobins is electrophoresis on cellulose acetate strips. This procedure is convenient, rapid, and inexpensive, but its limited sensitivity necessitates many complementary tests. The combination of several electrophoreses on agar and on cellulose acetate strips at different pH, in the presence or absence of urea, has seemed the most effective approach for the identification of several mutants and especially for the "S-like" electrophoretic variants. Nevertheless, this procedure necessitates several electrophoreses of each sample, and the results are sometimes difficult to interpret.

Isoelectric focusing in polyacrylamide gel offers an alternative approach. This method, as originally described for tubes, is inadequate for large-scale screening. This restriction can be overcome by isoelectric focusing in a thin layer, and high voltages provide the conditions required for the development of a technique applicable to screening.

In a previous report we described a thin-layer isoelectric focusing (TLIF) technique and employed it to study 31 hemoglobin variants. The present paper describes modifications to our original method that can now be applied to large-scale screening and are comparable in cost to electrophoresis on cellulose acetate strips. Seventy variants were studied, and the results are presented. In addition, the isoelectric focusing of normal hemoglobins modified in vivo and in vitro is shown.

*Most of the mutants are listed by Lehman H and Hunstman RG (Man's Haemoglobins; Amsterdam, North-Holland, 1974). Those not listed there can be found in References.

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MATERIALS AND METHODS

The structures of the hemoglobin variants used in this report were previously established in our laboratory or in others. Most of the blood samples were stored frozen in liquid nitrogen as hemolysates. Whole blood diluted with water was used for fresh samples as indicated below. Potassium cyanide was added to all samples except when the presence of abnormal ferrihemoglobins was suspected. The concentration of hemoglobin varied from 0.2\% to 3\%, the highest concentration allowed detection of trace amounts of various hemoglobins, while the lowest concentration was suitable for comparison of variants of adjacent pH.

The gel was prepared according to the method previously described, with the following modifications: Polymerization of the gel was carried out with ammonium persulfate (0.03\% final concentration), the pH range was 6.0-9.0 instead of 6.0-8.0, the thickness of the gel was reduced to 0.5 mm, and the gasket was of vinyl polychloride. The procedure for preparation of a gel of 250 x 115 x 0.5 mm was as follows: The stock solution of acrylamide (20 g/dl w/v) was prepared at intervals of 2 wk and stored in a dark bottle. This solution contained acrylamide (Fluka) (9.7 g), bisacrylamide (Fluka) (0.3 g), and distilled water (50 ml). To 4.5 ml of this solution 0.9 ml of 40\% ampholines pH 6-8 (LKB), 0.9 ml of 40\% ampholines pH 7-9 (LKB), and 11.7 ml of distilled water were added. The solution was degassed for 5 min, and 3.5 \( \mu \)l of \( N,N',N'',N''\)-tetramethylethylenediamine (TEMED) (Sigma) were then added. Finally, a 3\% ammonium persulfate (Merck) solution (0.18 ml), prepared daily, was added.

The final mixture was poured promptly between the plates with a pipette; one of these plates was glass; the other was of altuglass or plexiglass washed previously with detergent, distilled water, and ethanol. Polymerization occurred within a few minutes. After standing for 1 hr at room temperature, the gel was kept between the two plates (without clamps) in a humid chamber at 4°C for a further period of at least 2 hr and up to 1 wk prior to use. Immediately before use, the gasket and plexiglass plate were removed by gentle sliding.

Electrophoretic migration was performed at 10°C with the LKB Multiphor apparatus, using a constant power of 18 W (1.2 W/ml gel). The electrode strips (LKB) were impregnated on a glass plate with a Pasteur pipette containing sodium hydroxide (1 N) for the cathode or phosphoric acid (1 M) for the anode: these solutions were prepared at intervals of 2 wk. The strips were subsequently placed on the gel surface and a prerun of 10 min was performed before the addition of the samples.

For routine screening, 10 \( \mu \)l whole blood was placed in the wells of a microtitration plate containing 0.1 ml of 0.05\% KCN solution. Samples were transferred with a multiple syringe to a line of 48 squares of Whatman filter paper (3 x 3 mm) placed in a single line on a strip of celophane tape. The 48 samples were then placed on the cathodal side of the gel adjacent to the electrode strip.

For the precise study of hemoglobins, 6 mm x 3 mm papers were used. Thirty samples diluted up to 0.2\% could be compared simultaneously. The papers were not removed during migration, and the electrode strips were not changed. The duration of migration was 1 1/2 hr, depending on the concentration of the samples. When focusing had reached completion, the plate was placed in 12% trichloroacetic acid for 10 min, transferred to a sheet of Whatman paper (3 MM), and dried overnight at room temperature. The dried gels can be kept for at least 1 yr.

RESULTS

Thin-layer isoelectric focusing (TLIF) appears to be a very convenient method for the screening of abnormal hemoglobins. A gel of 2 mm thickness was originally recommended by LKB. Ideally one would like to focus in a thinner gel in order to obtain more efficient cooling. We have made 0.5-mm-thick gels without encountering the inconveniences previously reported. Very clean glass and plexiglass plates, however, must be used. Such very thin gels with less heating and better cooling allow more power/cc of gel and thus a shorter focusing time and superior band resolution. In addition, they are very economical in their use of ampholines. A mixture of equal quantities of ampholines of pH 6.0-8.0 and pH 7.0-9.0 allowed good resolution of bands.
Fig. 1. Screening of abnormal hemoglobins by isoelectrofocusing on slabs of polyacrylamide gels.
along the entire length of the gradient, even for hemoglobins with high isoelectric points. These hemoglobins focus in a position very close to the cathode with the pH 6-8 gradient commercially available and previously used. It is possible to use dried blood samples, and washing of the red blood cells is not necessary. Furthermore, numerous samples can be examined on the same plate; staining was not necessary and was replaced by a short fixation in trichloroacetic acid. Figure 1 shows the analysis of 48 samples on a single plate. Even with so many samples, good resolution was obtained, HbA\textsubscript{1c} and HbF thus being clearly distinguished from HbA.

The precise characterization of unusual variants, however, must be performed with very dilute hemolysates (0.2%\textsubscript{a} for mutations of a β chain), which can be compared side by side, or even by mixing with known samples having a similar focusing position. α and β chain variants may easily be distinguished by focusing of concentrated samples (2%\textsubscript{a}, 4%\textsubscript{β}). In addition to the major abnormal fraction, the duplication of HbA\textsubscript{2} is induced by abnormal α chains associated with δ chains. With the usual methods, distinction between α and β variants can be obtained only by specific benzidine staining of cellulose acetate strips. A comparison of these two methods is shown in Fig. 2.

The difference between the migration patterns obtained by cellulose acetate electrophoresis and by isoelectric focusing is illustrated by Fig. 2A. The migration of a double heterozygote for HbS and an α mutation was studied. Four major bands could be distinguished on focusing. In addition, a minor fraction...
Fig. 3. Characterization of 70 mutations of human hemoglobin. Hb\(\beta^{+}\) and Hb\(\beta^{-}\) are ferrous-ferric hybrids of HbA. \(\beta\), \(\alpha\) mutants.

resulting from the duplication of HbA\(\beta\) could be seen close to the cathode. Electrophoresis on cellulose acetate could separate only three major fractions. The additional minor fraction migrated with carbonic anhydrase B on cellulose acetate strips and could be detected only by specific staining with benzidine. Figure 2B also shows a comparison of the two methods. Four \(\beta\) variations possessing the same type of amino acid substitution, i.e., glycine replaced by
aspartic acid (J Baltimore, J Calabria, Fannin Lubbock, and Hope) were mixed with HbA and analyzed. Electrofocusing distinguished five fractions, only two of which were seen with electrophoresis on cellulose acetate. Furthermore, side by side comparison allowed us to conclude that the α variant shown in Fig. 2A could be either HbG Philadelphia or Hb Winnipeg (Fig. 3). Structural studies confirmed that the mutant was HbG Philadelphia.

We previously reported the study of 31 variants; we now present the characterization of 70 variants (Fig. 3). The most common mutations associated with pathologic states are among them. We expressed the different isoelectric positions of the mutants studied as distances (in mm) from the position of HbA. Under the conditions described above and with a migration period of about 1 hr, variations from one experiment to another were less than 10%. The 70 abnormal hemoglobins are in 39 different positions, thereby representing a great improvement compared to classical electrophoretic techniques. Overlapping of bands is not negligible in the region of the gradient between Hb Zurich and HbG Georgia, in which HbS and 32 other variants, including Hb Fort de France and Hb Arya, are located. This result is not surprising, since the distance between Hb Zurich and HbG Georgia under our technical conditions is only 5 mm.

HbS can be clearly distinguished from all of the mutants, with three exceptions. The three variants exhibiting the same isoelectric position as HbS are HbG Galveston, HbG Norfolk, and Hb Stanleyville II. The latter two can easily be distinguished from HbS because they are α variants. Shown in Fig. 4 is the separation obtained with artificial mixtures of hemolysates of some subjects AD, AG, or AP with HbS. None of these separations can be obtained by conventional cellulose acetate electrophoresis. Thus with TLIF alone, in the absence of any complementary test, only HbG Galveston may be confused with HbS among the 31 representative electrophoretic “S-like” variants we studied. Moreover, TLIF allows the identification of some clinically important variants as HbD Punjab (i.e., Los Angeles) and Korle Bu, both of which interfere with sickling (Fig. 5A); Hb Korle Bu may be confused only with Hb Mobile.
HbD Punjab can be distinguished from the other variants tested, as shown in Fig. 5B and confirmed in Fig. 5C, in which HbS was added to the hemolysates. It is not possible to distinguish such variants from each other on the basis of their pH measurements because such small differences in the isoelectric positions, as shown in Fig. 5D, are beyond the accuracy of pH measurements even with the more recent contact electrodes.

Figure 5E shows the separation obtained by focusing between HbA and variants that are not easily distinguishable by electrophoresis. However, we
were unable to detect any separation between HbA and three other variants tested, i.e., Hb Bethesda, Hb Brigham (two hemoglobins with high oxygen affinity), and Hb Hammersmith (a highly unstable hemoglobin).

Two other unstable mutants are shown in Figs. 5F and 5G: Hb Köln ($\beta^{98}$ Val $\rightarrow$ Met), whose $\beta$ chain loses heme$^{16}$ but can recover it in vitro, and Hb St. Etienne ($\beta^{92}$ His $\rightarrow$ Gln), which has no heme on its $\beta$ chain and consequently is a semihemoglobin.$^{17}$ In these two cases, a major abnormal fraction is in a position similar to HbE and corresponds to the abnormal fraction lacking heme. In the case of Hb Köln, the most frequent unstable Hb, an additional fraction can be distinguished in a slightly more cathodic position than HbA; it is probably Hb Köln prior to loss of heme. Hb St. Etienne, which is unable to bind heme, does not contain such a fraction.

In addition to these variants, we also analyzed two postsynthetic fractions, one derived from HbF, i.e., acetylated HbF, and HbA1c, derived from HbA. Acetylated HbF was identified with isoelectric focusing in tubes by Drysdale et al.$^{18}$ Acetylated HbF represents about 10$^{\circ}\%$ of HbF in normal subjects$^{19}$ and is thus not a negligible component of cord blood.

A major difficulty encountered in analysis of cord blood with cellulose acetate electrophoresis is the distinction of homozygous HbS disease from that of a hemoglobin S/A heterozygote or S/$\alpha$ thalassemia, since acetylated HbF and HbA give the same electrophoretic pattern. Such a distinction can be obtained with agar electrophoresis, but TLIF offers an alternative possibility for this diagnosis, as shown in Fig. 6A, in which normal cord blood and heterozygous and homozygous S cord bloods are compared. The TLIF separation of acetylated HbF, HbA, and nonacetylated HbF allowed us to conclude that sample (1) is AS and sample (4) is SS (no HbA). In contrast, it is difficult to determine by cellulose acetate electrophoresis whether or not patients (1) or (4) are heterozygous for $\beta$S.
HbA\textsubscript{IC}, the glycosylated derivative of HbA,\textsuperscript{20} cannot be evaluated by electrophoresis and is slightly more anodic than HbA with isoelectric focusing.\textsuperscript{18} However, previous studies performed in tubes were inadequate for quantitation and for screening purposes.\textsuperscript{20} Quantitation of HbA\textsubscript{IC} is of clinical interest because HbA\textsubscript{IC} is increased in diabetes mellitus\textsuperscript{23} and its level reflects the mean level of blood glucose over long periods. Until now, assay of HbA\textsubscript{IC} has been performed essentially by column chromatography on amberlite.\textsuperscript{22,23}

Although reliable and accurate, chromatography is time consuming and inadequate for routine measurements. We therefore attempted to determine if TLIF might be suitable for the quantitation of HbA\textsubscript{IC}. Blood from a normal adult and a diabetic patient were compared (Fig. 6B); HbA\textsubscript{IC} focused in a position very close to that of HbA. However, a pH gradient of 1 unit (pH 6.5 to 7.5), with ampholites prepared in our laboratory according to the method described by Righetti and Drysdale\textsuperscript{88} permitted a separation superior to that obtained with commercially available (LKB) ampholines (pH 6.0–9.0). Preliminary results indicated that with a gradient of 1 pH unit assay of HbA\textsubscript{IC} in a mixture of purified HbA and HbA\textsubscript{IC} may be accurately performed with a conventional scanner such as the Supercellomatic (Sebia, Issy les Moulineaux, France). This procedure could provide an alternative to the chemical method recently presented by Flückiger and Winterhalter.\textsuperscript{24}

**DISCUSSION**

TLIF appears to be not only a technique of high resolution but also a very convenient one for the routine detection of abnormal hemoglobins. The possibility of using whole blood, the short period of migration required, the absence of staining, and the analysis of nearly 50 samples on the same plate are decisive advantages for its application to screening. The limiting factor might be the high cost of commercial ampholytes. However, as we showed, very thin gels can be easily made and result in a cost per sample comparable to that of cellulose acetate electrophoresis. The use of inexpensive, “home-made” ampholytes thus opens an unlimited field for the application of TLIF.

The present results have to be compared to those obtained by the chromatographic procedure\textsuperscript{25,26} and by the combination of electrophoretic techniques described by Schneider and co-workers.\textsuperscript{12,27,28} As reported by Huisman’s group, chromatography is time consuming and is not adequate for screening. Chromatographic procedures have been of higher precision than isoelectrofocusing in columns.\textsuperscript{29} However, the resolution with TLIF is comparable to that obtained by Huisman’s group using CM-Sephadex\textsuperscript{25} and DEAE-cellulose\textsuperscript{26} chromatography. The real alternative for screening purposes is the combination of electrophoreses in urea described by Schneider. However, TLIF presents numerous advantages compared to this technique. First, the excellent resolution of TLIF allows side-by-side comparison of hemoglobins whose migrations are identical upon cellulose acetate electrophoresis in urea (e.g., HbG Galveston and HbG Coushatta or HbS, Hb Lepore, and Hb Osu Christianborg). Second, variants migrating similarly to HbA will not be detected by electrophoresis. It is possible to use screening TLIF to detect electrophoretically silent variants such as Hb Saki,\textsuperscript{20} Hb San Diego, Hb Creteil, Hb Strasbourg,\textsuperscript{31} or Hb Malmö (Fig. 5E). Furthermore, agar electrophoresis (or solubility tests) are always required
as complementary methods to cellulose acetate electrophoresis for the distinction of HbS from S-like variants. In contrast, only one variant (HbG Galveston) has been confused until now with HbS by the focusing technique. Small differences in mobility are useful in differentiating mutants only when standards are available.

It may be difficult to identify some of the 70 variants scattered in 39 different focusing positions. However, in many cases apparent ambiguities can be resolved. When α and β variants focus in the same position, the duplication of HbA2 characterizes the α variants. Other ambiguities can be resolved with clinical information. For instance, Hb Creteil (polycythemia) may be distinguished from Hb Hope (asymptomatic), Hb Castilla (hemolytic anemia) from HbG Ferrara, and Hb Hamadan or Hb Saint Louis (cyanosis and hemolytic anemia) from other variants exhibiting the same isoelectric position.

Some variants can be characterized by their instability in hemolysates of washed red blood cells or in erythrocytes in the presence of cresyl brilliant blue. Thus it is possible to overcome the ambiguity presented by Hb Malmö (polycythemia), Hb Fannin-Lubbock (instability in vitro), and HbK Woolwich, whose isoelectric positions are identical (Fig. 3). In this last example, demographic data can be taken into consideration because HbK Woolwich, in contrast to Hb Fannin-Lubbock, is encountered in blacks. However, this last criterion is ambiguous in mixed populations, and mutations can occur independently in different populations.

It is not always possible to differentiate the abnormal hemoglobins with TLIF, even by increasing the length of migration or with more refined gradients of 1 pH unit or less, which increase the resolution. Coincidence will occur for many of the mutants described to date, and structural studies have to be performed in such cases. If structural study is undertaken, TLIF can be a useful tool prior to “fingerprints.”

Finally, ampholytes made in the laboratory by simple techniques may considerably extend the applications of TLIF, particularly in large-population screening and for preparative purposes, which until now have been limited by the high cost of commercial ampholytes.

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