The Amino Acid Composition of Hemoglobin. III. A Qualitative Method for Identifying Abnormalities of the Polypeptide Chains of Hemoglobin

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Separation of the component chains of human and animal hemoglobins may be achieved by a variety of methods including chromatography on IRC-50 using a urea gradient at pH 1.9, differential precipitation in trichloracetic acid and by countercurrent distribution. It is frequently desirable to separate and characterize the polypeptide chains of hemoglobins in small quantities as a rapid test for the presence of abnormalities in one or the other pair of chains or to determine the purity of a preparation prior to further analysis. To this end, Muller developed a procedure for the electrophoretic separation of the chains of human hemoglobin in starch gel at pH 1.9 and Take and Kraus have described paper electrophoretic procedures for the same purpose using concentrated urea buffers at a more basic pH. This manuscript describes a technic for separating the individual chains of human hemoglobin in starch gel in a urea-barbital system at pH 8.0 in such a way as to make possible the identification of abnormalities in the individual polypeptide chains.

Methods and Materials

Hemoglobin solutions were prepared by standard technics of washing the erythrocytes with normal saline and lysing the red cells with either water and toluene or water and CCl₄. The hemoglobin solutions used in this study usually underwent at least one purification step, employing chromatographic separation on IRC-50, CMC or DEAE cellulose before the globin was harvested.

Globin preparation: Small scale globin preparation can be carried out on as little as 1 mg. of hemoglobin by precipitating the globin from solution with cold acid-acetone. In routine use, 5 to 10 mg. of hemoglobin are dissolved in approximately 0.5 to 1.0 ml. of water and added dropwise to a test tube containing 25 ml. cold acid-acetone while mixing on a vortex agitator. The acid-acetone is prepared by adding 3 ml. of 2 N HC₁ to a liter of acetone and chilling the mixture to -15 C. in an acetone-CO₂ bath. The chilled mixture of hemoglobin in acid-acetone is mixed intermittently over a period of about 10 minutes following which the precipitate is removed by centrifugation at 10,000 rpm. for 20 minutes at -5 C. The supernatent is discarded and the precipitated globin, often barely visible to the naked eye, is washed one to two times with fresh portions of cold acetone. The globin, adherent to the centrifuge tube as a thin, often invisible film, is dissolved in 1 to 2 ml. water and lyophilized for easier handling.

Buffers: 18.4 Gm. diethylbarbituric acid are dissolved in 500 to 600 ml. boiling H₂O and the mixture cooled somewhat by the addition of 200 ml. of water. 60 ml. 1 N NaOH are then added with mixing, the solution cooled to room temperature and the volume brought
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Fig. 1.—All figures are oriented in the same fashion with cathode to left. Gel stained with AB-10. Slots are numbered from top to bottom. (1) Hb A₂; (2) Hb F; (3) Hb A; (4) Hb S; (5) Hb C.

up to 1 liter. The pH is adjusted to a final value of 8.00 ± 0.05 by the addition of small quantities of 1 N NaOH. In the event the barbituric acid precipitates from solution during this preparation, the mixture must be reheated and additional quantities of sodium hydroxide added to insure solubility at the approximate pH indicated.

Gel preparation: 180 grams of urea* and 110 per cent of the usual amount of starch for 500 ml. of buffer (5.5 x the amount indicated for 100 ml. of buffer) are thoroughly mixed in the dry state. The dry mixture is added slowly to 300 ml. of the veronal buffer in a 2 liter erlenmeyer flask with constant stirring to prevent clump formation. The thoroughly mixed material is heated in a water bath at 70 C. for 5 to 7 minutes with constant and vigorous shaking until the material becomes relatively smooth, clear and can be easily poured. Degassing is carried out until boiling occurs. The starch is then poured into the gel mold and permitted to remain at room temperature for approximately 24 hours before use. Such gels may be used for up to 72 hours after pouring.

Preparation of globin for electrophoresis: 1 to 3 mg. of dry globin are dissolved in 0.1

*Purchased from J. T. Baker Chemical Co., Phillipsburg, N. J.
ml. of the 6 M urea—0.1 M veronal buffer containing 2-mercaptoethanol (50 lambda mercaptoethanol per 5 ml. of the urea-veronal buffer). The sample is mixed until dissolved and centrifuged to remove any insoluble material. Globin solutions so prepared are used immediately for analysis or may be stored for up to 3 to 5 days if kept at -20 C. At room temperature, deterioration of the sample takes place rapidly.

Electrophoresis: The vertical starch gel electrophoretic technic of Smithies has been used throughout these studies. Two commercially available types of electrophoretic chambers have been employed with little difference in the final result.* Following insertion of the globin samples into the slots, the latter are covered with melted vaseline and, in the case of the apparatus marketed by Hiller, covered with Saran Wrap. The gel is placed with the slots towards the top and the electrodes connected so that the anode is at the bottom. Electrophoresis is carried out in a cold room for 22 hours at 250 to 300 volts and 35 to 45 ma. The electrode chambers, using platinum electrodes, all contain the same urea-

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*Obtained from Otto Hiller, P. O. Box 1294, Madison, Wisc. and Buchler Instruments, Inc., Ft. Lee, N. J.
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Fig. 3.—See legend under figure 1. (1) Hb A; (2) Pure α chain preparation; (3) Hb A2; (4–5) Nil; (6) Unseparated hemoglobin mixture of Knoxville No. 1 and Hb A; (7) Same; (8) Hb A2; (9) α tetramer—pure α chains; (10) Hb A.

Results

Utilizing the technic described in this manuscript, advantage is taken of the ability of urea to cause dissociation of the polypeptide chains of hemoglobin\textsuperscript{1} and of mercaptoethanol to block reactive -SH groups\textsuperscript{7} which may, under certain conditions, cause disulfide bridging with resultant alterations in peptide mobilities and polymer formation. Although no disulfide bridges exist in native hemoglobin, we have found sharpened separation of the peptide chains of human globin prepared with mercaptoethanol. As is seen in figure 1, α and β chains move in opposite directions from the point of insertion under the conditions described, the α chains moving toward the cathode, the β

*Purchased from E. Merck AG, Darmstadt, Germany.
Fig. 4.—See legend under figure 1. (1) Hb A; (2) Hb New Haven No. 1; (3) Unseparated hemoglobin mixture of Hb Chicago No. 1 and Hb A; (4) Hb S; (5) Unseparated mixture of Hb A and Hb S; (6 and 7) Clean α chain preparations; (8) Clean β chain preparation; (9) Clean β chain preparation from Hb Durham No. 1; (10) Hb A.

toward the anode. Abnormal chains involving amino acid replacements in either of these polypeptides are easily detected (figs.1–5). Equally clearcut are the positions of the delta chains which migrate little if at all out of the starting slot and gamma chains which migrate slightly behind the position of the normal β chain (figs. 1–3). It is almost certain that alterations in the amino acid composition of the gamma and delta chains would affect their relative positions. However, at this time we have not had an opportunity to examine such hemoglobin variants. However, the non alpha chains of Hb New Haven No. 1, a Lepore-like hemoglobin which is presumed to have anomalous nonalpha chains composed of part β and part delta chains,10 have a mobility between those of normal β and delta chains (fig. 4).

As is seen in the accompanying figures, abnormalities of either the α chain (in this instance (figs. 2 and 3) Hb Knoxville No. 11 now shown to be identical with GPhiladelphia-α2 68 by β2) or of the β chain (figs. 4 and 5) are
readily demonstrable even when whole blood mixtures of unseparated hemoglobin types are used as the starting material. $\beta$ chain abnormalities with a mobility greater than Hb A on electrophoresis at pH 8.8 separate definitively (Hb R or Durham No. 1, Hb Chicago No. 1, and Hb New Haven No. 2 are illustrated in figures 2, 4 and 5). Those with a mobility less than Hb A are also easily identified (Hb S and C shown in figures 1, 4 and 5). Hb A$_2$, with an electrophoretic mobility essentially identical to that of Hb C, contains delta chains which have, as would be expected, a mobility approximately equal to the $\beta$ chains of the latter (fig. 1). Pure peptide chains of hemoglobin, prepared by either a combined technic of chromatography and countercurrent distribution or as found in $\alpha_2$ tetramers identified in normal hemolysates demonstrate how the technic can be utilized to test the purity of peptide chain preparations prior to amino acid analysis (figs. 3 and 4).

In view of the ease and specificity with which abnormalities of either peptide chain of hemoglobin can be identified with this technic, we no longer find it necessary to resort to dissociation and recombination studies for such information.

Fig. 5.—See legend under figure 1. (1) Hb A; (2) Hb New Haven No. 2; (3) Hb Durham No. 1; (4) Hb C (containing small amount Hb A); (5) Hb A.
SUMMARY

A relatively simple method is described which permits the identification of abnormalities of either polypeptide chain of hemoglobin. The procedure is based on the dissociation of hemoglobin by 6 molar urea and starch-gel electrophoresis in a barbital buffer at pH 8.0.

SUMMARIO IN INTERLINGUA

Es describite un relativemente simple methodo que permette le identificatio de anormalitates in le un o le altere del catenas polypeptidic de hemo-globina. Le technica se basa in le dissociation de hemoglobina per 6 M de urea e electrophorese a gel de amylo in un tampon de barbital a pH 8,0.

REFERENCES

10. Unpublished observations.

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