THE AMINO ACID COMPOSITION OF HEMOGLOBIN

I. AN IMPROVED METHOD FOR SEPARATING THE PEPTIDE CHAINS OF HUMAN HEMOGLOBIN*

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Hemoglobin dissociates into its component polypeptide chains when subjected to an environment of low pH and ionic strength[1-3]. Separation of the dissociated chains has been achieved by a number of techniques including electrophoresis[4], countercurrent distribution[5], fractional precipitation from acid acetone and ion exchange chromatography[6]. Most notable have been the studies of WILSON AND SMITH who separated the α and β chains of horse globin by elution from Amberlite IRC-50 resin with the use of a urea solution of increasing molarity at low pH[6]. Subsequently, similar techniques have been used to separate and isolate the α, β, and γ (and possibly δ) chains occurring in human hemoglobin[7,8] and N-terminal group analyses have permitted their more precise characterization. Although the technique of WILSON AND SMITH has proven highly satisfactory for clear cut separation of the α and β chains of horse globin, much less distinct separations were obtained when this method was applied to human globin. As a consequence of the overlap of the eluted chains and the resulting cross contamination, a significant amount of material must be sacrificed to ensure a modest degree of purity of the fractions, often a serious problem when dealing with the small amounts of protein available with some of the rare types of hemoglobin. A modification of the urea gradient method of WILSON AND SMITH, which results in almost complete separation of the two types of chains of human hemoglobin and their isolation in relatively pure form in high yield, is described in this communication. The chief modification involves elution by an "interrupted" or "intermittent" urea gradient in place of the continuously variable gradient of WILSON AND SMITH.

METHODS

Hemoglobin was prepared from washed, lysed erythrocytes and purified by one of several methods, including precipitation or crystallization from ammonium sulfate solutions, elution from starch after starch block electrophoresis[9] or by denaturation with highly alkaline solutions (for Hgb F isolation)[10]. Globin was prepared from either

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the crystallized or purified material by the acid-acetone method of ANSON AND MIRSKY[11]. To a 5 % solution of the hemoglobin, 10 to 15 vol. of acetone (containing 2.5 g of oxalic acid per 100 ml of acetone) were added slowly with constant stirring. Following filtration under gentle suction, the globin cake was washed repeatedly with acetone until free of heme and oxalate, air dried, and ground into a finely divided powder.

Amberlite resin, CG 50 type 2, in its acid phase, was prepared by the method of HIRS, MOORE AND STEIN[12] and suspended in 11.7 % formic acid. Chromatographic columns, 1.9 × 90 cm, were filled to a height of approximately 60 cm with the resin. From 500 to 700 mg of globin may be fractionated on a column of these dimensions. The globin was dissolved in 11.7 % formic acid (10 mg/ml), adsorbed with a fresh aliquot of resin suspended in 11.7 % formic acid and added to the top of the column. Approximately 1.5 ml of sedimented resin per ml of globin solution were used for adsorption. Chromatographic separation was carried out at room temperature.

Elution techniques

For comparative purposes, the technique of WILSON AND SMITH was followed with only minor modifications[6]. Approximately 1600 ml of 2 *M* urea, adjusted to pH 1.9 with concentrated HCl, were placed into a two liter bottle and 3000 ml of 8 *M* urea, pH 1.9, into a four liter bottle. Six hundred ml of the 2 *M* urea were passed through the column at the rate of approximately 2 ml/min and the effluent collected in 20 or 25 ml aliquots. Gradient elution was then started by the dropwise addition with stirring of the 8 *M* urea solution to the 1000 ml of solution remaining in the smaller bottle. After 2000 to 2500 ml of solution had passed through the column, the gradient was stopped and 8 *M* urea was utilized for the final phase of elution. The optical density of each tube was read at 280 mμ and the results graphed as in Fig. 1.

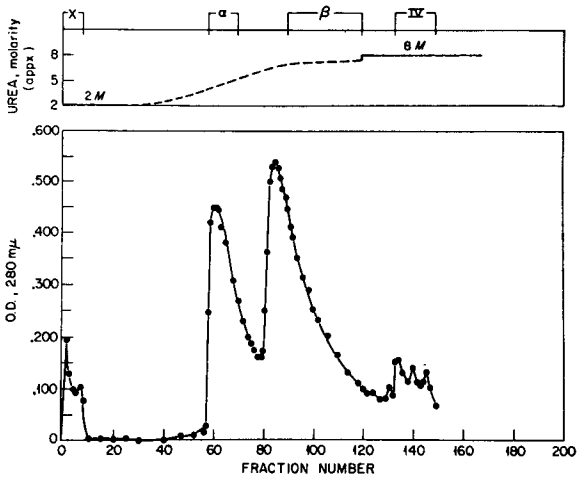

Fig. 1. Separation of the α and β chains of human globin by the method of WILSON AND SMITH[6]. 650 mg globin A; 20 ml fractions; pH 1.9; IRC-50 column; continuous urea gradient.

The modified technique employed in this study differs from that of WILSON AND SMITH in several respects. In general, solutions of 2 *M* and 8 *M* urea were prepared as above. 2000 ml of the 2 *M* urea and 3000 ml of the 8 *M* urea were introduced into appropriately sized bottles. The effluent from the column was continuously monitored by a direct recording U.V. detector (280 mμ)* (Fig. 2). For convenience, elution was

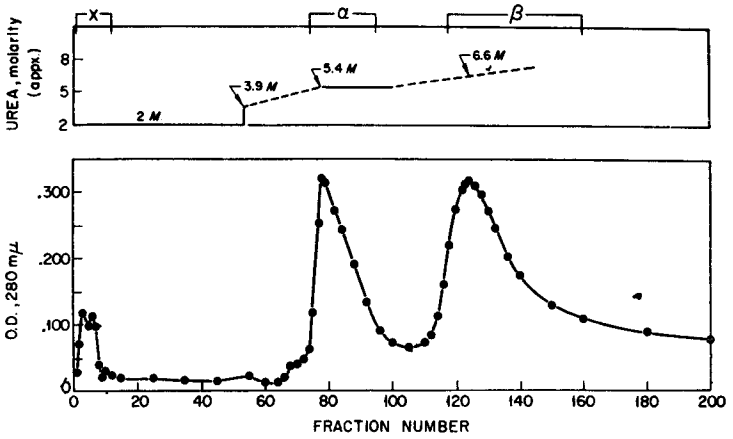

Fig. 2. Separation of the α and β chains of globin A by the interrupted gradient technique. 450 mg globin A; 25 ml fractions; pH 1.9; IRC-50 column; interrupted urea gradient.

started at the end of the working day and 1000 ml of the 2 *M* urea was permitted to wash through the column slowly overnight (approx. 1 ml/min). Similar results, however, were obtained by passing through the column only enough of the 2 *M* urea solution to elute the initial fractions which appear at the beginning of each run (usually 300 to 400 ml) at which time the absorption curve of the effluent will have returned nearly to its base line value. Appropriate adjustment of the starting volume of the 2 *M* urea should, however, be made. Five hundred ml of the 8 *M* urea solution were then added to the remaining 1000 ml of 2 *M* urea, resulting in a solution of 4 molar concentration, and a dropwise gradient begun. Elution was carried out at the rate of 2 ml/min until the peak of the α chain had eluted, at which point the gradient was interrupted and the solution of constant molarity (approximately 5.3 *M* urea) continued until the nadir had been reached. At this point, the gradient was resumed and continued until the β chain was almost completely removed after which 8 *M* urea was permitted to elute a final, sharp, pigmented peak from the column.

More recently we have found that 3 *M* urea can replace the 2 *M* solution as the initial eluting agent without causing any loss of the α chain. One may, after the initial phase of the procedure, shift directly to a urea gradient starting with the 3 *M* solution, or jump the urea concentration to approximately 4.0 to 4.2 before starting the gradient, as described above, the latter procedure shortening the total time of the run. Similarly, since the β peak and the material eluted with 8 *M* urea are identical on peptide analy-

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sis, one may switch over to 8 *M* urea as soon as it is apparent that the β peak is coming off the column.

Protein determinations were carried out by the method of LOWRY *et al.*[13] on 0.2 ml aliquots. The presence of heme was ascertained by the benzidine method of CROSBY AND FURTH[14] on 0.5 ml samples. Approximate urea molarities were calculated by standard mathematical techniques utilizing the known initial pot volume and urea concentration and determining the molarity at any point after the addition of the specified amount of 8 *M* urea.

The isolated fractions were dialyzed against 8 to 10 changes of distilled water at 4° until urea was no longer detected by the diacetyl monoxime method[15]. Overnight dialysis versus cold running tap water resulted in the precipitation in the dialysis bags of considerable material which was poorly digested by trypsin and yielded inadequate fingerprints. Hence, this method was abandoned. Peptide analyses of the separated, lyophilized fractions were carried out by techniques described in a separate communication[16].

RESULTS AND DISCUSSION

Comparative elution curves of the α and β chains of human hemoglobin resulting from the two methods of separation are presented in Figs. 1, 2 and 3. In general, their characteristics are remarkably similar. The series of peaks, eluted by 2 or 3 *M* urea within the first 250 to 350 ml of effluent (fraction X in Figs. 1 and 2), strongly absorb U.V. light at 280 mμ. This material, however, does not react with ninhydrin, with Folin reagent or with benzidine and probably represents formic acid washed out of the column by the advancing buffer front. The α peaks of both Hgb A and Hgb F make their appearance at urea concentrations as low as 3.8 *M*, although maximal elution occurs at approximately 4.9 to 5.1 *M*. β chain elution starts at a urea molarity of

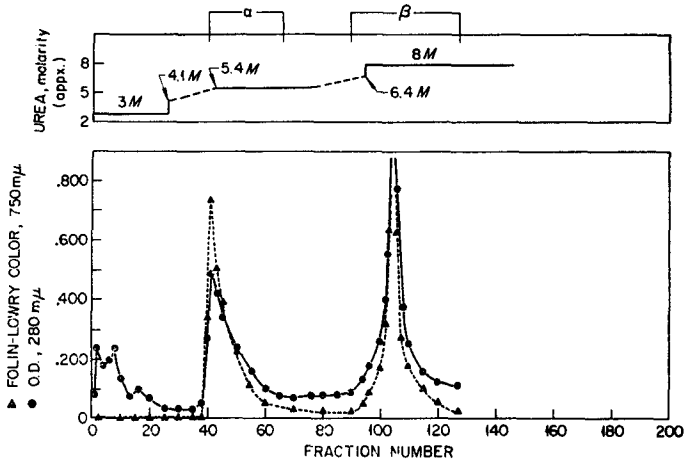

Fig. 3. Separation of the α and β chains of globin A by the interrupted gradient technique. 650 mg globin A; 25 ml fractions; pH 1.9; IRC-50 column; interrupted urea gradient. Also shown are the intensities of Folin–Lowry color and approximate urea molarities.

approximately 5.8 while maximal elution occurs in the range of 6.2 to 6.4 M. The γ chain characteristically is washed from the Amberlite resin at somewhat lower molarities, elution starting at 5.4 to 5.5 M with peak recovery at approximately 5.9 to 6.1 M urea. These figures are at considerable variance with those reported by HUISMAN AND SEBENS[17,18] who noted elution for all three chains at significantly higher molarities. We can offer no ready explanation for the inconsistencies noted, but it is possible that, because of the differences in techniques in our respective laboratories, such variations are more apparent than real.

Certain characteristics of the types of peaks obtained by the "interrupted" or "intermittent" gradient system deserve mention. The α and β curves may be separated by as great a distance as the operator desires by appropriate adjustment of the starting volumes of solution, provided one knows at approximately what molarity of urea maximal elution of the chains occurs. Furthermore, the sharpness of the graphed peaks provides an indication of the cleanness of separation—an abrupt rise and slightly slower decrease characterizing the elution of relatively uncontaminated peptide fragments. Since the gradient is interrupted just after the α peak is detected by the recorder, it is apparent that a urea molarity somewhat higher than indicated above (because of dead space and holdup volume) will "wash" the column until the gradient is resumed. Continuing the "wash" with the urea solution of constant molarity permits the curve to return almost to the base line before reinstituting the gradient for β chain elution. In the technique of WILSON AND SMITH, the "trough" between the two peaks remains considerably above the base line due to considerable mixing of the respective chains in this area. Visual inspection of the recording curve also permits one to assess the completeness of α chain removal from the resin. On resumption of the gradient, an additional small peak before the main β chain elutes or marked skewing of the curve of β chain elution suggests incomplete separation of the two polypeptide chains. Although rarely a problem in dealing with β chains, complete separation may be more difficult to achieve with the γ chains of Hgb F. Peptide patterns of the tryptic digests of the α chains prepared by the "interrupted" method reveal virtually no contamination with β chains, while only very slight contamination of the β chain by α chain peptides has been observed. Our data on the γ chain contamination is not yet sufficiently detailed to determine the degree of admixture.

The final peak eluted by 8 M urea, and designated IV in the figures, is highly pigmented, has a peptide pattern identical with that of the β or γ chains and probably represents aggregation of these polypeptides. It is of interest that most of the heme positive material is present in this region, although the total amount of benzidine reacting substances is very small.

Protein determinations by the technique of LOWRY *et al.*, reveal no reacting material in fraction X, while the α, β and IV peaks contain amounts equivalent, in general, to those expected from the U.V. absorption. As mentioned above, the benzidine positive material is associated almost entirely with the initial portion of fraction IV although, occasionally, traces may be seen in the early parts of the α and β fractions.

Following the separation, dialysis and lyophilization of the α and β chains as

described above, approximately 60 to 65 % of the starting globin is recovered. In the technique of WILSON AND SMITH, approximately 30 % of the total recovery represents α chains, 25 % β chains, 10 % fraction IV and 35 % the mixed material between the α and β peaks. In the interrupted gradient method, the corresponding values are 40 to 45 % for α, 5 to 10 % for the intermediate zone, 30 to 35 % for β and 10 to 15 % for fraction IV.

SUMMARY

A modification of the gradient elution method of WILSON AND SMITH for the preparation of the individual polypeptide chains of human globin is described. The chief advantage of the modified procedure relates to the improved separation between the α and β or γ chains.

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