THE AMINO ACID COMPOSITION OF HEMOGLOBIN

IV. THE PREPARATION OF PURE POLYPEPTIDE CHAINS OF HUMAN HEMOGLOBINS*

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Pure polypeptide preparations are vital to the successful amino acid analysis of any protein material. For those dealing with the analysis of hemoglobin, large scale separation of the protein into its component polypeptide chains has been carried out by means of column chromatography on Amberlite IRC-50 utilizing a urea gradient at pH 1.9[1,2], by elution from carboxymethyl cellulose (CMC) with pyridine buffers[3], by fractional precipitation with trichloroacetic acid (TCA)[4,5] and by countercurrent distribution[6]. Each of these methods presents serious difficulties or shortcomings. The IRC-50 technique, though easiest to do, fails to provide clean non-α-chains in the case of human hemoglobin, although essentially pure preparations of the α-chains are easily obtained. The CMC technique is highly dependent on the individual batch of cellulose employed, is thus difficult to standardize, and, in our hands, has not given sufficiently clean preparations of the peptide chains. At first glance, the fractional precipitation method of HAYASHI[4] would seem to provide ease and reliability for such separations, but we have never been able to achieve pure non-α-chain preparations while on only rare occasions have we been able to prepare relatively clean α-chain material using this technique. The countercurrent method requires equipment not available in many laboratories as well as very careful attention to details. Furthermore, clean α-chain preparations are not uniformly obtained by this procedure. In view of these difficulties, we have attempted to incorporate the better aspects of several of these techniques into a method which would provide pure α- and non-α-chain preparations of human hemoglobins, would be simple to perform, easy to standardize and would utilize unspecialized equipment readily available in most laboratories.

METHODS AND MATERIALS

Hemoglobin solutions were prepared by standard techniques of washing the erythrocytes with saline and lysing by water and toluene or water and carbon tetrachloride. Hemoglobin preparations were purified by chromatography on CMC[7] or DEAE cellulose[8]. Frequently, rechromatography on one or the other ion exchanger or successive passage through both kinds of cellulose was carried out until the preparations were judged to be homogeneous by starch gel electrophoresis.

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Globin preparations were obtained from the pure hemoglobin fractions by precipitation with acid acetone in a scaled up version of a technique previously described[9], which is based on the method of ANSON AND MIRSKY[10].

Chromatography on IRC-50

Amberlite columns, prepared essentially as previously described[2], were utilized for the initial phase of the separation of the α- from the non-α-chains. The resin, CG-2, was purified as described by HIRS, MOORE AND STEIN[11] and sized by the hydraulic technique of HAMILTON[12] to obtain a more uniform particle range and thus improve the flow rates. Columns, 1.9 × 65 to 70 cm, resin height, were poured in one successive batch by permitting a slurry of Amberlite to enter the top of the column as the suspending buffer flowed slowly from the bottom. Columns poured in this fashion have flow rates of from 100 to 150 ml/h at room temperature. The clean, sized resin in its acid phase was equilibrated with 11.7 % formic acid before pouring, and the completed column was further equilibrated with 500–1000 ml of this same material. From 500 to 800 mg globin were dissolved in 11.7 % formic acid at approximately a 1 % concentration and the clear solution added directly to the top of the column. 500 ml of 2 *M* urea, brought to pH 1.9 with concentrated HCl, were allowed to pass through the column slowly overnight, during which time non-heme proteins and non-protein materials are eluted. The elution buffer was then changed to 5 *M* urea, pH 1.9, and the effluent monitored at 280 mμ utilizing a Gilson Medical Electronics instrument. Fraction I consists of the effluent containing α-chain material which was collected from the time of the initial rise of the absorption at 280 mμ until the recording graph demonstrated a plateau effect on the descending limb of this peak (Fig. 1). Approximately 300–400 ml of 5 *M* urea suffice to recover the α-chains, following which 8 *M* urea, pH 1.9 was utilized for the elution of the remaining material. Fraction II consists of a mixture of approximately equal amounts of α- and β-

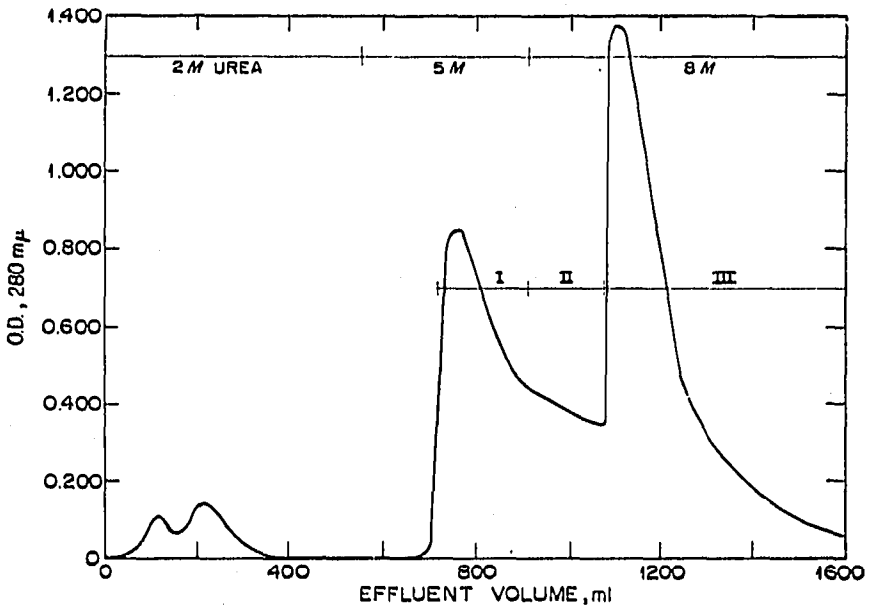


Fig. 1. Chromatography of globin from Hgb A into α- and non-α-polypeptide chains. Amberlite IRC-50, pH 1.9; column dimensions: 1.9 × 65 cm; 500 mg globin A; elution buffers: 2 *M*, 5 *M* and 8 *M* urea, pH 1.9; optical densities at 280 mμ. See text for definition of fractions I, II and III.

chains and represents the material collected from the time the elution buffer was changed from 5 *M* to 8 *M* urea until the sharp rise in optical density signals the elution of the β-chains. Fraction III consists of α- and β-chains in a ratio of approximately 1:3 and comprises the remaining effluent from the column. From 600 to 800 ml of the 8 *M* urea are sufficient to clear the column of α- and β-chains.

Fraction I

This material contains 95–98 % α-chains and may be dialyzed *versus* repeated changes of distilled water in the cold until free of urea (14 to 18 changes of the dialysis fluid will be required). The dialyzed material is then freeze dried. A yield of 35 % of the starting material is usually obtained. An alternative method of preparation eliminates the long dialysis procedure mentioned above and depends on the precipitation of the protein by TCA followed by washing with acetone to remove excess reagents. After reducing the urea content with 2 to 4 changes of the dialysis fluid, the polypeptide is precipitated by the addition of sufficient TCA to constitute an approximately 0.6 *M* solution (10 g per 100 ml of solution). The resulting precipitate is washed 3 times by centrifugation with acetone and dried under vacuum. This procedure yields a product indistinguishable in behavior and amount from that obtained by the method described above.

Fractions II and III

These fractions must be repurified to obtain satisfactory β-chain preparations. Dialysis to remove much of the urea is carried out against 2 to 4 changes of distilled water and the peptide mixtures are precipitated by the TCA technique described above. The dried samples, representing approximately 15 % of the starting material in fraction II and 40 % in fraction III are subjected to a modified countercurrent separation according to the technique of BOWMAN AND INGRAM[13]. 200 ml of 1 % aqueous dichloroacetic acid are mixed several times with 200 ml of reagent grade *sec.*-butanol in a separatory funnel and allowed to equilibrate for 1 h at room temperature. The two phases are drawn off separately. 8 ml of the lower phase are introduced into a series of 18 test tubes, 15 × 125 mm. To tube No. 1 are added up to 125 mg of fraction II or III and the mixture agitated until maximal dissolution is achieved. 8 ml of the upper phase are then added to tube No. 1 and the mixture again shaken, at which time any undissolved material will have been noted to go into solution. The stoppered tube is centrifuged at 2000 r.p.m. for 5 min at 5°, which results in a clean separation of the two phases. The upper phase of tube No. 1 is transferred to tube No. 2 and approximately 8 ml of fresh upper phase are added to tube No. 1. In transferring the upper phase of tube No. 1 to tube No. 2, any whitish suspension should be allowed to remain with tube No. 1 and care should be taken not to transfer any of the lower phase to tube No. 2. The tubes are mixed, centrifuged and transferred as before to each successive tube until the 18th transfer has been achieved. Protein determinations on the well mixed contents of each tube are carried out on a small aliquot by the method of LOWRY *et al.*[14]. A curve of the optical densities at 750 mμ will demonstrate three peaks, the initial two containing variable quantities of α- and β-chains, the third, 93–98 % β-chains (Fig. 2). The latter material is harvested by washing fraction C with ether in a separatory funnel and freeze drying the aqueous layer. Peaks A and B may be repurified by an additional passage through

the 18 tube transfer system. One may thus obtain a yield of approximately 35 % purified β-chains in addition to a like amount of similarly purified α-chains.

Starch gel electrophoresis of the individual fractions was carried out in urea–veronal buffer, pH 8.05, by methods previously described[9].

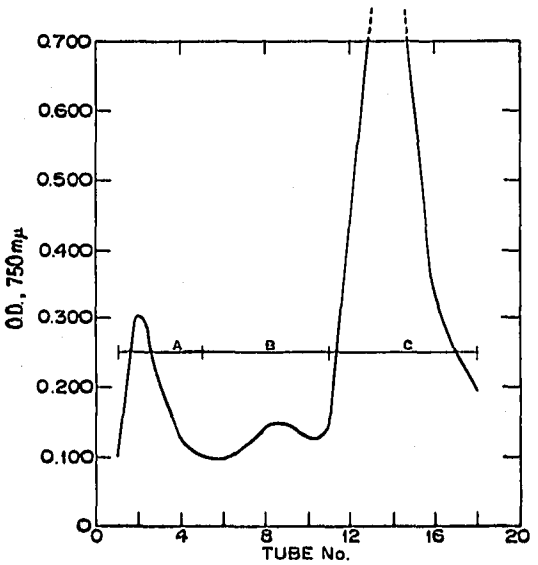


Fig. 2. Separation of non-α- from α-chains of globin A by countercurrent distribution. Protein determinations done on 0.2 ml aliquots from each of the 18 tubes by the method of LOWRY *et al.*[14]. See text for definition of fractions A, B and C.

Peptide mapping of the tryptic digest of the individual purified chains was carried out by chromatography in a butanol–acetic acid–water (4:1:5) system and electrophoresis at pH 3.7 or 6.4 in pyridine–acetic acid–water buffers by techniques previously described[15,16]. A determination of the amino acid composition of the individual peptides or of the purified polypeptides was carried out by hydrolysis with 6 *N* HCl in sealed evacuated tubes for 24 or 72 h at 108° followed by analysis on an automatic amino acid analyzer by the method of MOORE, SPACKMAN AND STEIN[17].

DISCUSSION

Of the various methods described for the preparation of hemoglobin polypeptide chains of sufficient purity for further analysis, the countercurrent distribution technique yields the best material. Even with this technique, however, the α-chain peptides frequently display excessive contamination with non-α-components. Furthermore, highly specialized, expensive equipment is needed to carry out a sufficient number of transfers to promote separation of the two types of chains. Although the Amberlite technique is the easiest with which to deal, sufficiently pure β-chain preparations cannot be obtained. By combining the better characteristics of these two procedures, it is possible to obtain good yields of sufficiently pure polypeptide chains to carry out quantitative amino acid studies. If one adds to this technique the simplification suggested by the method of HAYASHI[4], that is, the precipitation of the chains by TCA and extraction of the excess reagent by acetone or ether, one has a

reliable, relatively uncomplicated procedure which yields highly purified fractions in satisfactory quantities for further study.

It is unfortunate that the TCA method of HAYASHI[4] has not been found to work adequately in our laboratory since it would appear to be ideal for the purposes at hand. Among the reasons for this difficulty may be mentioned the following. Perhaps the most critical problem in using TCA for differential precipitation is the difficulty in preparing precisely similar TCA solutions in view of the hygroscopic nature of the chemical. Absolute control of the temperature of precipitation seems to be needed and the end point of titration is exceedingly difficult to visualize. Even in our best preparations, gross (10–20 %) contamination of one chain with another could be seen on starch gel analysis. Nevertheless, precipitation with TCA does permit one to deal with the purified material obtained in the method described in this manuscript in a more expeditious way.

DINTZIS[3] has pointed out that different batches of CMC yielded different degrees of separation of the polypeptide chains of animal hemoglobins[3]. We have tried four different preparations of CMC, including one home-made batch, without success in achieving a clean-cut separation. The reasons for our lack of success may well reside in the fact that only specific lots of the exchanger seem to work.

Our experience with the technique described in this presentation suggests that it is more than adequate to achieve a satisfactory product. Starch gel patterns of the isolated fractions obtained reveal no visible contamination of one chain by the other (Figs. 3, 4 and 5) although quantitative amino acid analysis suggests the possibility of traces of such contamination, amounting to significantly less than 5 % in most preparations. This degree of contamination is insufficient to show up on peptide mappings as can be seen in Figs. 6 and 7. We have used the method with equal success with hemoglobins possessing abnormal α-, β- or δ-chains. To date we have not had an opportunity to study abnormal γ-chains but we presume they would act in

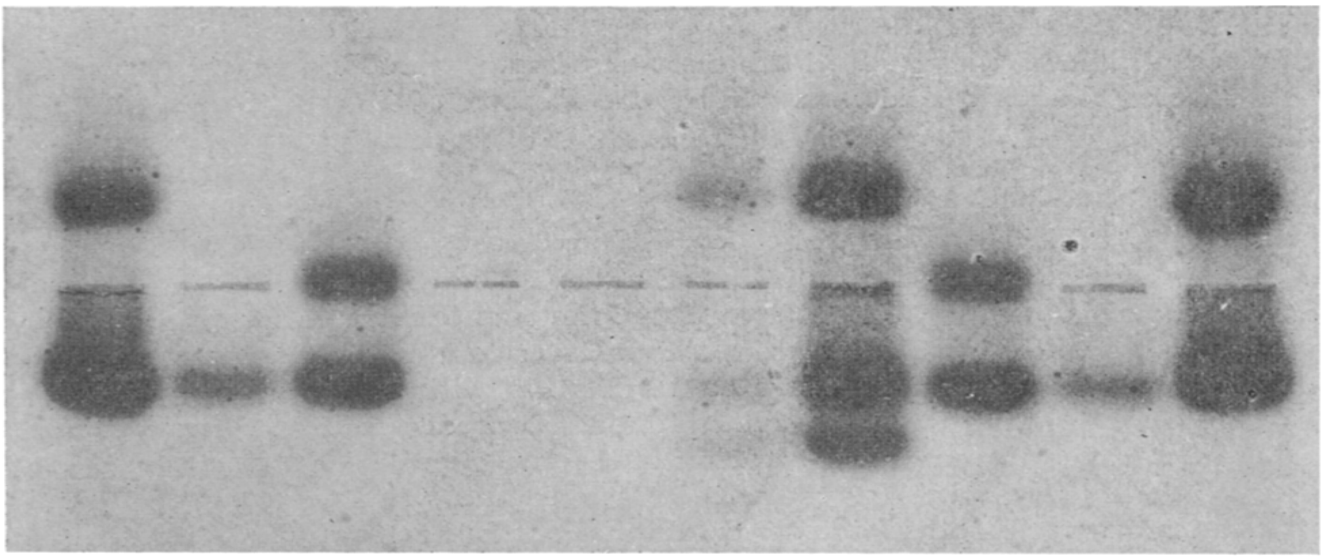


Fig. 3. Starch gel electrophoresis of polypeptide chains of human globin in urea–veronal buffer, pH 8.6[9]. Anode at the top. Slots numbered from left to right. Stained with AB-10. Pure α-chain preparations in slots 2 and 9. Normal globin (α- and β-chains) in slots 1 and 10. Globin from Hgb A_2 (α- and δ-chains) in slots 3 and 8. Globin from a mixture of normal hemoglobin and hemoglobin with abnormal α-chains in slots 6 and 7.

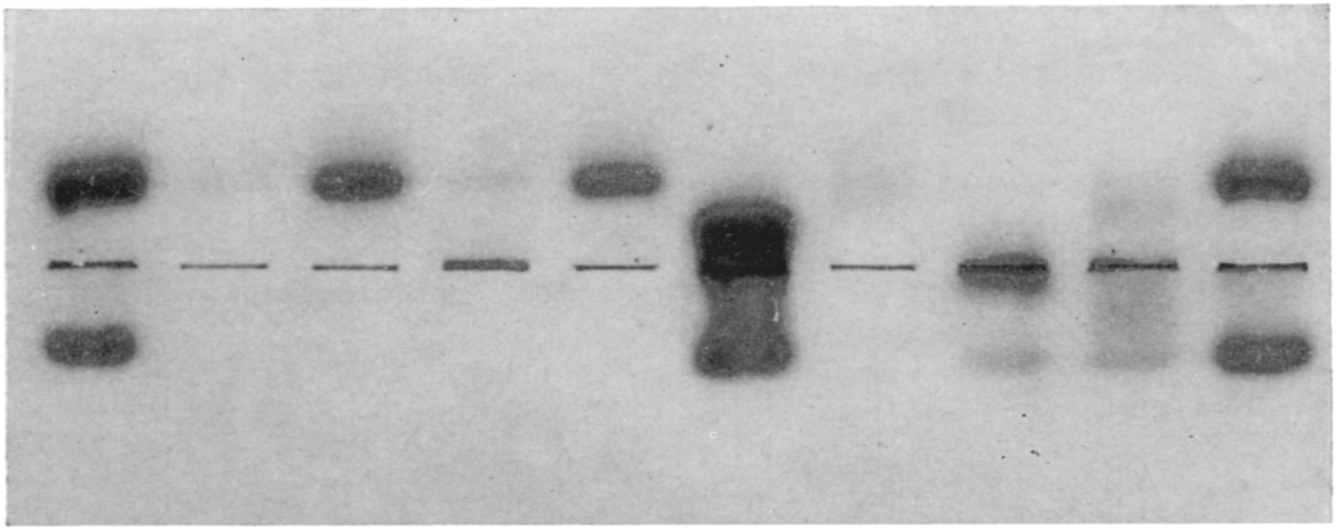


Fig. 4. See legend of Fig. 3. Purified β-chains from fraction C seen in slots 2, 3, 4 and 5.

a comparable fashion. In evaluating this procedure, several aspects are worthy of comment. By pouring the Amberlite column in a continuous slurry, sharper peaks seem to have been achieved. It is also likely that the hydraulically sized resin has contributed to the cleaner separation of the α-chain in comparison to previous work with this system. Certainly, the flow rates, faster than previously observed, are a reflection of the uniformity of the resin particles. Because no gradient is involved in the ion exchange portion of the procedure, the technical set-up of the column has become simpler. We have adopted a urea molarity sufficient to remove most but not all of the α-chains in the resin, but insufficient to start the elution of the non-α-

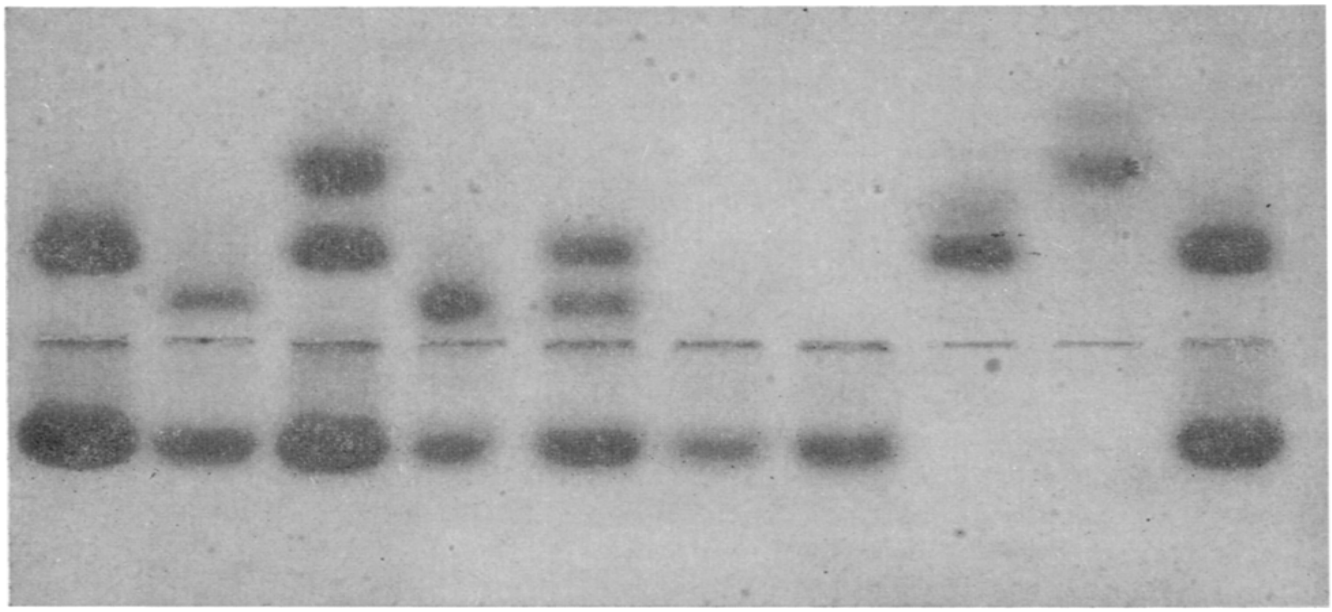


Fig. 5. See legend of Fig. 3. Pure α-chains from Hgb A (from fraction I) in slots 6 and 7. Pure β-chains from Hgb A (from fraction C) in slot 8. Pure β-chains from abnormal Hgb Durham No. 1 (from fraction C) in slot 9. Remaining slots contain globins from a variety of unseparated hemoglobins.

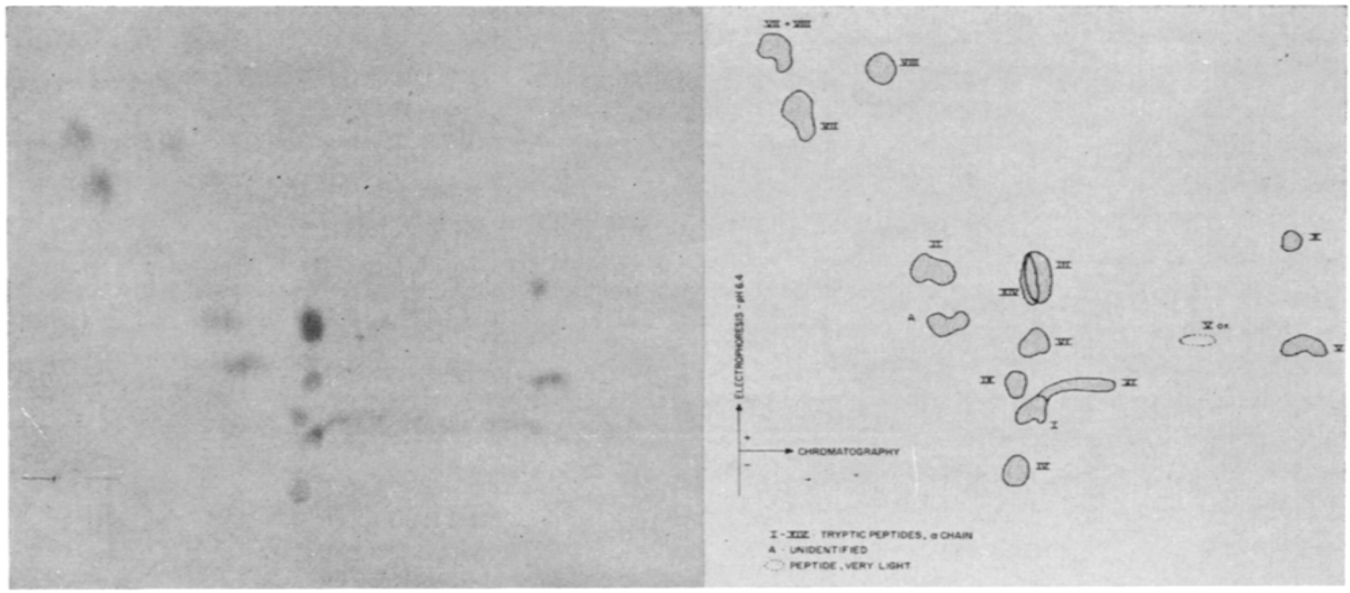


Fig. 6. Tryptic peptide map of α-chain of Hgb A harvested from fraction I of IRC-50 column. 0.5% ninhydrin in 100% ethanol used for stain. The line drawings of the peptides indicate their position and official numbers.

chains. The latter requires 5.3–5.6 *M* urea before the initial traces are eluted. Should one wish to increase the purity of the α-chain preparation, fraction I may be cut off just beyond the point of peaking noted in Fig. 1, with, of course, a corresponding decrease in yield.

Points of note related to the countercurrent distribution aspect of the procedure are as follows. The relatively limited number (18) of transfers employed has proven to be sufficient to provide the degree of purification sought because of several reasons. First, the starting material is already enriched in terms of the non-α-chains and has

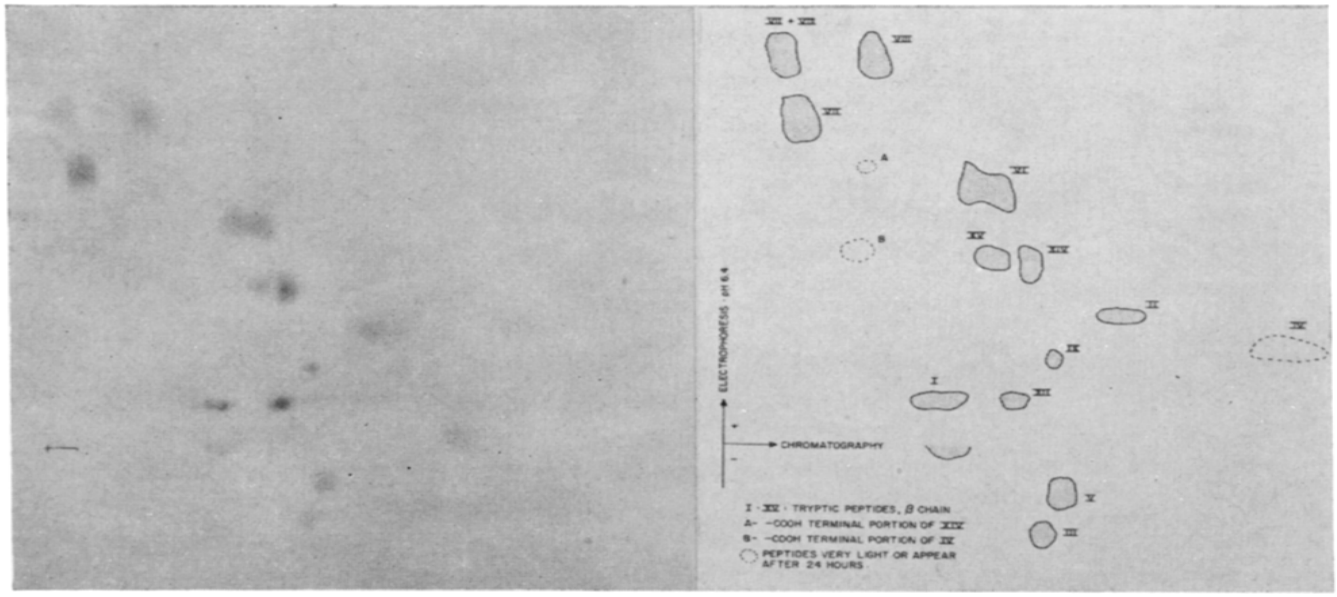
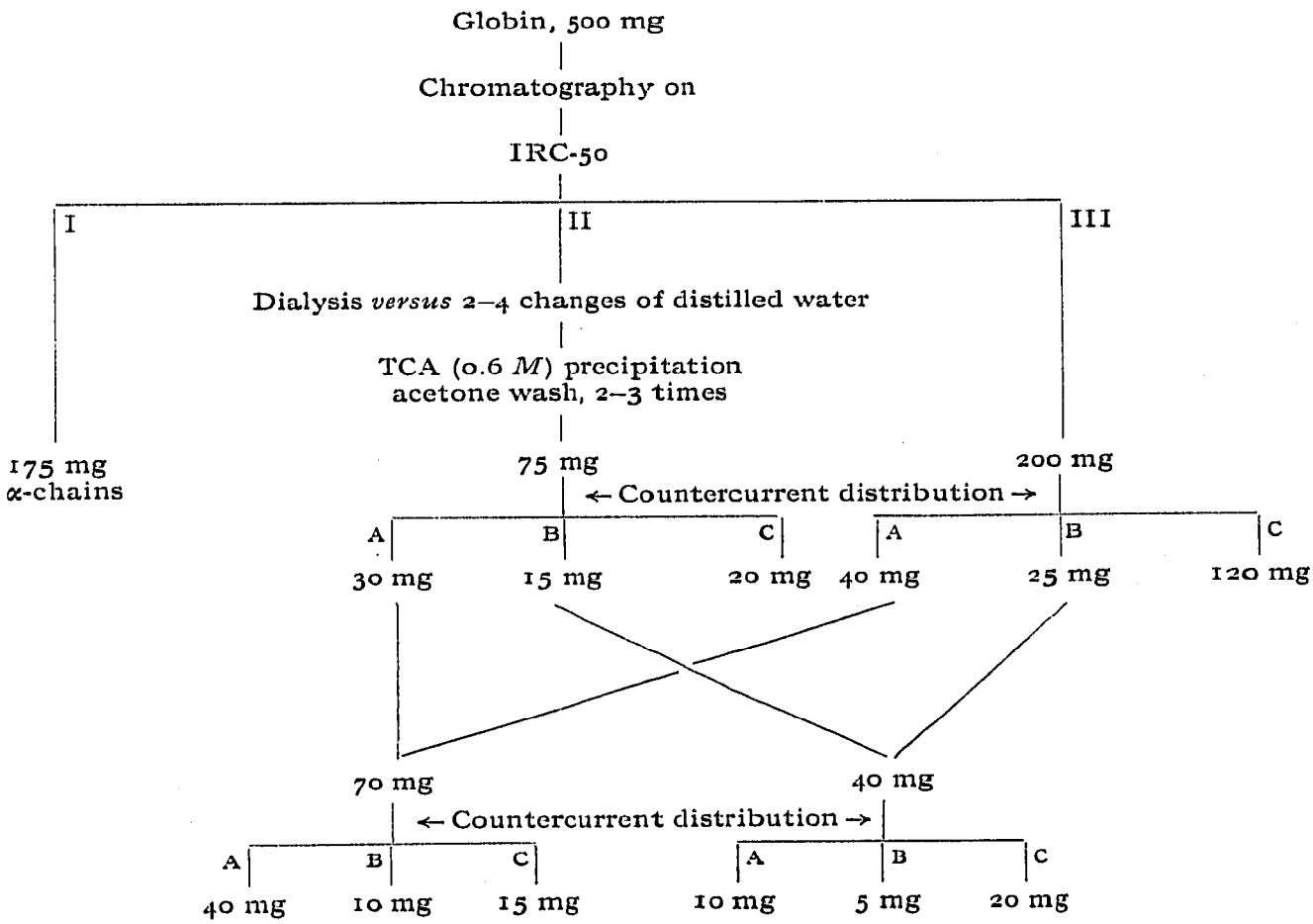


Fig. 7. Tryptic peptide map of β-chain of Hgb A harvested from fraction C of countercurrent distribution technique. Other conditions as in Fig. 6.

relatively little contaminant to separate. Second, by centrifuging the tubes, very clean separation between the two phases is achieved and this separation is weighted in favor of obtaining purer β-chains by leaving a trace of the upper phase undisturbed in each transfer. Such methods cannot, of course, be utilized to determine distribution constants, but for the purposes of separation and purification work admirably. Repurification of the non-α-chains may be achieved by re-cycling through the countercurrent procedure and increased yields are thus possible. The method will easily accommodate larger samples by upgrading the volumes used. Finally, it should be noted that a single individual can complete the entire procedure of countercurrent distribution in one working day.

A flow pattern with representative recoveries is outlined below.

SCHEME I



Combined C total = β-chains, 175 mg

SUMMARY

A technique for the preparation of polypeptide chains of human hemoglobins of a high degree of purity is described. The method involves the combined use of ion exchange chromatography and countercurrent distribution.

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