ADVANCES IN PROTEIN CHEMISTRY

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VOLUME 19

1964

ACADEMIC PRESS • *New York and London*

ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK, N. Y. 10003

United Kingdom Edition
Published by
ACADEMIC PRESS INC. (LONDON) LTD.
Berkeley Square House, London, W.1

Library of Congress Catalog Card Number 44-8853

PRINTED IN THE UNITED STATES OF AMERICA

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THE HEMOGLOBINS

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I. Introduction

The term "hemoglobin" came into being one hundred years ago, when Hoppe-Seyler (1864) first used it in referring to the pigments of blood. Today, we understand this term to include the respiratory (oxygen-carrying) proteins of the vertebrates. Studies in recent years have shown, however, that the hemoglobins and the myoglobins have very similar structures and that this is probably also true for the erythrocruorins. These data suggest that there is a phylogenetic relationship between these molecules and the transport and storage of oxygen. It also appears probable that these molecules have evolved from a common precursor. Therefore, it seems justified to consider these biopolymers as a special subgroup of the hemoproteins.

The hemoglobins were first investigated many years ago; particularly important were the studies of Küster (1912) and Willstätter (1913) on the porphyrins and the systematic investigation of Hans Fischer, which culminated in the complete synthesis of protoheme (Fischer and Zeile, 1929).

Work on the structure of the intact hemoglobin molecule began about 25 years ago, when physicists first began to apply the technique of X-ray diffraction to this problem. These investigations were begun with great optimism, because at that time the prevalent concepts of protein structure were extremely simple. There is no doubt that the advanced state of our knowledge today evolves from these circumstances, because the problem would otherwise not have been attempted until much later. After many setbacks, a fundamental breakthrough was achieved in 1954 which pointed the direction toward the solution of protein structure (Green *et al.*, 1954). Four years later, a molecular

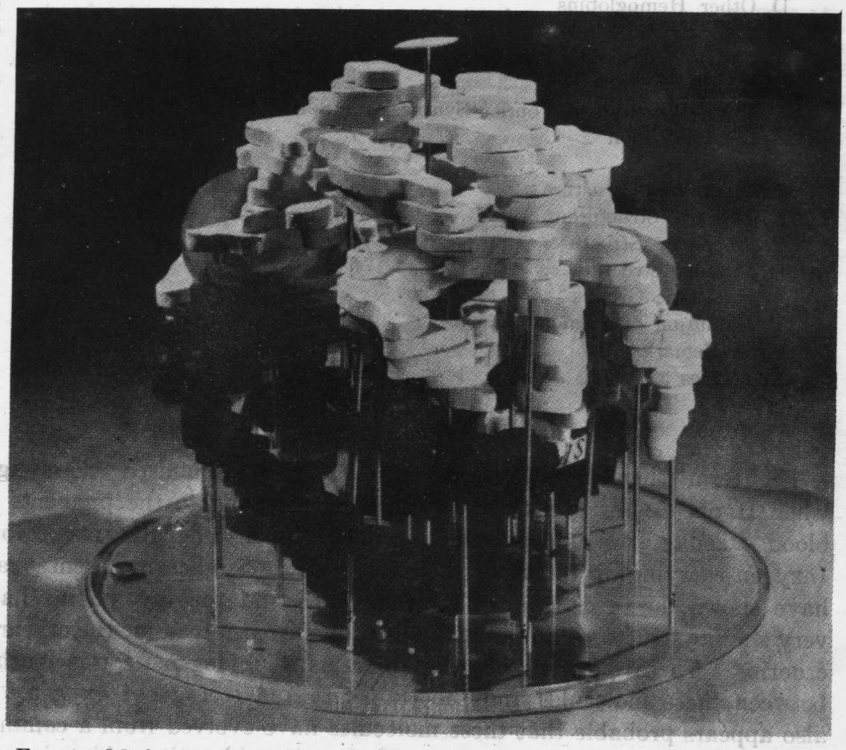

FIG. 1. Model of horse hemoglobin at 5.5 A resolution. The chains are arranged in pairs opposite one another; α-chains in white, β-chains in black (Perutz, 1960).

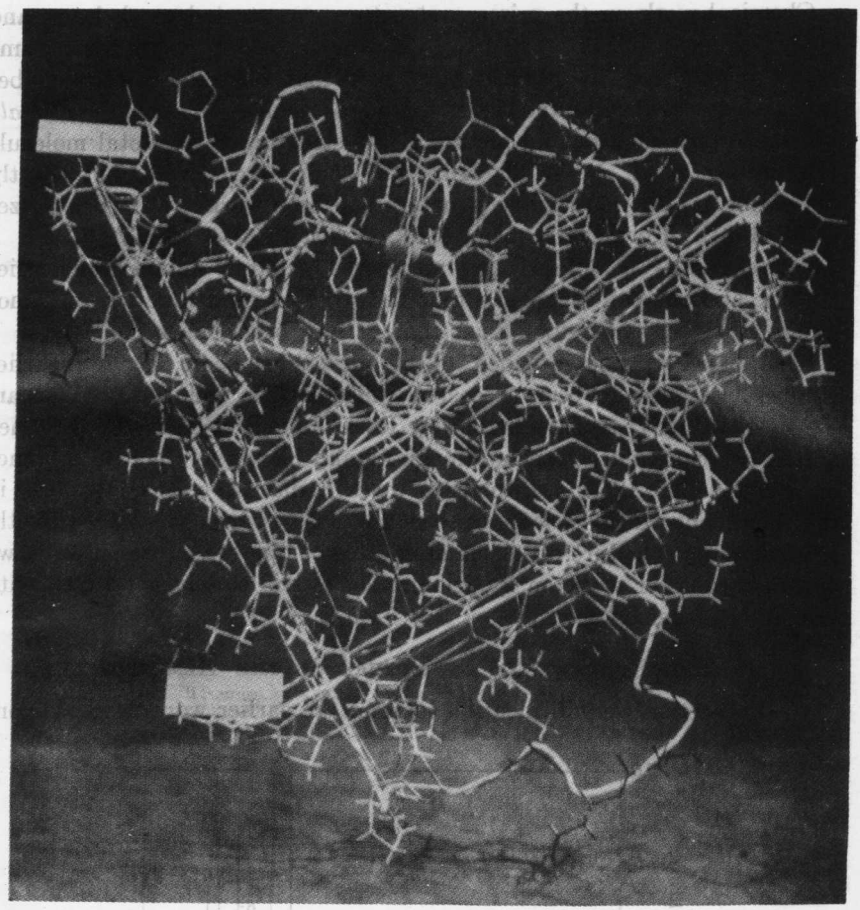

FIG. 2. Atomic model of whale myoglobin at 2.0 A resolution. For better visibility the course of the peptide chains is marked by a thread. The straight segments represent right-hand α-helices (according to Kendrew *et al.*, 1961).

model of myoglobin had been derived (Kendrew *et al.*, 1958), and two years after that, a similar model was available for horse hemoglobin (Perutz *et al.*, 1960). In 1961 the atomic model of sperm whale myoglobin at 2 A resolution was presented (Kendrew *et al.*, 1961) (Figs. 1 and 2).

The investigation of hemoglobin received a strong impetus from studies on sickle-cell anemia and the discovery of hemoglobin S (Pauling *et al.*, 1949). The observation that the abnormality of this hemoglobin is caused by the exchange of a single amino acid (Ingram, 1958), opened one of the most fascinating areas in human genetics.

Chemical work on the primary structure was started much later, and many technical difficulties had to be overcome before the results became really meaningful. It was not until 1960 that sufficient correlation between chemical studies and X-ray data was achieved (Braunitzer *et al.*, 1960b) to provide a partial picture of the structure of the total molecule (Braunitzer *et al.*, 1960c, d; Hill and Konigsberg, 1961); but shortly thereafter, the entire primary structure had been worked out (Braunitzer *et al.*, 1961b; Konigsberg *et al.*, 1961; Goldstein *et al.*, 1963).

These results completed and correlated a large number of earlier studies, and one can safely say that the structure and function of hemoglobin is better understood than that of any other macromolecule.

This paper is not intended to be an encyclopedic review; earlier studies have already been extensively discussed in this series (Wyman, 1948; Itano, 1957; and the article by Rossi Fanelli *et al.* in this volume) and in two conference reports (Roughton and Kendrew, 1949; Conference on Hemoglobin, 1958). Primarily, we will discuss recent advances in this field, but will restrict ourselves to those studies that are directly related to the structure of hemoglobin. To complete the presentation we will also consider work on myoglobin, since it is closely connected with the study of hemoglobin.

II. The Fundamental Structure of the Hemoglobins

In this section we will mention some of the earlier work that has con-

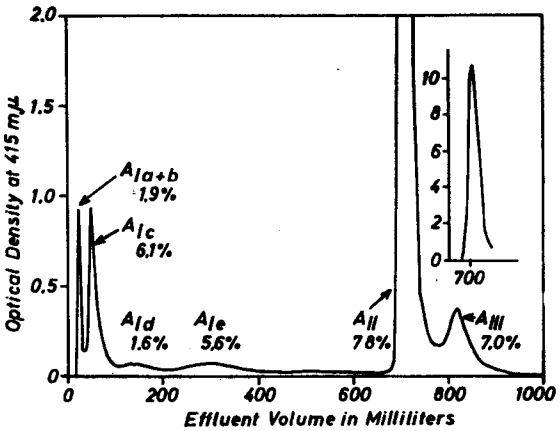

FIG. 3. Chromatogram of whole adult oxyhemoglobin on a 1 × 35-cm column of Amberlite IRC-50 with Developer No. 5. The inset shows that portion of the chromatogram near 700 ml of effluent volume on a reduced vertical scale. The chromatogram was warmed from 5-6°C to room temperature (28°C) after 675 ml of effluent (Allen *et al.*, 1958).

tributed fundamentally to our present concepts of the structure of hemoglobin.

In order to present this material chronologically, we have found it useful to subdivide it into three parts. In doing so we have consciously omitted mention, particularly in the chemical section, of work not directly connected with the fundamental structure of the molecule, and of studies which will be discussed in later sections. This is particularly true for the many studies dealing with the heterogeneity of the blood pigment which led to the discovery of multiple hemoglobins in most species (Fig. 3).

A. Physicochemical Investigations

The first accurate determination of the iron content in hemoglobin gave a value of 0.35 % (Engelhart, 1825), a value which has been essentially confirmed by later studies (Drabkin, 1957). From this value the equivalent weight of hemoglobin was calculated to be 16,700. In 1907 Huefner and Gansser measured the osmotic pressure of solutions of hemoglobin in electrolyte-free media and found a molecular weight of similar value, but later studies could not reproduce these results. On the contrary, Adair (1923–1925), using the same technique, found values between 60,000 and 68,000, four times that of the minimal molecular weight. A similar value of 66,800 was obtained by Svedberg and Fahreus who used horse hemoglobin in the first ultracentrifugal analysis of a protein in 1926. In succeeding years Svedberg and Hedenius (1934) in Uppsala, as well as other authors, measured the sedimentation constants of many vertebrate hemoglobins in the ultracentrifuge. These data showed the molecular weights of vertebrate hemoglobins to be in the order of 64,000 to 68,000; the only exception was the hemoglobin of the cyclostomes, which gave a molecular weight of about 17,000 (Svedberg and Eriksson-Quensel, 1934).

Pedersen (1933) observed that the results of some of these determinations depended on hemoglobin concentration. He found that the sedimentation constant of horse hemoglobin showed a maximum only within a very definite concentration range. More specific information was obtained by Tiselius and Gross (1934) from their measurements of diffusion constants. They showed that with increasing dilution of the hemoglobin solution the diffusion constant increased markedly. Their results suggested dissociation of the hemoglobin molecule at high dilution. The osmotic pressure measurements on fetal sheep hemoglobin (McCarthy and Popják, 1947), which showed a marked increase in osmotic pressure with increasing dilution of the hemoglobin, could be

similarly interpreted. Finally, Gutfreund (1946, 1949) obtained conclusive evidence of the splitting of the hemoglobin molecule into subunits in his measurements on the concentration dependence of the sedimentation constants of horse and fetal sheep hemoglobin. He also observed that at very high dilutions the molecules split first into halves and then into quarters.

Gralén (1939) was the first to study the stability of the hemoglobin molecule at various pH values. He found that the sedimentation constant of horse carbonmonoxyhemoglobin was constant from pH 6 to pH 9.5, but decreased at higher and lower levels. A few years later Moore and Reiner (1944) isolated two distinct components from human hemoglobin by electrophoresis at low pH and showed that these two components had different sedimentation constants. However, exact information about the events which occur at low pH was first obtained by Field and O'Brien in 1955. From measurements of sedimentation and diffusion velocities for human hemoglobin these workers were able to show that between pH 6 and 3.5 the hemoglobin molecule dissociates reversibly into units having one-half the molecular weight of the intact molecule. Only a single component could be found by ultracentrifugal measurements, but there was a broadening of the sedimentation boundary, which was interpreted as being caused by the rapidly occurring dissociation–association equilibrium expected from theoretical grounds. Below pH 3.5 these changes became irreversible because of denaturation. Reichmann and Colvin (1956) focused their studies on this low pH range. Using light-scattering and osmotic pressure measurements they arrived at a mean molecular weight of 20,000 for horse hemoglobin at pH 1.8–2.0. Two components could be separated electrophoretically at pH 1.5. It was concluded that under these conditions the hemoglobin molecule split into four fragments of about equivalent size which were electrokinetically different, however. In 1959 Hasserodt and Vinograd observed that the hemoglobin molecule could also dissociate at high pH levels. By measuring the sedimentation constants of human carbonmonoxyhemoglobin at basic pH they were able to show that between pH 10–11 there is a marked, but reversible, dissociation of the hemoglobin molecule. These data were confirmed for horse hemoglobin by Kurihara and Shibata (1960).

In addition to studies on the effects of dilution and pH extremes on the hemoglobin molecule, there has been considerable work on the effect of concentrated salt solutions upon hemoglobins from a variety of species. Burk and Greenberg (1930) observed that horse hemoglobin split into fragments of molecular weight of 34,000 in concentrated urea, an observation which has been amply confirmed. However, the hemoglobins of

various species differ considerably in their tendency toward dissociation. Whereas beef hemoglobin also splits into halves, dog and adult sheep hemoglobin (Wu and Yang, 1932) and human hemoglobin (Gutter *et al.*, 1956) do not dissociate under the experimental conditions of these authors. The exact pattern of dissociation in concentrated solutions of urea or guanidine hydrochloride is apparent from the recent studies of Kurihara and Shibata (1960), who measured the changes with salt concentration in sedimentation constants of horse hemoglobin. They were able to show that dissociation in urea begins at a concentration of 1.5 *M* and is complete in 8 *M* urea. At this point the molecules have split in half. On the other hand, in guanidine hydrochloride solutions, the molecule is already split in half at a concentration of 2.5 *M*. With a further increase in the concentration, the half-molecules split into quarter-molecules and this dissociation is complete in 6 *M* guanidine hydrochloride solution.

The first molecular weight determinations on globin, i.e., hemoglobin with the heme removed, were performed by Roche *et al.* (1932). From osmotic pressure measurements at pH 5.6 they arrived at molecular weights of 37,000 for beef globin and 29,000 for horse globin. These data were confirmed for horse globin by Gralén in 1939 using sedimentation measurements in the ultracentrifuge. Recently, Rossi-Fanelli *et al.* (1958, 1959) used measurements of sedimentation, diffusion, and light scattering at pH 7 to determine the molecular weights of adult and fetal human globin. At moderate ionic strengths they found molecular weights of 41,000 to 42,000, but in salt-free media they found a particle weight of 18,000. They interpreted these data to indicate that at low ionic strengths there is an equilibrium between quarter- and half-molecules, at high ionic strengths between half-molecules and their dimers.

The situation is even more complicated at acid pH as indicated by electrophoretic studies on horse globin. Reichmann and Colvin (1956) showed that in the transition to a very low pH there is a clear-cut change in the electrophoretic components. Once the pH has dropped as low as 1.5, two electrophoretic components appear, although ultracentrifugal studies indicate that all the particles have a molecular weight of about 16,000. Smith and co-workers (Haug and Smith, 1957; Smith *et al.*, 1957) then demonstrated that there were two chemically distinct units, each with molecular weight of 16,000.

The final conclusion reached from these many experiments is that the hemoglobin molecules with molecular weight of 68,000 may, under certain conditions, split into halves or quarters. Under appropriate circumstances this dissociation may be reversible.

B. Early Chemical Studies

It was recognized very early that hemoglobin consists of a protein and a chromophore and that the latter could easily be split off from the protein. In 1852 Teichmann was able to isolate the prosthetic group of hemoglobins in the form of hemin chloride. In 1898 Schulz reported on the isolation of the protein component, the globin, without running into particular difficulties; however, his preparation may have contained denatured protein. Hill and Holden (1926) were the first to obtain native globin which could recombine with heme to form hemoglobin under reducing conditions.

The interest of chemists was then focused almost exclusively on the low molecular weight chromophore which had such extraordinary properties. The culmination of a great many workers' efforts on the heme was the elucidation of its structure by Küster and, finally, the structural proof obtained from the synthesis of protoheme by Hans Fischer and his associates.

Once the structure of the prosthetic group was known, it became possible to examine the quantitative relationships of the two components of the hemoglobin molecule. From the measurements of Adair (1925a, b) and the known iron content of the molecule, it was concluded that in a particle with molecular weight of 68,000 there are four heme groups.

As early as 1866 Koerber noticed differences in the patterns of denaturation of the various hemoglobins by strong acids or alkali. This species specificity has been confirmed by modern physicochemical methods and also by immunological techniques. The studies of the chemical derivatives of heme (Haurowitz, 1928) strongly suggested that the prosthetic groups of all hemoglobins were identical. Polderman (1932) arrived at a similar conclusion on the basis of his spectroscopic studies. Thus, it became apparent that the differences in the hemoglobins of the various species were due to the globin portion of the molecule. Shortly thereafter (1934), Roche and co-workers provided conclusive proof for this assumption by their demonstration of differences in the amino acid content of various animal hemoglobins. The experimental techniques available at that time were not adequate for detailed and systematic investigations of globin. This became possible only after the introduction of partition chromatography in its various forms and the technique of chemical end-group analysis in about 1940.

The first end-group analyses on hemoglobin were performed by Porter and Sanger (1948). Using the fluorodinitrobenzene method these authors examined human hemoglobin and a number of other mammalian hemoglobins. They showed that in adult hemoglobin and in

horse hemoglobin the terminal amino group was contributed by valine. In beef, sheep, and goat hemoglobin the terminal amino groups were valine and methionine. These qualitative results have been confirmed by all later workers. However, the quantitative data from these experiments did not permit any structural conclusions, because different numbers of terminal groups were found in the various species and, even with human hemoglobin, the results were often contradictory. The precise number of terminal amino groups in the molecule was determined only at a much later date.

The sulfhydryl groups of hemoglobin have also been extensively studied. Hughes (1950) was the first to report the reaction of human hemoglobin with mercury derivatives. Thereafter, a number of workers tried to measure the number of free sulfhydryl groups per molecule by amperometric titration with silver salts and with mercuric and mercurous salts (Ingram, 1955; Benesch *et al.*, 1955; Hommes *et al.*, 1956; Murayama, 1957; Allison and Cecil, 1958). These techniques suggested differences in the cysteine content of the hemoglobins of various species, but the differences in the sulfhydryl content of human hemoglobin reported by several workers appeared to be due to the limitations of the method. However, Ingram (1955) observed that the various sulfhydryl groups of the molecule reacted differently. Furthermore, Ingram's data on horse hemoglobin (1955) and Benesch's data on human hemoglobin (1955) seemed to exclude the presence of disulfide linkages in human hemoglobin.

At about this time the first vague outlines of the structure of the hemoglobin molecule began to become apparent. In 1956 Ingram showed that the hydrolysis of human hemoglobin with trypsin produced only thirty peptides. This corresponded to only one-half of the split products, which one would expect on the basis of amino acid analyses, and indicated the existence of two identical half-molecules. This concept was soon confirmed by the work of Rhinesmith *et al.* (1957, 1958) and Braunitzer (1958) who re-examined the nature and number of the terminal amino groups and then inferred the number of peptide chains in the hemoglobin molecule. By quantitative study of the products of partial acid hydrolysis of dinitrophenyl-(DNP-)globin quite unambiguous results were obtained. Human DNP-globin yielded, on the average, 2 moles of DNP-Val-Leu and 2 moles of DNP-Val-*im*-DNP-His-Leu[1] per mole of globin. Horse globin yielded 2 moles of DNP-Val-Leu and DNP-Val-Glu-Leu and beef and sheep hemoglobin 2 moles each of DNP-Val-Leu and DNP-Met-Leu (Braunitzer, 1958). From these

[1] *im*-DNP-His: imidazole-dinitrophenyl-histidine.

results it became clear that the hemoglobins are composed of four polypeptide chains, two of which are identical.

Rhinesmith *et al.* (1958) and Ingram suggested that the two polypeptide chains of human hemoglobin be designated as α- and β-chains, and this nomenclature has been extended to the hemoglobins of other species. According to this usage the hemoglobin molecule can be defined chemically as $\alpha_2\beta_2$.

C. X-Ray Studies

In 1938 the Cambridge group of crystallographers began their work on hemoglobin with the purpose of using X-rays to provide information about the external and internal structure of this protein molecule (Bernal *et al.*, 1938). This effort, which was interrupted by the war, was taken up anew in 1946 by Perutz and his co-workers, who devoted themselves primarily to the study of horse hemoglobin.

The symmetry relationships of horse hemoglobin were soon clarified, and in 1942 Perutz showed that this protein had a twofold axis of symmetry. The same was shown to be true for human hemoglobin (Perutz *et al.*, 1951). The space-group symmetry derived from the X-ray patterns of monoclinic horse hemoglobin indicated that the molecule is composed of two structurally identical halves (Boyes-Watson *et al.*, 1947). A similar result was obtained with tetragonal human oxyhemoglobin which is also composed of asymmetric units of molecular weight 34,000 (Perutz *et al.*, 1951). It was also shown that adult human hemoglobin and one of its abnormal variants, hemoglobin S, have nearly identical X-ray diffraction patterns, indicating that despite their different chemical properties their structures are very similar (Perutz *et al.*, 1951). Shortly thereafter, Perutz (1951) demonstrated the presence in horse hemoglobin of a weak 1.5 A reflection which could be assigned to an α-helical structure previously postulated by Pauling and Corey. Finally, a comparison of the Patterson projections of horse and human hemoglobin led to the conclusion that these two proteins had a very similar inner structure (Perutz *et al.*, 1955).

Although X-ray diffraction patterns had already provided information concerning the external shape of the molecule and its dimensions (Bragg and Perutz, 1952 a, b), major obstacles were encountered in attempts to learn more about internal structure. Initial efforts to solve the diffraction patterns by "analysis by trial" without experimental determination of phase were unsuccessful. It was only after Perutz and co-workers had succeeded in introducing the technique of isomorphous substitution to proteins and had obtained suitable heavy metal

derivatives of hemoglobins that the problem of phase determination could be solved.

Following an observation of Riggs (1952) Green *et al.* (1954) reacted horse methemoglobin with *p*-chloromercuribenzoate and silver ions and obtained derivatives which crystallized isomorphously with monoclinic horse methemoglobin. Localization of these heavy metal atoms showed that they were symmetrically substituted in the molecule (Bragg and Perutz, 1954; Ingram, 1955). It was also possible to determine the phase angles for the majority of the reflections. Six other isomorphously crystallizing, heavy metal derivatives of hemoglobin were then prepared (Perutz *et al.*, 1960; Perutz, 1960). Aiming at 5.5 A resolution, Perutz and co-workers then measured the intensities of 1,200 of the 31,000 possible reflections and derived the necessary phase information. The three-dimensional Fourier synthesis obtained with these data made it possible to construct a model showing the main aspects of the hemoglobin molecule. The outstanding feature of this model was the unique quaternary structure; the molecule is composed of four units tetrahedrally arranged with two identical pairs.

Thus, the X-ray data provided a picture of the structure of hemoglobin which was entirely consistent with the information obtained by chemical and physicochemical techniques.

III. Chemical Investigation of the Primary Structure

A. Methods

1. Separation of the Peptide Chains

The first separation of the peptide chains was carried out by Haug, Smith, and Wilson (Haug and Smith, 1957; Smith *et al.*, 1957), who showed that globin dissociates almost completely into units with molecular weight of 17,000 at very low pH (pH 1–2). Using Tiselius electrophoresis, these authors demonstrated the presence of two components in these preparations (α- and β-chains) and separated and characterized them.

The first important preparative procedure for the separation of the α- and β-chains was column chromatography. Wilson and Smith (1959) showed that the peptide chains of horse hemoglobin could be readily separated on a cation-exchange resin (Amberlite IRC-50) using gradient elution with acidic urea (pH 2.1). The peptide chains of adult and fetal human globin which are more similar in composition than those of horse globin were only partially separated by this procedure (Hunt, 1959). Better results were obtained using somewhat different

conditions (Hilse and Braunitzer, 1959; Chernoff, 1961). Excellent results were obtained with beef globin using stepwise elution with increasing concentrations of urea (Sasakawa, 1961).

The best preparative technique for the fractionation of the peptide chains is that of countercurrent distribution (Hill and Craig, 1959). Very favorable partition coefficients are obtained by using a mobile phase of 2-butanol and a stationary phase of dichloroacetic acid, the latter also providing the necessary low pH. Good separation can be obtained only when the heme group has been previously removed from the globin. Emulsion formation can be prevented by addition of acetic acid (Hill *et al.*, 1962) (Fig. 4). The distribution curves obtained using this tech-

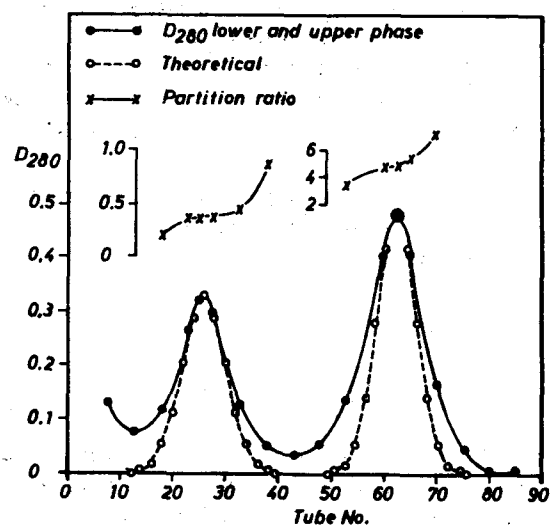

FIG. 4. Countercurrent distribution pattern of the globin from hemoglobin (Hill and Craig, 1959).

nique deviate only slightly from the theoretical, and the technique provides not only satisfactory separation of the peptide chains but also a purification of the material. Countercurrent distribution has been successful in the separation of the α- and β-chains of horse globin (slow component), of pig globin, and of the globins of the rabbit, the llama, the carp, and the lamprey (Braunitzer *et al.*, unpublished observations). Experimental conditions require only minor modifications for the various materials. In general, lower concentrations of dichloroacetic acid are used for the globins with low histidine content. Yields are in the range