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Studies on the Chemistry of Human Hemoglobin

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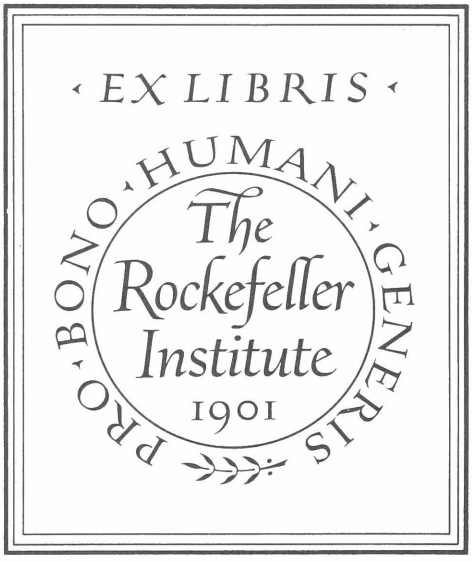

STUDIES ON THE CHEMISTRY OF HUMAN HEMOGLOBIN

A thesis submitted to the Faculty of The Rockefeller Institute

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

by

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It is a pleasure to thank President Detlev W. Bronk for the opportunity to study at the Rockefeller Institute.

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To my many teachers at the Rockefeller Institute, I am appreciative for their efforts; I thank especially Dr. Norman Sutin for allowing me to spend one summer in his laboratory at the Brookhaven National Laboratory, and Dr. Henry G. Kunkel, who was my faculty advisor, for his counsels.

I am happy to acknowledge the kindness of the Editor of Nature and of Dr. Max F. Perutz for their permission to reproduce Fig. 3. Dr. Perutz also allowed me to quote some of his unpublished work.

To Mr. Gerhard Bitterlich, I am thankful for his help with the amino acid analyses. Finally, I express my gratitude to the people who helped in the preparation of the manuscript: Miss Margaret Costanzo, whom I especially thank, for typing the manuscript and Mrs. Georgia T. Fisher for typing the Appendix.

Abstract

This dissertation is concerned with the conformation of adult human hemoglobin in solution. It is now well established that one form of the hemoglobin molecule is composed of four polypeptide chains, two α chains and two β chains, held together by non-covalent bonds. The linear sequence of amino acids in both the α chains and the β chains of human hemoglobin and the three-dimensional arrangement of the polypeptide chains in the crystal of horse hemoglobin have been recently elucidated. Nevertheless, the relationships between the structure and the function of the molecule, although subjected to numerous investigations, are yet to be well understood especially on the basis of the Adair hypothesis.

The function of hemoglobin is closely related to the state of the SH groups in the molecule. It was shown that of the six SH groups in the hemoglobin tetramer, the two reactive ones in the native molecule at neutral pH are those of cysteine residue 93 on each β chain. When these SH groups have reacted with either iodoacetamide or N-ethylmaleimide, the chromatographic properties (and by inference the conformation) of the hemoglobin changes dramatically. The lack of reactivity of the other four SH groups in the tetramer, one on each α chain and one on each β chain, was attributed to the interactions between the polypeptide chains.

The mechanism of dissociation of hemoglobin into subunits was then studied both by analyzing the reactivity, and chromatographic behavior of the products of the reaction, of hemoglobin with iodoacetamide and N-ethylmaleimide under various conditions of pH and ionic strength and by the technique of thin film dialysis. The dissociation was found to be symmetrical according to the equations:

$$\alpha_2\beta_2 \overset{K_1}{\rightleftharpoons} 2\alpha\beta \overset{K_2}{\rightleftharpoons} 2\alpha + 2\beta$$

where K_1 and K_2 are the dissociation constants. By the method of thin film dialysis, the values of the constants K_1 and K_2 were found for a variety of solvent conditions. The pH dependence of the dissociation in solutions of moderate ionic strength was found to resemble closely the data in the literature. However, in strong salt solutions at neutral and acid pH's, no evidence for a shift of the equilibrium in favor of dimers was found.

It was concluded that under all conditions hemoglobin is to be regarded as a solute in rapid association-dissociation equilibrium. The implications of the dynamic feature of the molecule with regard to its function were discussed as a possible alternative to the Adair hypothesis.

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Errata

Wherever "configuration" appears, one should read "conformation".

page iii, line 17 - " ... changes dramatically." should read "... change dramatically."

page 2, line 18 - "It consists of ... " should read "The presentation has been divided into ... ".

page 6, line 12 - "At pH's below 5, ... " should read "Below pH 5, ... ".

page 14, line 14 - " ... molecular weight of 16,000 in salt solutions ... " should read " ... molecular weight of 16,000, in salt solutions ... ".

page 14, line 28 - " ... for n would ... " should read " ... for the value of n would ... ".

page 20, line 5 - " ... of whether ... " should read " ... as to whether ... ".

page 20, line 26 - " ... since n is 1.4." should read " ... since the value of n is 1.4."

page 26, line 29 - " ... procedure of CO hemoglobin ... " should read " ... procedure for the purification of CO hemoglobin ... ".

page 27, line 10 - " ... previous described ... " should read " ... previously described ... ".

page 29, line 15 - " ... S-carboxyamidomethylcysteine ... " should read " ... S-carboxamidomethylcysteine ... ".

page 30, line 2 - " ... decrease in absorption at 300 mμ ... " should read " ... decrease in the absorbancy of NEM at 300 mμ ... ".

page 31, line 26 - " ... in prechromatography dialysis conditions." should read " ... in dialysis conditions used before chromatography."

page 36, line 7 - " ... have been described." should read " ... has been described."

page 37, line 17 - " ... have been ... " should read " ... has been ... ".

page 42, line 17 - " ... to be 2 ... " should read " ... to be 2 (in c.g.s. units x 10^{13}) ... ".

page 48, line 5 - "The experimental procedures have been largely described in Part III. In these experiments, ... " should read "Most of the experimental procedures have been described in Part III. In the present experiments, ... ".

page 52, line 24 - " by by chromatography ... " should read " ... by chromatography ... ".

page 82, line 9 - "k_{obs} was plotted again ... " should read "k_{obs} was plotted against ... ".

PART I

General Introduction

"There are two prominent pigments of living matter on the earth. One is the green pigment, chlorophyll. The other is the red pigment, heme." (Granick, 1949). Compounds containing heme and protein, the heme proteins, are of vast antiquity and of almost universal occurrence in nature. They are highly adapted to the performance of specific physiological functions: the transport and storage of oxygen, the transport of electrons, the regulation of the internal environment, and the catalysis of H_2O_2 decomposition. Those heme proteins whose main function is the transport and storage of oxygen are found in almost all vertebrates, in many species of different phyla of invertebrates, in a few ciliates among the protozoa, in some yeasts and moulds, and in the root nodules of leguminous plants (Redfield, 1933; Keilin, 1956). They are a class of compounds which includes the respiratory pigment found in the red blood cells of vertebrates called hemoglobin; the pigment occurring in muscle cells called myoglobin; and the pigments found in the blood and tissue fluids of invertebrates called erythrocruorins and chlorocruorins.

This dissertation will concern itself with the hemoglobin of man. It consists of four main parts. In Part II, a general view of the molecule of normal adult human hemoglobin will be presented. This summary, although pointing out the essential features which endow the molecule with the specific function of oxygen transport, will be necessarily brief; as it is not my purpose to review in detail all the studies on hemoglobin. In Part III, the identification of the reactive SH groups of normal adult human hemoglobin will be described. The relationships of these SH groups to the function of the molecule, and in particular to the oxygenation, heme-heme interactions, and Bohr effect, are to be found in Part II. In Part IV, studies on the conformation of the hemoglobin molecule in solution and the phenomena of association-dissociation of the subunits will be presented. Finally, in Part V, the implications of these studies will be considered.

PART II

The Structure and Function of Hemoglobin

A. Composition of the Molecule

Hemoglobin is composed of two constituents, heme and protein, joined in the native molecule in a highly specific manner. Only when an exquisitely delicate balance between the components is maintained does the molecule function to the limit of its capabilities in reversibly combining with oxygen. Any alteration whatever in the nature of the components or in their relative conformation seems to be reflected in alterations in the oxygenation capacity.

1. Heme.

The heme in hemoglobin, though treated here as a constituent, is itself a complex of ferrous ion and protoporphyrin IX both of which have unique properties.

Iron belongs to the transition metals, a group with an atomic structure favorable to the formation of compounds in which the outermost set of five stable 3d electron orbitals is only partially filled. These unfilled d orbitals are responsible for the ability of the ferrous ion to form octahedral complexes and for the colors and paramagnetism which these complexes demonstrate.

Protoporphyrin IX is a derivative of the parent ring system, porphin, which is made up of four pyrrole rings linked together by methene bridges. Different porphyrins are distinguished by the nature of the side chains attached to the ring. In protoporphyrin these groups are methyl, vinyl, methyl, vinyl, methyl, carboxyethyl, carboxyethyl, and methyl. There are fifteen possible ways to arrange these side chains around the ring; however, the only protoporphyrin that occurs in nature, protoporphyrin IX, has the order of the substituents given above (Granick, 1949; Lemberg and Legge, 1949).

In the heme molecule, the iron atom fits in the space between the four pyrrole nitrogens and in so doing displaces two hydrogen atoms. Of the six coordination valencies of the iron, four lie in one plane and link it to the nitrogen atoms of the pyrrole nuclei; but the remaining two valencies, the fifth and sixth, project one on each side of the flat heme molecule and link it to molecules of water. Complexes of heme in which the water molecules are replaced by nitrogenous bases, CN^-, or CO occur readily and are called hemochromogens. These derivatives have been

extensively studied largely due to their relationship to the heme proteins (Keilin, 1960; Lemberg and Legge, 1949).

In combining with bases, the electronic structure of the iron atom changes from a state in which there are four unpaired electrons to one in which all the electrons are paired (Pauling and Coryell, 1936a). This change has been attributed either to a shift in bond type from ionic to covalent (Pauling, 1960) or to a change in the strength of the ligand field (Orgel, 1960) depending on the viewpoint.

One of the most striking features of the hemochromogens and of heme is the ease with which the iron is oxidized to the ferric state, even by molecular oxygen (Lemberg and Legge, 1949). The property of reversible combination with oxygen seems to be conferred on the heme complex in hemoglobin by virtue of its association with the protein moiety. Only in exceptional circumstances do hemochromogens exhibit this property (Corwin and Reyes, 1956; Wang, 1958).

2. Globin.

When the heme is carefully removed by extraction from a solution of dissociated hemoglobin, the protein component, globin, can be obtained in a more or less native form. The criterion of a native molecule here is the ability of the globin to recombine with heme and thus reform a functioning hemoglobin molecule. Although some evidence of the production of native globin was obtained by Bertin-Sans and de Moitessier as early as 1892, the first preparation of really native globin was that of Hill and Holden (1926), who extracted hemoglobin with an ether-kieselguhr mixture. However, the most widely used method for the preparation of native globin is that of Anson and Mirsky (1930) in which the extraction of the heme was made with acid acetone at low temperatures. Since the introduction of this method, globin has been studied by many investigators.

These studies had two main objectives. One was the characterization of the native globin molecule as a physical entity in its own right. This knowledge would then help to understand its interactions with heme. The other was the chemical characterization of the globin molecule. This problem was related to the fact that as early as the turn of the century it had been established that the minimal molecular weight of various hemoglobins based on their iron content was about 17,000 (for review, see Cohn et al., 1925),

whereas one form of the hemoglobin molecule was shown to have a molecular weight of 68,000 (Adair, 1925a; Svedberg and Fahraeus, 1926). Thus, it became of interest to establish whether or not the larger hemoglobin molecule was made up of subunits; and if so, whether or not these subunits were identical.

In more recent years, Rossi-Fanelli et al. (1958) prepared, by a modification of the Anson and Mirsky method, human globin which strictly adhered to the criteria of the native molecule mentioned above. These authors found that between pH's 5 and 9 human globin was homogeneous both by electrophoresis and by sedimentation velocity but that it had a molecular weight of 42,000 ($s_{20,w}$ = 2.55, $D_{20,w}$ = 5.49 x 10^{-7} cm^2/sec, $\bar{V}$ = 0.75, f/f_o = 1.76).[1] The isoelectric point was 6.95. At pH's below 5, the sedimentation coefficient decreased to a minimum value of 1.5 at pH 2, indicating a decrease in molecular weight, presumably to units one fourth the size of the intact hemoglobin molecule; a result also obtained with globin in salt-free solutions (Rossi-Fanelli et al., 1959a). These studies are in general agreement with the earlier ones of Roche et al. (1932), Gralen (1939), and Benhamou (1956b) on the molecular weights of globins and with those of Havinga and Itano (1953) and of Munro and Munro (1943) on the electrophoretic mobility of human globin. The isoelectric point found in these earlier studies was slightly higher than pH 6.95. The differing results obtained by other authors (Wu and Yang, 1932; Moore and Reiner, 1944) are most probably due to technical difficulties with the preparation of the globin and with the measurements. The studies of Rossi-Fanelli et al. (1958) indicated that the molecules of globin and of hemoglobin differ by more than just the absence or presence of the heme component. The heme obviously mediates substantial changes in the conformation of the globin molecule, as manifested by the shift in the frictional coefficient (1.76 $\longrightarrow$ 1.15) concurrent with the greater degree of aggregation.

[1] $s_{20,w}$ stands for the sedimentation coefficient in c.g.s. units x 10^{13}, $D_{20,w}$ for the diffusion coefficient, $\bar{V}$ for the partial specific volume, and f/f_o for the frictional coefficient.

The chemical characterization of the globin molecule has been one of the major achievements of protein chemistry during the past five years. After the demonstration by Reichman and Colvin (1956) that the minimal molecular weight of horse hemoglobin ($\sim$ 17,000) indeed did correspond to the measured molecular weight of its globin at very acid pH, it was clear that the smallest individual polypeptide unit of the hemoglobin molecule obtainable without breaking covalent bonds was a polypeptide chain of molecular weight $\sim$ 17,000. Almost at the same time, Schramm et al. (1956) found by the Edman method that both horse and human hemoglobin had four NH_2-terminal valine residues per molecule (M.W. 68,000). Later, Rhinesmith et al. (1957a, 1957b, 1958) conclusively demonstrated that adult human hemoglobin contains per molecular weight 68,000 two chains with the NH_2-terminal sequence valyl-leucyl- and two with the sequence valyl-histidyl-leucyl-. It was at this time that the terminology α (Val-Leu) and β (Val-His-Leu) chain was introduced into the hemoglobin field. This work clearly superseded all the previous work on the NH_2-terminal groups of hemoglobin which had produced varied results (Porter and Sanger, 1948; Havinga, 1953; Huisman and Drinkwaard, 1955; Brown, 1956).

Haug and Smith (1957) and Wilson and Smith (1959) were able to separate the chains of horse globin by electrophoresis in concentrated urea solutions and by fractional precipitation with acid acetone or by column chromatography on the resin IRC-50 in the acid form using a urea gradient, respectively. Later, both Hunt (1959) and Ingram (1959a) partially separated the α and β chains of adult human hemoglobin by the chromatographic procedure. Finally, Hill and Craig (1959) obtained completely pure samples of the α and β chains by the method of countercurrent distribution. These crucial studies gave impetus to the groups in Pasadena, New York, and Munich who have during the past year successfully completed the work on the entire sequence of the α and β chains (Schroeder et al., 1961; Braunitzer et al., 1961; Konigsberg and Hill, 1962; Konigsberg et al., 1963). The data which will be described now are those obtained by Dr. Craig's group at the Rockefeller Institute. Table I shows the amino acid composition of the α and β chains of normal adult human hemoglobin. The α chain is composed of 141 amino acids and has a molecular weight of 15,121; the β chain is composed of 146 amino acids and has a molecular weight of 15,866. Figs. 1 and 2 show the sequence of the amino acids in these two chains. The α chain is characterized by the NH_2-terminal sequence valyl-

TABLE I

Amino Acid Composition of the α and β Chains

Amino acid	Amino acid residues per molecule of protein	
	α chain	β chain
Lysine	11	11
Histidine	10	9
Arginine	3	3
Aspartic acid	8	7
Asparagine	4	6
Threonine	9	7
Serine	11	5
Glutamic acid	4	8
Glutamine	1	3
Proline	7	7
Glycine	7	13
Alanine	21	15
Valine	13	18
Methionine	2	1
Leucine	18	18
Tyrosine	3	3
Phenylalanine	7	8
Cysteine	1	2
Tryptophan	1	2
Total	141	146
Molecular weight	15,121	15,866

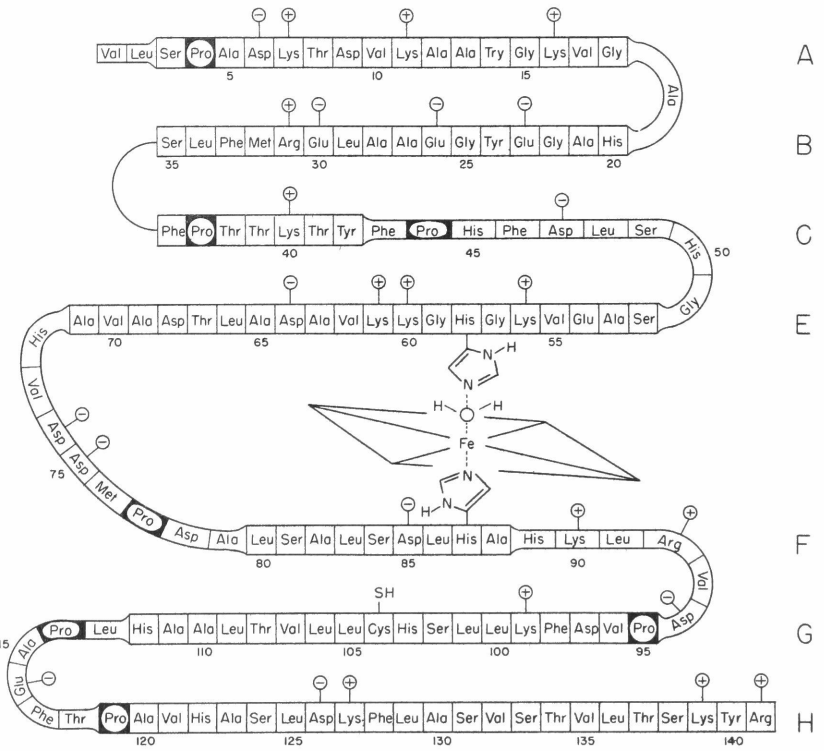


Fig. 1. The amino acid sequence of the α chain. A two-dimensional representation of the three-dimensional structure of the polypeptide chain derived from the data of Watson and Kendrew (1961). The square boxes represent helical parts of the molecule; the rectangular boxes represent non-helical regions. The letters on the right designate the helices. The heme is shown with a molecule of water in the sixth coordination position of the iron atom.

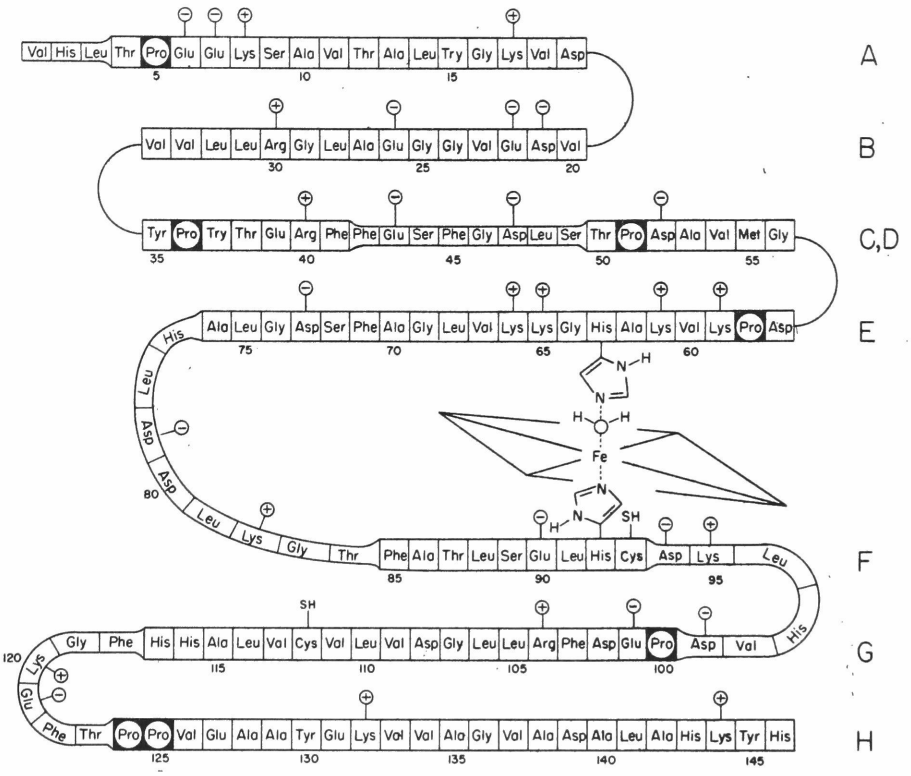


Fig. 2. The amino acid sequence of the β chain. A two-dimensional representation of the three-dimensional structure of the polypeptide chain derived from the data of Watson and Kendrew (1961). The square boxes represent helical parts of the molecule; the rectangular boxes represent non-helical regions. The letters on the right designate the helices. The heme is shown with a molecule of water in the sixth coordination position of the iron atom.

leucyl-; by the COOH-terminal sequence -tyrosyl-arginine; by having one cysteine residue at position 104 and a tryptophan residue at position 14. The β chain is characterized by the NH_2-terminal sequence valyl-histidyl-; by the COOH-terminal sequence -tyrosyl-histidine; by having two cysteine residues, one at position 93 and one at position 112; and two tryptophan residues, one at position 15 and one at position 37. I emphasize that these pictures represent the fruits of three years of work by a team of investigators, as does of course also the excellent work of the groups of Braunitzer and of Schroeder.

It is in general accepted nowadays that the primary sequence of a polypeptide chain uniquely determines the configuration of the chain (Haber and Anfinsen, 1961). Theoretically, the information in Figs. 1 and 2 is sufficient to allow the formulation of the three-dimensional structure of the chains if the nature of the interactions between groups were known. But we are far from this knowledge at present. The most reliable method of determining one configuration of a protein remains the X-ray crystallographic approach, but whether or not the fixed configuration held in the crystal persists in solution is an open question.

The snake-like representation of the α and β chains in Figs. 1 and 2 derives from a two-dimensional representation of the three-dimensional structure of the α and β chains, respectively. These figures were constructed from the data of Watson and Kendrew (1961) which are based on the analogy of human hemoglobin with horse hemoglobin (Braunitzer and Matsuda, 1961) and of the chains of the latter with myoglobin (Perutz et al., 1960). The details of the molecule of myoglobin achieved by the resolution at 2 A of the X-ray diffraction patterns have been recently presented by Kendrew et al. (1960, 1961). In the figures, the amino acid residues in the square boxes represent those parts of the molecule in the α-helical configuration; and the residues in rectangular boxes represent those parts of the molecule in non-helical configuration. The helices are designated by the capital letters at the side of the figure and the coiling of the polypeptide chains by the bends in the linear arrangement of the residues. Clearly, the three-dimensional picture differs from the one shown in these figures, because the chains are tightly wound upon themselves; however, certain of the features are obtainable from this representation. The proline residues are present

at points not more than three residues in from the NH_2-terminal sections where the helices turn or in non-helical regions. Tryptophan residues 14α and 15β are the ones responsible for the photodissociation effect of CO from hemoglobin (Perutz, 1962). The cysteine residue, 93β, is located on a part of the β chain close to the heme-linked group. The same general conformation of the individual chains in solution has been suggested on the basis of measurements of the helix content of the molecule by optical rotatory dispersion (Doty and Klemperer, 1961), but this conclusion should be accepted with reservations.

B. The Heme-globin Linkage

In the previous section, the chemical composition and structure of the α and β polypeptide chains and the physical characteristics of the globin molecule have been described. It was pointed out that the property of reversible combination with oxygen is bestowed on the heme group by its combination with globin and that the complex shows a strong tendency to aggregate. Since heme will combine with other proteins, like albumin and denatured globin, (Lemberg and Legge, 1949) without gaining the ability to combine reversibly with oxygen, the relationship between heme and globin must be unique indeed.

The recent evidence presented by Kendrew et al. (1961) has been taken to indicate almost certainly that the groups which link the iron atoms to the polypeptide chains of hemoglobin are histidine residues 87α and 92β called the proximal histidines (Perutz, 1962). On the distal side of the iron atom, there are other histidine residues in each chain, histidine residues 58α and 63β, which are not close enough to be in direct contact with the iron atom. In myoglobin, a water molecule is seen on the distal side of the iron atom, supporting the contention of Haurowitz (1949) that the iron in reduced hemoglobin is coordinated to a water molecule (see Figs. 1 and 2). The inner part of the pocket of the polypeptide chain into which the heme fits and in which the vinyl groups of the heme molecule are buried is predominantly made up of amino acid residues with non-polar side chains. In contrast, the polar (carboxyethyl) groups of the heme lie on the outside of the polypeptide chain. One is probably linked to arginine.

Undoubtedly, these data give definitive evidence for the group directly

coordinated with the iron atom, and they vindicate the imidazole hypothesis first proposed by Conant (1933) and supported by the extensive studies of Wyman (1948) and Coryell and Pauling (1940). The view that the iron is coordinated to groups other than imidazole is then definitely superseded (for review, see Keilin, 1960).

That the heme is bound to globin not only by its iron atom but also by the porphyrin ring was suggested a long time ago by Granick (1949). A large body of evidence now suggests that the iron bond though essential for oxygenation may be of secondary importance in the binding of heme to globin, which is to be attributed to the relationship between the structure of the porphyrin and the configuration of the polypeptide chains. This concept is supported by the ability of protoporphyrin IX to combine stoichiometrically with globin (Hill and Holden, 1926; Teale, 1959) and to form in competition with heme a stable molecule with physical characteristics identical to those of hemoglobin (Rossi-Fanelli et al., 1959b) and by the instability (O'Hagan, 1960) and the altered oxygenation properties (Rossi-Fanelli et al., 1959c; Rossi-Fanelli and Antonini, 1959a, 1959b; Antonini and Gibson, 1960) of artificial hemoglobins made from hemes lacking the carboxyl groups and the vinyl groups, respectively. In any case, the affinity of heme for globin is very great (Gibson and Antonini, 1960).

It is, therefore, reasonable to conclude that the heme is linked to a highly specific portion of the polypeptide chains mainly by interactions of the latter with the porphyrin ring and that the iron-histidine bond is necessary mainly for oxygenation.

Notwithstanding this rather detailed picture of the heme-globin linkage, the mechanism by which oxygen combines passively with the heme in the hemoglobin molecule rather than oxidizing it is still not at all clear. The only pertinent evidence bearing on this problem indicates that differences in structures and solvations of ferro- and ferrihemoglobin (Sutin, 1961) or the presence of a barrier of hydrophobic amino acid residues in front of the heme group (Wang, 1958) are responsible for the behavior of this group. The latter hypothesis is supported by the theories of the crevice configuration for the heme proposed by St. George and Pauling (1951) and expanded by George and Lyster (1957). The X-ray crystallographic data

would seem to disagree with this theory, since the hemes are seen to be located on the surface of the polypeptide chains. However, it should be remembered that this state of affairs does not necessarily apply to the molecule in solution.

C. The Physical Properties of Hemoglobin

1. The molecular weight.

Any discussion of the molecular weight of a protein requires that the meaning of the word "molecule" be clearly defined. This is not an easy task for systems of solutes in rapid association-dissociation equilibria, which are characteristic for many proteins. In these cases, the molecule cannot be defined as a group of atoms covalently linked but is often considered as the "molecular kinetic unit in solution ... the unit which is observed to move in a diffusion or sedimentation experiment." (Edsall, 1953). The molecular weight of a protein molecule defined in this way merely indicates the size of the average molecule in solution. Only detailed studies will demonstrate whether all the molecules are of identical size or not. These considerations are very pertinent to the following discussion on hemoglobin.

Hemoglobin is the first protein whose molecular weight was determined accurately. The first attempts were by osmotic pressure measurements. Thus, for the molecular weight, Reid (1905) found a value of 48,000; Hufner and Gansser (1907) a value of 15,000 to 16,000 for a 1% solution of hemoglobin in distilled water; and Roaf (1909) a value of 32,000 for hemoglobin in distilled water and of 16,000 for hemoglobin in dilute Na_2CO_3 solution. It remained for Adair (1925a, 1928) to reduce to order these disparate results. He found, by accurate osmotic pressure measurements, that the molecular weight of oxyhemoglobin in both physiological salt solution and in distilled water was 67,000 over the concentration range 1% to 20%.

The extensive and detailed data of Adair have been confirmed for solutions of oxy-, aquo-, and carbonmonoxyhemoglobin over a wide concentration range and at neutral pH by a variety of physical methods such as surface films (Laporta, 1931; Michel and Benhamou, 1949; Benhamou, 1956a, 1956b), diffusion (Lamm and Polson, 1936), osmotic pressure (Bourdillon, 1939), and light scattering (Benhamou and Weill, 1957; Benhamou et al., 1960;

Rossi-Fanelli et al., 1961a). It is remarkable that all these data agree within narrow limits notwithstanding the technical difficulties inherent in these methods.

With the advent of the high-speed ultracentrifuge (Svedberg and Pedersen, 1940), this technique has been repeatedly applied to the various hemoglobin systems shown in Table II. It is of interest that the first successful ultracentrifugal experiments on a protein were carried out by Svedberg and Fahraeus (1926) on horse hemoglobin with the result that the molecular weight corresponded to an iron content of four atoms for each molecule of hemoglobin. Thus, human hemoglobin at neutral pH and at moderate ionic strength can reasonably be said to have a molecular weight of $\sim$ 67,000.

2. The size and shape of the molecule.

Twenty years of work by the group of Perutz in Cambridge, England, have been recently rewarded by the ability to display a very detailed structure of one form of the molecule of oxy- and ferrihemoglobin of the horse resulting from the resolution at 5.5 A of the X-ray diffraction patterns of the crystals of these hemoglobins (Perutz et al., 1960; Cullis et al., 1962).

Fig. 3 is a picture of the hemoglobin molecule taken from the published work of Perutz et al. (1960). The black units represent the β chains (Smith and Perutz, 1960; Braunitzer and Matsuda, 1961) and the white units the α chains. The large discs are the heme groups with a molecule of oxygen attached to the reactive side of the heme. The notation SH indicates the reactive SH group of the β chains of horse hemoglobin, and the notation N indicates the NH_2-terminal end of the β chains. From these data, the hemoglobin molecule appears as a spheroid with a length of 64 A, a width of 55 A, and a height of 50 A. It has one true dyad axis of symmetry through the top of the model in Fig. 3 and two pseudo-dyad ones at right angles to the true one. The molecule is made up of four separate chains or two identical pairs. When each chain is matched with its symmetrical partner, little contact between them is seen--suggesting little affinity between identical chains.

The size of the molecule has also been estimated from calculations

TABLE II

The Molecular Weights of Human Hemoglobin

Author	Hemoglobin	Concentration g %	Solvent	pH	$s_{20,w} \times 10^{13}$	$D_{20,w} \times 10^{7}$	M.W. s,d	M.W. eq	f/f_o	M.W. l.s.
Pedersen (Svedberg and Pedersen, 1940)	CO				4.48					
Kegeles and Gutter, 1951	CO	0.12 - 2.01	0.042 M phosphate	7.07	4.35					
Field and O'Brien, 1955	CO	0.71	0.033 M phosphate	8.0	4.24	6.91	59,400		1.18	
Gutter et al., 1956	CO	0.7	0.1 M KCl	7	4.1	6.0	64,500		1.3	
			+ 4 M urea		3.1	4.3	69,000		1.8	
			+ 1.4 M mercaptoethanol		3.3	7.0	45,000		1.3	
			+ 4 M urea and 1.4 M mercaptoethanol		2.2	5.4	40,000		1.7	
Schumaker and Schachman, 1957	CO	0.005 - 1.0	0.042 M phosphate	7.07	4.6					
Itano and Singer, 1958	CO	0.7	0.1 M sodium acetate	6.73	4.08					
Hasserodt and Vinograd, 1959	CO	0.7	0.25 M Tris HCl-NaCl	8	4.3					
				7.1	4.45		66,500		1.21	
Ingram, 1959a			sodium dodecylsulfate	8.6	2.0					
Benhamou et al., 1960	O_2	0.0064 - 1.2	0.02 M phosphate	7	4.6	6.86	65,000			68,000
	O_2	0.01 - 1.2	+ 1 M NaCl	7	2.5	7.90	32,000			36,000
Rossi-Fanelli et al., 1961a	O_2	0.02 - 1.0	0.05 M phosphate	7	4.4					67,500
	H_2O	0.02 - 1.0	0.05 M phosphate	7	4.5					66,900
	O_2	0.02 - 1.0	+ 2 M NaCl	7	3.6					40,000
	H_2O	0.02 - 1.0	+ 2 M NaCl	7	3.6					45,500
R. E. Benesch et al., 1962	O_2	0.4 - 1.2	0.05 M phosphate	7				65,600		
	H_2O	0.4 - 1.2	0.05 M phosphate	7				66,000		
	O_2	0.25 - 0.8	+ 2 M NaCl	7				36,000		
	H_2O	0.25 - 0.8	+ 2 M NaCl	7				45,000		

$s_{20,w}$ is the sedimentation coefficient in c.g.s. units reduced to water at 20°.

$D_{20,w}$ is the diffusion coefficient in c.g.s. units reduced to water at 20°.

M.W. s,d is the molecular weight by sedimentation and diffusion.

M.W. eq is the molecular weight by sedimentation equilibrium.

f/f_o is the frictional ratio.

M.W. l.s. is the molecular weight by light scattering.

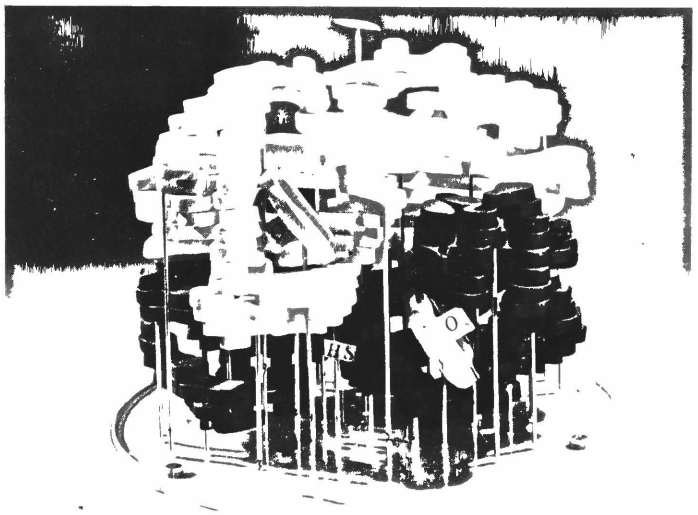


Fig. 3. The three-dimensional structure of the hemoglobin molecule taken from the published work of Perutz et al. (1960). The black chains are the β chains; the white ones are the α chains. The flat disks are the heme groups shown with a molecule of oxygen attached to the sixth coordination position of the iron atom. The letters N and SH stand for the NH_2-terminal residue and for the reactive sulfhydryl group of the β chain, respectively.

based on ultracentrifugal data (Svedberg, 1930) and by direct measurements with the aid of electron microscopy (Sadhukhan et al., 1962) with results comparable to the more accurate ones of the X-ray diffraction.

From the X-ray data, Wyman (1948) calculated that a molecule of the dimensions of hemoglobin, which resembles an oblate ellipsoid of revolution with an axial ratio of 2.0, would have a molecular weight of 67,000, $\overline{V}_{20} = 0.75$, $s_{20} = 4.6$, $D_{20} = 6.7$, $f/f_o = 1.18$, and $h = 0.45$, where $\overline{V}_{20}$ is the partial specific volume, s_{20} the sedimentation coefficient in c.g.s. units x 10^{13}, D_{20} the diffusion coefficient in c.g.s. units x 10^{7}, f/f_o the frictional ratio, and h the hydration (volume fraction). These values are in good agreement with the experimental data.

3. Conclusions based on the preceding data.

The predominant form of the molecules of human hemoglobin in solutions of neutral pH and of moderate ionic strength has the formula $\alpha_2\beta_2$, a notation introduced by Schroeder (1959). This conclusion is based on the demonstration that a solution of hemoglobin contains equal amounts of each chain (Rhinesmith et al., 1958; Hill and Craig, 1959); on the calculated molecular weights for the α and β chains (Hill et al., 1962); on the fact that the measured molecular weight of hemoglobin is close to four times that for the individual chains and that it is constant over a wide concentration range (see Part II, C. 1.); and on the high degree of homogeneity (80 to 90%) of the normal adult red cell hemolysate by electrophoresis and chromatography (Kunkel and Wallenius, 1955; Schnek and Schroeder, 1961; Huisman and Dozy, 1962; Huisman et al., 1958). The $\alpha_2\beta_2$ molecule has a molecular weight of 64,450 calculated from the amino acid composition.

D. The Functional Properties of Hemoglobin

1. The reaction with oxygen.

The most important functional property of hemoglobin is its ability to combine reversibly with oxygen. Only this property and the phenomena immediately attending it will be considered here.

The reaction of hemoglobin with oxygen has been extensively studied since Hufner's (1901) first investigations. The reaction of a hemo-

protein with oxygen may be described by the equation

$$\text{hemoprotein} + O_2 \underset{\longleftarrow}{\overset{K'}{\longrightarrow}} \text{hemoprotein} - O_2$$

where $K' = \frac{(HpO_2)}{(Hp)(O_2)}$.

In the case of myoglobin, which has a molecular weight of 17,000 and has one heme group per molecule, the dissociation curve indeed follows this equation which is characteristic for a rectangular hyperbola (Theorell, 1934).

Although Barcroft and Roberts (1909-1910) [for review, see Adair, 1925b; Barcroft, 1928] found that dialyzed hemoglobin solutions also had a hyperbolic oxygenation curve, Bohr (1903-1904) first described the sigmoid oxygenation curve for hemoglobin solutions containing salt. In an attempt to explain this difference in behavior between dialyzed and salt-containing solutions of hemoglobin, A. V. Hill (1910) suggested that while in salt-free solutions hemoglobin had a molecular weight of 16,000 in salt solutions these units polymerized; and, therefore, the reaction between hemoglobin and oxygen could be described by the equation

$$Hb_n + nO_2 \underset{\longleftarrow}{\overset{K}{\longrightarrow}} (HbO_2)_n$$

where

$$K = \frac{(HbO_2)_n}{(Hb_n)(O_2)^n}$$

If y is taken to represent the fraction of hemoglobin in the oxygenated form, then the equation reduces to

$$y = \frac{Kp^n}{1 + Kp^n} \qquad (1)$$

where p, the partial pressure of oxygen, replaces the concentration of oxygen and n represents the degree of polymerization. The value of n was 1 in dialyzed hemoglobin solution and reached a maximum of 3 in solutions of $(NH_4)_2CO_3$. With the discovery by Adair (1925a) and by Svedberg and Fahraeus (1926) that the molecular weight of one form of hemoglobin was ~68,000 and, therefore, contained four heme groups per molecule, the Hill hypothesis was necessarily dropped, for n would have to be 4. It is, however, remarkable that this equation describes the oxygenation curves

with relative accuracy and is still used today for an empirical way of fitting the dissociation curves.

As Adair suggested (1925a, 1925b), it is now generally accepted that the equilibrium between oxygen and hemoglobin is expressible in terms of four intermediate reactions:

$$Hb + O_2 \rightleftharpoons HbO_2 \qquad K_1 = \frac{(HbO_2)}{(Hb)(O_2)}$$

$$HbO_2 + O_2 \rightleftharpoons HbO_4 \qquad K_2 = \frac{(HbO_4)}{(HbO_2)(O_2)}$$

$$HbO_4 + O_2 \rightleftharpoons HbO_6 \qquad K_3 = \frac{(HbO_6)}{(HbO_4)(O_2)}$$

$$HbO_6 + O_2 \rightleftharpoons HbO_8 \qquad K_4 = \frac{(HbO_8)}{(HbO_6)(O_2)}$$

where K_1 to K_4 are the equilibrium constant for the various reactions.

If y is the fractional saturation of hemoglobin with oxygen,

$$y = \frac{K_1p + 2K_1K_2p^2 + 3K_1K_2K_3p^3 + 4K_1K_2K_3K_4p^4}{4(1 + K_1p + K_1K_2p^2 + K_1K_2K_3p^3 + K_1K_2K_3K_4p^4)} \qquad (2)$$

which for the case of independent heme groups reduces to

$$y = \frac{Kp}{1 + Kp}$$ where K is

the constant for the overall reaction. This equation is identical to (1) in the case of $n = 1$. But the value of n in (1) is 2.8 for human hemoglobin (Riggs, 1961). From this fact ($n > 1$), it follows immediately that there must be stabilizing interactions between the hemes; which is to say that the binding of one molecule of oxygen facilitates the binding of the next, and so on (Wyman, 1948; Tanford, 1961).

One of the main objectives in the study of the reaction of hemoglobin with oxygen has been to explain the nature of the interactions between the heme groups. Necessarily, any theory by which a value for the interaction constant, n, in the Hill equation is obtained must also satisfy the observed relation between the constants K_1 to K_4 in equation (2)

in order to have any meaning. The theoretical considerations of Pauling (1935) and Wyman (1948) based on the apparent identity of the four oxygen-combining centers (from the data of Ferry and Green, 1929, and Roughton, 1936) and those of Allen et al. (1950) based on the symmetry of the oxygenation curves, from all of which spatial arrangements for the heme groups which would give the appropriate value of n were deduced, are not in agreement with the beautiful data of Roughton et al. (1955). The latter obtained values for the constants K_1 to K_4 by very accurate measurements of the oxygen-dissociation curves, $K_4 = 6\ K_1$ for human hemoglobin (Lyster, 1957), and found that these values did not vary equally with pH and temperature so that the oxygen-combining centers are not identical and indistinguishable. Further evidence for this last point stems from the different pH dependence of the velocity constants for the combination of the first and the fourth molecule of CO with sheep hemoglobin (Ainsworth and Gibson, 1957). Thus, no theory at present has explained the shape of the oxygenation curve.

As far as the actual physical meaning of the heme-heme interactions, there is general disagreement. The suggestion (St. George and Pauling, 1951) that the heme groups are sufficiently buried in the body of the protein that the combination of a ligand with the iron requires a dilation of the protein appears to be the most likely, despite criticisms on the grounds that the iron atoms bind some rather large molecules (Keilin, 1953) and that the X-ray diffraction data show the hemes to be on the surface of the molecule and separated from one another by 25 A or more (Cullis et al., 1962). With regard to these considerations, it is to be emphasized that the conformation of hemoglobin in solution need not resemble that of the crystalline state. This point was first suggested by George and Lyster (1957) and is a very important one for the discussion presented in Part V.

2. The Bohr effect.

The Bohr effect refers to the change in pH of an unbuffered hemoglobin solution during the course of oxygenation. The effect is reversible so that a change in pH of a hemoglobin solution changes the extent of oxygenation at a given partial pressure of oxygen. It was first described by Bohr et al. (1904) and by Christiansen et al. (1914), but Henderson (1920) was the first to propose that it resulted from the change in pK of oxygen-linked groups.

Wyman (1948) and Coryell and Pauling (1940) inferred from considerations based on the differential titration of aquohemoglobin and oxyhemoglobin (German and Wyman, 1937), on the effect of temperature on the titration curve of oxyhemoglobin (Wyman, 1939a) and on the Bohr effect (Wyman, 1939b), and on the change in electronic structure and bond type of the iron atom of aquohemoglobin and oxyhemoglobin (Pauling and Coryell, 1936b) that there were two oxygen-linked groups and that both these were the imidazole groups of the histidine residues linked to the heme. One had a change in pK from 5.25 to 5.75 and the other a change from 7.93 to 6.68 on oxygenation at 25° (Wyman, 1948). Subsequently, it has been suggested that the group which becomes more acid on oxygenation need not be the heme-linked imidazole group of histidine, but a group, either imidazole of histidine (R. Benesch and R. E. Benesch, 1961; Riggs, 1961) or amino (ε or α) (Riggs, 1961), closely related to the oxygenation centers. The hypothesis that this group is the SH group of cysteine (Riggs, 1960) is no longer acceptable (R. Benesch and R. E. Benesch, 1961).

Thus, the variations in pK values of the oxygen-linked groups are now thought to be related to alterations in the configuration of the hemoglobin molecule during oxygenation rather than to locally transmitted electronic effects due to binding of oxygen by the iron atom. This view was proposed by Wyman and Allen (1951) and is supported by a large body of evidence as will be shown in the next section.

As far as the detailed mechanism of the Bohr effect is concerned, there are currently two theories, both based on the fact that the Bohr effect is decreased by one half in hemoglobin in which two of the six SH groups have reacted with N-ethylmaleimide (NEM). R. Benesch and R. E. Benesch (1961) attribute this effect to the spreading apart of the chains in the molecule during oxygenation, with the result that an imidazolium group of histidine which can hydrogen bond to a nucleophilic group in aquohemoglobin no longer can do so in oxyhemoglobin. The effect of NEM is interpreted as the ability of the N-ethylsuccinamido derivative of the cysteine residue (produced by imidazole-catalyzed opening of the imide ring of the N-ethylsuccinimido derivative) to hydrogen bond at all times to the implicated imidazolium group of histidine. Riggs (1961) has developed a simpler theory based on the effect of the environment of ionizable groups on their pK. Thus, he proposes that the major part of the Bohr effect is to

be attributed to the change in the environment of an ionizable group on each β chain, be it an imidazole or an amino group, between aquo- and oxyhemoglobin, and that the SH groups of the reactive cysteine residues play an important role in this environment. When the SH groups are covered by NEM, the environment of the ionizable group becomes similar in oxy- and aquohemoglobin--thus the decrease in the Bohr effect.

The upshot of this discussion is that, in spite of these very ingenious theories, the Bohr effect is most probably related to changes in the overall configuration of the molecule during oxygenation with a change in pK values of several ionizable groups rather than to localized effects on one group.

E. The Configuration of Hemoglobin and Oxygenation

There are several striking changes in the physical properties of hemoglobin when it combines with oxygen. The changes in absorption spectra (Lemberg and Legge, 1949) and those in the electronic structure of the iron atom from paramagnetic in aquohemoglobin to diamagnetic in oxyhemoglobin (Pauling and Coryell, 1936b) are well known. However, there is considerable recent evidence suggesting that there is also a profound rearrangement in the conformation of the molecule during oxygenation. Reciprocally, changes in the conformation of the molecule alter the oxygenation parameters (oxygen affinity, heme-heme interaction, Bohr effect). These parameters are thus directly related to the configuration of hemoglobin, as in fact has been suggested by St. George and Pauling (1951) and Wyman and Allen (1951).

1. The effect of oxygenation on the molecular conformation.

The first evidence for a difference in the structure of aquo- and oxyhemoglobin derived from the crystal type, monoclinic and ortho-rhombic, respectively, (Jope and O'Brien, 1949) and the solubility--aquo-hemoglobin being less soluble than oxyhemoglobin (Jope and O'Brien, 1949). Other reflections of the molecular alterations of oxyhemoglobin are the greater sensitivity to hydrolysis by subtilisin (Ottesen and Schroeder, 1961); the faster rate of reaction of the reactive SH groups with iodoacetamide $\left[INH_2\right]$ (R. E. Benesch and R. Benesch, 1962) and with NEM (Riggs, 1961; Morell et al., 1962); and the smaller value for the pK of fluorescent dyes covalently linked to the molecule (Klotz and Tosi, 1962) compared to aquohemoglobin. On a deeper level (Muirhead and Perutz, 1963),

the β chains in the crystal of oxyhemoglobin have been shown to be displaced by 7 A with respect to their positions in the crystal of aquohemoglobin.

The large and positive entropy change of 35 cal per degree per mole at 25° between the first and last oxygenation steps (George and Lyster, 1957) is indicative of a change to a more disordered structure in oxyhemoglobin. It is, however, pertinent to notice that while the overall entropy change is positive, that for the first and second oxygenation steps is negative--in the direction of a more ordered molecule (Table III).

TABLE III

Thermodynamic Data for the Oxygenation of Sheep Hemoglobin at pH 9.1 and 25°*

	K (l/mole)	H (kcal/mole)	S (e.u.)
First oxygen	1.1×10^4	-12.1	-22.2
Second oxygen	5.8×10^4	-7.8	-4.4
Third oxygen	1.2×10^5	-4.2	+9.2
Fourth oxygen	3.9×10^6	-5.1	+13.0

*George and Lyster, 1957.

This state of affairs would not have been expected on the basis of the hypothesis that the heme-heme interactions are related to steric effects.

The most dramatic evidence is provided by the measurements of the dielectric increment and relaxation times (Takashima and Lumry, 1958) and of the viscosity (Lumry and Matsumiya, 1958) of hemoglobin in solution during oxygenation. These parameters go through two maxima, one at 25% and the other at 75% oxygenation, and through a minimum at 50% oxygenation, and then return to values close to those for the unoxygenated molecule. These data are compelling evidence for the dramatic changes which occur during the course of oxygenation; and they point out, especially, that while the two end states may be very similar, the path from one state to the other is attended by remarkable rearrangements in the configuration of hemoglobin.

2. The effect of changes in conformation on oxygenation.

There appear to be several inconsistencies in the interpretations of the effects of presumed conformational changes of the molecule on the oxygenation parameters. These inconsistencies are related to the uncertainty of whether or not the proposed changes have actually taken place and to the different experimental approach of the various investigators.

Thus, while for hemoglobin in 6 M urea the heme interactions are decreased (Rossi-Fanelli et al., 1959d), they are slightly increased in strong salt solutions (Rossi-Fanelli et al., 1961b); yet in both situations, the hemoglobin molecule is supposed to dissociate partly into units of molecular weight $\sim$ 32,000. In the first instance, the data followed expectations, for the value of n in the Hill equation (1) can never be greater than the number of interacting groups. This contrasts with the second case where the value of n was found to be 3. Moreover, it turns out that human hemoglobin in concentrated urea actually does not dissociate (Gutter et al., 1956) nor does it in strong salt solutions, as will be shown later in this dissertation. So it is now the effects of urea on oxygenation which are difficult to explain instead of those in strong salt solutions. In any case, strong salt solutions may be expected to decrease the Bohr effect by changing either the configuration of the protein or the ionic atmosphere of the ionizable groups (Antonini et al., 1962a).

Better correlation between theory and experiments derives from the fact that both in very dilute hemoglobin solutions (Rossi-Fanelli et al., 1961c) and in very dilute salt solutions (Rossi-Fanelli et al., 1961b; Takashima, 1955) the oxygen affinity is increased, and there is indication of dissociation of the molecule since n is 1.4.

Some of the effects obtaining when the reactive SH groups of hemoglobin are blocked have been discussed with regard to the Bohr effect. The oxygen affinity increases in hemoglobin reacted with both INH_2 and NEM, but the value of n and the Bohr effect are decreased by one half only by NEM. The relationship of the SH groups to the configuration of the molecule has already been suggested in the previous section.

More drastic alterations in the molecular structure, such as those resulting from vigorous acetylation (Bucci et al., 1963) and from treatment with carboxypeptidases (Antonini et al., 1961), are also attended by large

changes in the oxygenation parameters. The limiting case of this type of experiment has been performed by Ross (1939), who showed that hemoglobin digested with pancreatic extracts is completely non-functional.

More interesting is the information obtained from the abnormal hemoglobins and from the minor components of the red cell hemolysate. The ones that have been studied are the fetal hemoglobin $\alpha_2\gamma_2$ (Schroeder and Matsuda, 1958; Schroeder et al., 1962a); the minor A_2 component, $\alpha_2\delta_2$ (Ingram and Stretton, 1962); hemoglobin S, $\alpha_2\beta_2^{6\ Glu \rightarrow Val}$ (Ingram, 1959b); and hemoglobin H, β_4, a hemoglobin which under physiological conditions is a tetramer (R. Benesch et al., 1962; Rigas et al.,1956) composed of four β chains (Jones et al., 1959a) and thus quite different from all other hemoglobins. The differences between the normal β chain and the γ, δ, and altered β chains have been worked out in all the cases mentioned. The oxygen affinity of hemoglobin A_2 is slightly increased (Huisman et al., 1962) while the oxygenation parameters of the hemoglobins S and fetal are essentially similar to those of normal adult hemoglobin (Schruefer et al., 1962). The most dramatic changes, however, have been observed with hemoglobin H in which the oxygen affinity is greatly increased, the Bohr effect is abolished completely, and there is apparently no interaction between the hemes (R. E. Benesch et al., 1961). In other words, it behaves like a myoglobin molecule.

The hemoglobins M (Gerald and Efrom, 1961) represent an altogether different situation because in these hemoglobins a mutation has occurred either at the position of the distal histidine residue in the α or β chains (histidine residue 58α and 63β) or at a position close to these residues. These hemoglobins are characterized by the tendency of the iron atom to be in the ferric state, presumably because the side chain of the amino acid which has substituted the one present in normal hemoglobin is in the right position to bond directly to the iron atom. Thus, these hemoglobins are unable to function.

From all these data, it might be possible to deduce which amino acid residues are important for the function of the molecule, much as the groups of Moore and Stein, Hofman, Anfinsen, and Richards are doing with ribonuclease. However, the problem is considerably more complicated with hemoglobin, for it will be shown that it consists of a system in

extemely mobile dissociation equilibrium. Thus, a distinction has to be made between alterations in the molecular structure which change the equilibria of the dissociation reactions and those that change the reactivity of the heme in all the forms of the molecule. It is best, therefore, to include at present all the effects under overall conformational changes in the molecular architecture. Similarly, the inability to explain the oxygenation of hemoglobin in terms of the Adair hypothesis is likely to be related to the profound molecular rearrangements which take place during the oxygenation process.

PART III

The Reactive Sulfhydryl Groups of Hemoglobin

A. The SH Groups of Hemoglobin

1. The number and reactivity of the SH groups.

Interest in the SH groups of human hemoglobin can stem from two considerations: 1) that only two of the six SH groups are reactive in the native molecule and 2) that these reactive SH groups play an important role in the function of the molecule. In order to further correlate structure with function, an attempt has been made to determine which are the reactive SH groups in the molecule. The data pertaining to the functional relationships of the SH groups have been described in Part II. Treatment in this section will be limited to a survey of the studies pertaining to the reactivity of the SH groups. An excellent general review on the reactivity of SH groups has recently been presented by Cecil and McPhee (1959).

Work on the amino acid composition (Hill et al., 1962) and sequence of the chains of hemoglobin (Konigsberg and Hill, 1962; Konigsberg et al., 1963; Braunitzer et al., 1961) clearly shows that there is only one SH group in each α chain (cysteine 104α) but two in each β chain (cysteine 93β and cysteine 112β). Therefore, the $\alpha_2\beta_2$ tetramer of hemoglobin contains a total of six SH groups. These more conclusive data agree with those of several previous workers. Thus, Cole et al. (1958), who determined the cysteine content in adult human hemoglobin both as cysteic acid and S-carboxymethylcysteine, reported values of five to six residues per molecule; and Brown (1957) and Stein et al. (1957) found 5.8 and approximately five cysteic acid residues per molecule, respectively, after performic acid oxidation of hemoglobin.

Attempts to determine the number of SH groups directly are of interest in this connection. Many determinations have been done both on the native and denatured molecule by amperometric titration with Ag^+ and Hg^{++} and by the spectrophotometric methods of Boyer (1954) [PCMB][2] and Alexander (1958) [NEM]. Cecil (1950) and Cecil and McPhee (1959) have described the difficulties pertaining to amperometric titrations with Ag^+ and Hg^{++} at the dropping electrode. The varied results obtained by many early investigators

[2]PCMB stands for parachloromercuribenzoate.

are now understandable in view of the difficulty of these methods and the now known content of SH groups of the hemoglobin molecule. The high values obtained by Ingram (1955), Hommes et al. (1956), Hommes and Huisman (1958), Murayama (1957, 1959), and R. E Benesch et al. (1955) by amperometric titrations of both native and denatured hemoglobin are to be taken in the light of the statements above.

If, however, the experiments which show that denatured hemoglobin contains six SH groups per molecule are taken as representative of the right conditions for the particular determination, then it appears that in the native molecule only two of the six SH groups are reactive. Thus, Ingbar and Kass (1951) found two SH groups in the native molecule and four to five in the denatured molecule by Ag^{+} titration; Ingram (1955) found 2.2 and two SH groups by Hg^{++} titration and PCMB titration, respectively, in the native molecule and six in the denatured molecule; and Allison and Cecil (1958) identically found 2.2 and six SH groups, respectively, in the native and denatured molecule by both Hg^{++} titration and phenylmercuric hydroxide titration. Hughes (1949), by equilibration with methylmercuric iodide, Murayama (1957), and Taylor (1955), by PCMB titrations, also found two SH groups per molecule of native hemoglobin.

These results by many workers and the more recent ones obtained by Riggs (1961) and R. Benesch and R. E. Benesch (1961) by alkylation of native hemoglobin with NEM and with NEM and INH_2, respectively, show conclusively that only two to 2.2 SH groups are reactive in the native molecule. The lack of reactivity of the other four groups cannot be ascribed to disulfide bonds, because of the ability to titrate all the SH groups in the denatured molecule.

2. The problem of differential reactivity.

The reason for the difference in reactivity of functional groups in proteins is not a simple one. Clearly, the first point to be established in a system which shows differences in the reactivity of a number of groups is whether this a) is related to individual functional groups, b) is the result of slow rates of reaction of all the groups, or c) is the consequence of an equilibrium involving all the groups. The distinction between cases a) or c) and case b) is simple; it is made by determining the sharpness of the end point of reaction. The distinction between cases a) and c) can only

be achieved by identification of the chemically altered functional groups. Hemoglobin, then, represents an example of cases a) or c); and it was for this reason that the identification of the reactive SH groups was undertaken. At the time this study was begun, there was already evidence that hemoglobin belonged to class a); for Cullis et al. (1962) had identified the reactive SH groups of horse hemoglobin by X-ray diffraction studies and Riggs (1961) had shown that NEM was found bound only to the β chain of human hemoglobin after reaction. Since the β chain of human hemoglobin contains two SH groups, chemical identification of the reactive SH group was clearly indicated.

B. Experimental

1. Methods.

The details pertaining to the preparation of the carbonmonoxy-hemoglobin (CO hemoglobin) solutions, preparation of globin, countercurrent distribution of globin, amino acid analysis, chromatography of CO hemoglobin, hydrolysis of the β chains by trypsin, and separation of the tryptic peptides by chromatography have been published (Hill et al., 1962; Guidotti et al., 1962).

Hemoglobin solutions were prepared from the freshly drawn blood of one individual (G.G.) by the method of Drabkin (1946). The clear hemoglobin solutions were dialyzed against three changes of 10^{-4} M EDTA adjusted to pH 7.4 over a period of 24 hours at 5°. The dialyzed hemoglobin solutions were saturated with CO and kept under a CO atmosphere in the cold. All experiments were done with CO hemoglobin solutions not over six days old. Although I agree with Allison and Cecil (1958) that storage of CO hemoglobin at 4° does not change its SH titer, the results of Ingram (1957) and R. E. Benesch and R. Benesch (1962) on oxyhemoglobin, indicating changing SH titers with time, convinced me to take this precaution.

The chromatographic procedure of CO hemoglobin was identical to that previously described, except that the hemoglobin solutions were dialyzed for 48 hours prior to chromatography against a sodium phosphate buffer, which was 0.016 M Na^+ and pH 6.5 (137.5 g $NaH_2PO_4 \cdot H_2O$ and 76 g $Na_3PO_4 \cdot 12\ H_2O$ per liter of deionized water diluted 1:100). This buffer of lower ionic strength did not change the chromatographic pattern to any extent, and it was of advantage when the pH of the hemoglobin solution was to be changed rapidly

by stronger buffers. The CO hemoglobin (350 to 700 mg as a 3.5% solution) was applied to a column of Bio-Rex 70 (4.0 x 30 cm), a resin with carboxylic acid exchange groups, equilibrated with the developing buffer. The buffer was made by dissolving 52.2 g of Na_2HPO_4 and 114.8 g of $NaH_2PO_4 \cdot H_2O$ in 8 liters of deionized water; it was 0.196 M Na^+ and pH 6.42. The chromatographic procedure was carried out at 5°. The flow rate was 32 ml per hour. Fractions of 8 ml each were collected and analyzed by measuring the absorbancy at 415 mμ.

The globin was prepared by extracting the heme from an acid solution of CO hemoglobin with methyl ethyl ketone as previous described (Hill et al., 1962). The distribution equipment consisted of a 1020-tube automatic machine with a 3-ml capacity. The system had the following composition: sec-butanol, 9; 0.5 M acetic acid, 10; and 10% dichloroacetic acid (v/v), 1.

The hydrolysis of the β chain with trypsin was carried out at 25° for 8 hours as previously described (Guidotti et al., 1962).

The chromatography of the tryptic peptides was carried out on columns (0.9 x 50 cm and 0.9 x 15 cm) of Dowex 50-X2, a resin with sulfonic acid exchange groups. The buffers used for elution were pyridine acetate buffers of the following composition: a) the starting buffer, 0.17 M, 14 ml of pyridine and 15 ml of acetic acid per liter, pH 4.7; b) 0.40 M, 32 ml of pyridine and 37 ml of acetic acid per liter, pH 4.6; c) 1.07 M, 86 ml of pyridine and 30 ml of acetic acid per liter, pH 5.4; d) 2.23 M, 180 ml of pyridine and 60 ml of acetic acid per liter, pH 5.4. The buffer changes were made at the elution volumes shown in the figures. The chromatograms were analyzed by the ninhydrin method with 0.1- or 0.2-ml aliquots, depending on the sample size.

Thioethers are sensitive to oxidation on columns of Dowex 50 resins (Moore and Stein, 1951) unless an antioxidant is added to the chromatographic system. Thus, thiodiglycol in a concentration of 0.5% was added to all the pyridine acetate buffers of the chromatographic system, following the method of Spackman et al. (1958). The thiodiglycol did not change the chromatographic behavior of the peptides. After collection of the cuts containing the separated peptides, the pyridine acetate buffers were removed by lyophilization; but of course a considerable amount of thiodiglycol was not removed by this method. Aliquots of these peptide solutions were hydrolyzed

for 24 and 72 hours in 6 N HCl at 110° in carefully evacuated and sealed glass tubes. In spite of the fact that thiodiglycol under these conditions is converted to mustard gas which has been shown to react with the sulfur of methionine (Stein and Moore, 1946) and with the amino and carboxyl groups of amino acids (Moore et al., 1946), good recoveries of the amino acids based on the overall yields of the peptides were obtained. Therefore, removal of thiodiglycol prior to hydrolysis was not attempted. Care, however, was taken on evaporation of the hydrolysates on a rotary evaporator to prevent contact with any fumes of mustard gas.

In all cases where thioether derivatives of cysteine were subjected to acid hydrolysis, very careful evacuation of the tubes was necessary to prevent any oxygen from remaining in the tube. Even small traces of oxygen caused decomposition of the derivatives. For this reason, the tubes containing the protein or peptide dissolved in 1 to 2 ml of 6 N HCl were evacuated for 10 minutes with a high vacuum pump during constant shaking of the tube on a Vortex mixer.

2. The alkylation of hemoglobin.

Monohaloacetates and monohaloacetamides react readily with thiol groups (Dickens, 1933; Smythe, 1936) and have been used extensively for inhibition of enzymes (Dixon, 1948) and to a smaller extent for the measurement of SH groups in proteins (Cecil and McPhee, 1959). Of these compounds, the iodo derivatives are the most reactive (Bartlett and Barron, 1947; Dickens, 1933). Kinetic studies show that these reagents react with the ionized form of the SH group (Smythe, 1936; Cecil and McPhee, 1959). It is apparent from the reactions

$$RSH \overset{K}{\rightleftharpoons} RS^- + H^+$$

$$RS^- + R'I \xrightarrow{k_1} RSR' + I^-$$

(where K is the dissociation constant, RSH and RS^- represent the unionized and ionized form of the thiol, and R'I is the alkylating agent) that the observed rate constant is a function of hydrogen ion concentration:

$$k_{obs} = k_1 \frac{K}{K + (H^+)}$$

When the reaction is done at a pH below the pK of the SH group,

$$k_{obs} = k_1 \frac{K}{(H^+)}$$

which shows that for each decrease in pH unit the observed rate constant will decrease by a factor of 10. It also shows that at a given pH, k_{obs} varies directly with the dissociation constant of the SH group, as experimentally observed by Cecil and McPhee (1959).

Iodoacetic acid and INH_2 also react with thioethers, amino and imidazole groups; but in general the pH dependence of these reactions is different from that with SH groups (Gundlach et al., 1959).

The different methods of estimating the reaction of thiols with these reagents involve measurement of the decrease of the SH titer, titration of the iodide ion released (R. Benesch and R. E. Benesch, 1961), or titration of the hydrogen ion liberated (R. Benesch and R. E. Benesch, 1957). The method used in this work was the precise quantitation of S-carboxymethylcysteine by amino acid analysis (Spackman et al., 1958). The product of the reaction of cysteine with INH_2 is S-carboxyamidomethylcysteine, which is completely converted to S-carboxymethylcysteine by hydrolysis in 6 N HCl at 110° for 22 hours. INH_2 instead of iodoacetic acid was chosen because iodoacetic acid was found not to react with hemoglobin at neutral pH (R. E. Benesch and R. Benesch, 1962; Konigsberg, 1962).

NEM has not been used as extensively as INH_2 as a reagent for sulfhydryl groups, although it reacts rapidly with thiols (Friedmann et al., 1949). Very preliminary measurements of the rate of reaction of NEM with glutathione have shown that it is 100 to 1000 times more reactive than either iodoacetic acid or INH_2 and that the rate constant is not very sensitive to pH. This finding is in disagreement with the statement of Cecil and McPhee (1959) that NEM also reacts with the ionized form of the thiol group. The lack of pH dependence may be taken as an indication that the transition state of the reaction is a four-centered one. NEM also reacts with imidazole and amino groups (Smyth et al., 1961) but at a slower rate than with SH groups. The mechanism of the reaction with amino and imidazole groups has not been clearly determined.

There are three main techniques to measure the reaction of NEM with thiol groups. The decrease in the titer of SH groups, estimated as heavy

metal-binding sites (Riggs, 1961), nitroprusside color (Tsao and Bailey, (1953), or iodine titer (Friedmann et al., 1949), and the decrease in absorption at 300 mμ (Gregory, 1955; Alexander, 1958) have been widely used. More recently, Smyth et al. (1960) identified the product of the reaction of cysteine with NEM as S-(N-ethylsuccinimido)-cysteine. Smyth et al. (1961) have shown that prolonged hydrolysis (72 hours) of this compound in 6 N HCl at 110° produces equal amounts of S-succinylcysteine and ethylamine in a yield of 88%. These compounds are accurately identified and estimated by amino acid analysis. This method was used here to measure the reaction of hemoglobin with NEM.

NEM, chromatographically pure, and INH_2 were obtained from the Mann Research Laboratories. The INH_2 was recrystallized from hot water prior to use. The reactions were carried out in a sodium phosphate buffer (11 g $NaH_2PO_4 \cdot H_2O$ and 15.2 g $Na_3PO_4 \cdot 12\ H_2O$ per liter of deionized water), which was 0.2 M Na^+ and pH 7.15.

All the reactions were performed in an atmosphere of CO obtained by repeatedly flushing the buffer containing the reagent before adding the CO hemoglobin solutions. The concentration of CO hemoglobin, estimated by measuring the absorbancy at 418 mμ on a Beckman DU Spectrophotometer ($\varepsilon_M^{418\ m\mu} = 6.16 \times 10^5$, Lemberg and Legge, 1949), was 6×10^{-4} M, that of INH_2 was 3.6×10^{-2} M, and that of NEM 1.8×10^{-3} M. After allowing the reaction to proceed for 1 hour at room temperature, the reagent was removed by dialysis in Visking 23/32 casings for 48 hours at 5° against four changes of four liters of 0.016 M sodium phosphate buffer, pH 6.5. After dialysis, the hemoglobin solutions were stored under CO. Small amounts of precipitate which formed during the reaction or during dialysis were removed by centrifugation at 20,000 g for 20 minutes. In all cases, standard hemoglobin solutions were carried through all the procedures, except that no reagent was used.

The absorption spectrum (300-700 mμ) of a sample of each derivative of hemoglobin was obtained on the Perkin-Elmer Spectracord and found to be identical to unreacted hemoglobin.

3. Method of reporting the data.

In all cases, total amino acid analyses were performed on the samples of hemoglobin, globin, and the isolated chains. Only the values for

the cysteine derivatives are reported, because under the conditions used no change was observed in the other residues. The reported values are averages of at least three determinations.

The results of the analyses performed on the globin are reported in terms of residues of S-carboxymethylcysteine or of S-succinylcysteine and ethylamine per $\alpha\beta$ unit, since it is easier to compare the data with those obtained with the isolated α and β chains. In order to obtain values for the tetrameric molecule, the reported values for the globin must be multiplied by two. No corrections were made in reporting the values of the S-carboxymethylcysteine and S-succinylcysteine.

C. Results

1. The chromatography and countercurrent distribution of the hemoglobin derivatives.

Fig. 4 shows the results obtained with CO hemoglobin reacted with INH_2. On the left side of the figure are the chromatographic patterns and on the right side the curves obtained from the countercurrent distribution of globin. The separated chains have been labeled α and β. In the top panel are the patterns typical for normal hemoglobin; in the bottom panel, those for the INH_2 derivative.

The chromatographic purification of the red cell hemolysate of a normal adult and the attending modification of the countercurrent distribution patterns of the globin have been previously described (Hill et al., 1962). The chromatographic pattern shown in the top panel of Fig. 4 is fairly typical of the results previously obtained, except that the first and minor component is no longer split into a doublet. This result can be due to the change in prechromatography dialysis conditions. Since, however, this particular component is not of interest here, the lack of resolution is not a disadvantage.

The striking retardation of the main component of the CO hemoglobin reacted with INH_2 was unexpected, since no positive charge was introduced into the hemoglobin molecule by the reaction. The possibility that the difference in charge between the normal and the INH_2-reacted CO hemoglobin might be attributed to the ionization properties of the SH groups themselves is unlikely since the pK of these groups would have to be well below pH 6.42. This of course is unlikely. Thus, the observed change is best attributed

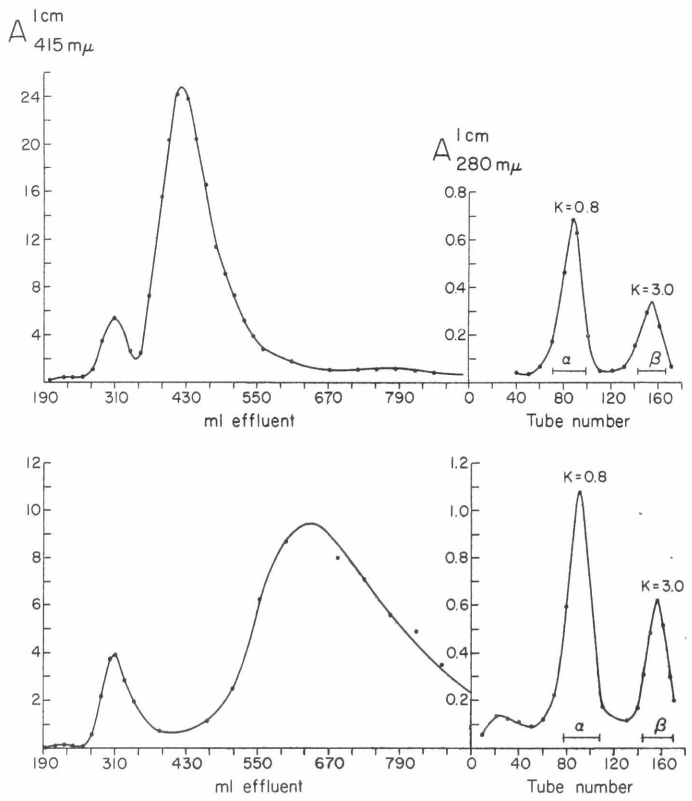


Fig. 4. Chromatography on Bio-Rex 70 (left side) and countercurrent distribution (right side) of normal CO hemoglobin, 350 mg, (top panel) and CO hemoglobin reacted with INH_2 at pH 7.15, 350 mg, (bottom panel). The reaction conditions were: CO hemoglobin 6×10^{-4} M; INH_2 3.6×10^{-2} M; 1 hour at 25°C. Countercurrent distribution of peak 2 for 200 transfers, 3 ml in each phase; system: sec-butanol, 9; 0.5 M acetic acid, 10; 10% (v/v) dichloroacetic acid, 1. Only the concentrations in the lower phase are shown.

to a change of the hemoglobin configuration resulting in the production of a molecule more basic than the normal one by about one to two units of charge. This estimate is very rough and is based on a comparison with the chromatographic behavior of sickle cell hemoglobin. The countercurrent distribution pattern for the globin of the INH_2 derivative is very similar to that of normal globin, except for a slight increase in partition coefficient of the β chain too small to be shown in the figure.

Fig. 5 shows the results obtained with the CO hemoglobin reacted with NEM. The arrangement of the figure is the same as Fig. 4 with the normal hemoglobin in the top panel. The main component of the NEM derivative is slightly advanced compared to that of the normal CO hemoglobin. This result can be interpreted in two ways. If it is assumed that the chromatographic behavior of all CO hemoglobins with reacted SH groups is the same as that of the INH_2 derivative, then the behavior of the NEM derivative must be caused by the presence of approximately two more negatively charged groups per molecule compared to the INH_2 derivative. This could come about by the opening of the imide ring of the S-(N-ethylsuccinimido)-cysteine residues, as suggested by R. Benesch and R. E. Benesch (1961). Or it may mean that the configuration of the NEM-reacted CO hemoglobin is different from that of the INH_2-reacted CO hemoglobin and that this change makes the molecule only slightly more acidic than normal hemoglobin. The latter hypothesis is the more likely one, as it will be shown later that the imide ring of the S-(N-ethylsuccinimido)-cysteine residues is closed. In any case, the chromatographic behaviors of the INH_2- and the NEM-reacted CO hemoglobins are not likely to be caused by changes in the average size of the molecules of these derivatives at the concentrations used for chromatography (Cecil and Snow, 1962b).

The countercurrent distribution pattern for the globin of the NEM-reacted CO hemoglobin shows that the partition coefficient of the β chain is again slightly increased relative to that of the normal β chain. These slight changes in the partition coefficients of the β chain must be ascribed to the reacted cysteine residues, but the physical basis for these shifts is not readily apparent.

Table IV shows the results from the amino acid analyses performed on the hemoglobins after chromatography and on the separated α and β chains.

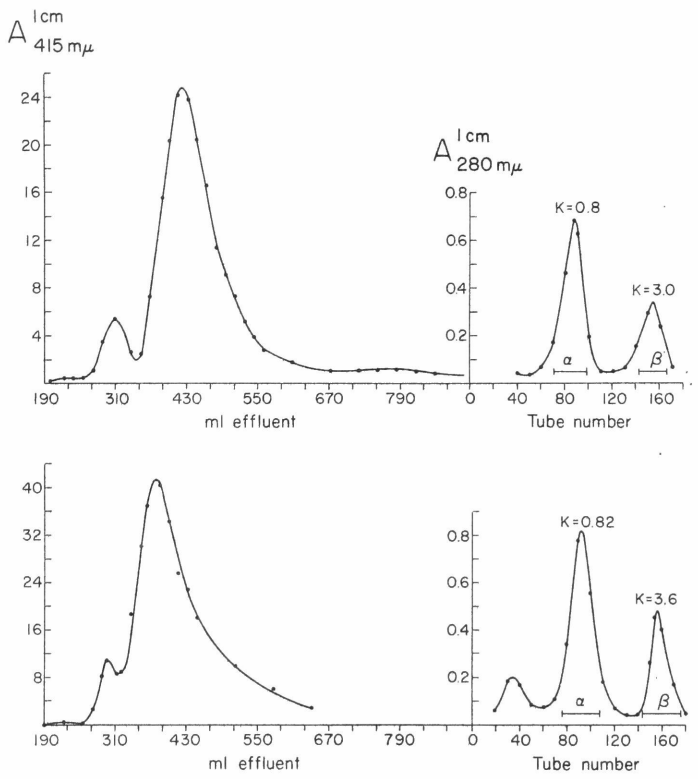


Fig. 5. Chromatography on Bio-Rex 70 (left side) and countercurrent distribution (right side) of normal CO hemoglobin, 350 mg, (top panel) and CO hemoglobin reacted with NEM at pH 7.15, 700 mg, (lower panel). The reaction conditions were: CO hemoglobin 6×10^{-4}; NEM 1.8×10^{-3} M; 1 hour at 25°C. Countercurrent distribution of peak 2 for 200 transfers, 3 ml in each phase; system: sec-butanol, 9; 0.5 M acetic acid, 10; 10% (v/v) dichloroacetic acid, 1. Only the concentrations in the lower phase are shown.

TABLE IV

Cysteine Derivatives from the Amino Acid Analyses of the INH_2- and NEM-reacted CO Hemoglobins

CO hemoglobin derivative	Residues per molecule of protein		
	S-carboxymethylcysteine	S-succinylcysteine	Ethylamine
INH_2-reacted CO hemoglobin			
globin[1]	0.95		
α chain	-		
β chain	0.97		
NEM-reacted CO hemoglobin			
globin[1]		0.85	0.87
α chain		-	-
β chain		0.87	0.85

[1]The globin values are reported for an αβ dimer. Only the results of the analyses done after chromatography of the CO hemoglobins are shown. Conditions: CO hemoglobin 6×10^{-4} M; INH_2 3.6×10^{-2} M; NEM 1.8×10^{-3} M; 1 hour at 25° and pH 7.15.

It is clear from these results that the reactive cysteine is located exclusively in the β chain. The agreement between the values for the S-succinylcysteine and the ethylamine in the case of the NEM derivative is good and is further proof that the only residues that react with NEM are cysteine residues. The values for S-carboxymethylcysteine and for S-succinylcysteine are close to one residue per αβ unit or per molecule of β chain but never over one residue. In this they differ from the results obtained by other investigators (see A. 1.) who find that two to 2.2 SH groups are reactive in the native molecule. The specificity of mercury compounds for SH groups does not make it very likely that these compounds are reacting with groups other than SH groups. This possibility, of course, is not excluded in the alkylation reactions with INH_2 and NEM, especially since their quantitation was not by direct identification of the products. Since, however, Cecil and Snow (1962a) have shown that mercury compounds will eventually react with all the SH groups in hemoglobin, the values of 2.2 SH groups per molecule probably reflect this property of the mercury compounds.

2. Identification of the reactive SH groups.

The β chains, isolated by countercurrent distribution of the globins of the INH_2- and NEM-reacted hemoglobins, were subjected to hydrolysis with trypsin in the ammonium bicarbonate system previously described (Guidotti et al., 1962). The hydrolyses were carried out for 8 hours, instead of the usual 4 hours, in order to insure complete splitting of the most resistant bonds in spite of the known slow chymotryptic-like activity of trypsin. Thus, small amounts of chymotryptic peptides were expected and were indeed found.

The preliminary fractionation with 5% dichloroacetic acid, with both digests, produced an insoluble fraction which contained no S-carboxymethyl-cysteine or S-succinylcysteine; these compounds were found only in the soluble fraction. The dichloroacetic acid precipitates were oxidized with performic acid as previously described (Guidotti et al., 1962).

Fig. 6 shows the chromatographic separation of the twelve dichloro-acetic acid-soluble peptides of the normal β chain. The peptides are labeled as Tβn, where T indicates that trypsin was used for the hydrolysis of the chain, β indicates the polypeptide chain, and n the order of elution

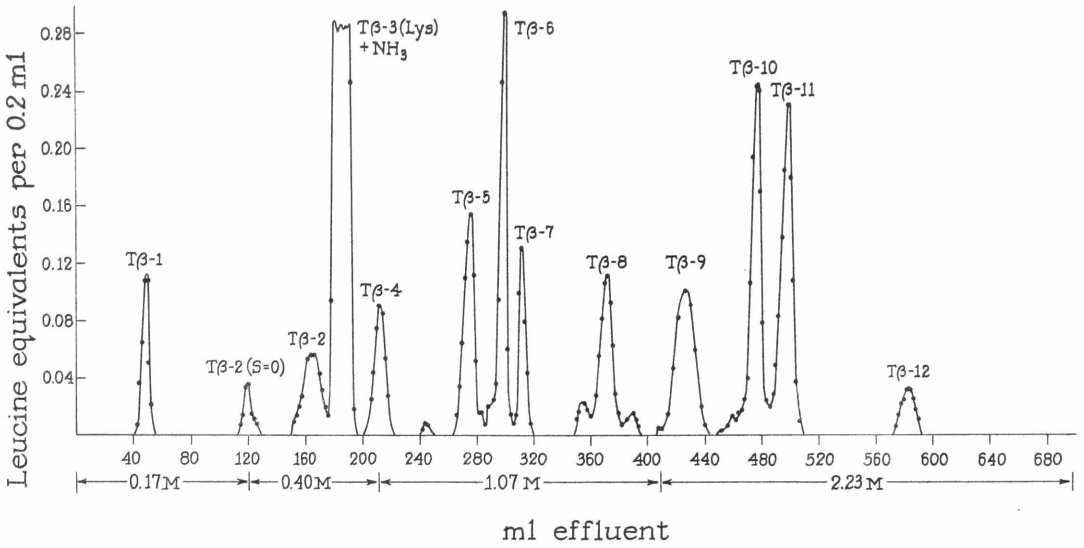


Fig. 6. Chromatography of the dichloroacetic acid-soluble peptides of the normal β chain (100 mg) on a column of Dowex 50-X2 (0.9 x 50 cm). The flow rate was 15 ml per hour; 2-ml fractions were collected; the column temperature was 35°C. The elution schedule and the molarity of the pyridine acetate buffers are shown at the bottom of the figure. S = O indicates methionine sulfoxide.

from the column. Fig. 7 shows the chromatographic separation of the two peptides in the dichloroacetic acid precipitate after oxidation with performic acid. Each of these peptides contains one residue of cysteic acid per molecule, accounting for all the cysteine in the β chain. There are eleven lysine residues and three arginine residues in the β chains. Since none of these is COOH terminal, the theoretical number of tryptic peptides is fifteen. Only fourteen were obtained. The designation of the first peptide in the chromatogram of the dichloroacetic acid-insoluble peptides as Tβ13,14 indicates that this peptide contains, in addition to the COOH-terminal arginine residue, a lysine residue which is resistant to trypsin. The reason for this insensitivity to trypsin will be shown later. The sum of the amino acid residues of the individual tryptic peptides equals that for the intact β chain. Thus, these fourteen peptides account for the entire β chain.

Fig. 8 shows the chromatographic separation of the dichloroacetic acid-soluble peptides of the β chains derived from the INH_2-reacted (top panel) and the NEM-reacted (bottom panel) hemoglobins. In both patterns, there are two peaks more than in the pattern for normal hemoglobin. One, labeled Tβ13, is at the position of Tβ7; the other, labeled Tβ14, is between Tβ8 and Tβ9. The peaks Tβ6, Tβ13, and Tβ7 were pooled and rechromatographed on the same column, using only the 0.40 M and 1.07 M pyridine acetate buffers, as shown in the inset of the top panel. Adequate separation of the components was thus obtained. The small peak between Tβ4 and Tβ5 in the bottom panel is not a tryptic peptide; it is a consequence of the chymotryptic-like activity of the trypsin.

Fig. 9 shows the chromatographic separation of the dichloroacetic acid-insoluble peptides of the β chain derived from the INH_2-reacted hemoglobin. The pattern for the same fraction of the β chain digest derived from the NEM-reacted hemoglobin was identical to this one and is not shown. This chromatogram shows that only Tβ15 is now present while Tβ13,14 has disappeared (thus the labeling of the peaks in Fig. 8).

Fig. 10 shows the relationship between the state of the SH group of cysteine residue 93β and the sensitivity to trypsin of lysine residue 95β. Presumably, in the normal β chain the SH group of cysteine residue 93β can, at the pH of the digestion, form a disulfide bond and thus render lysine 95β

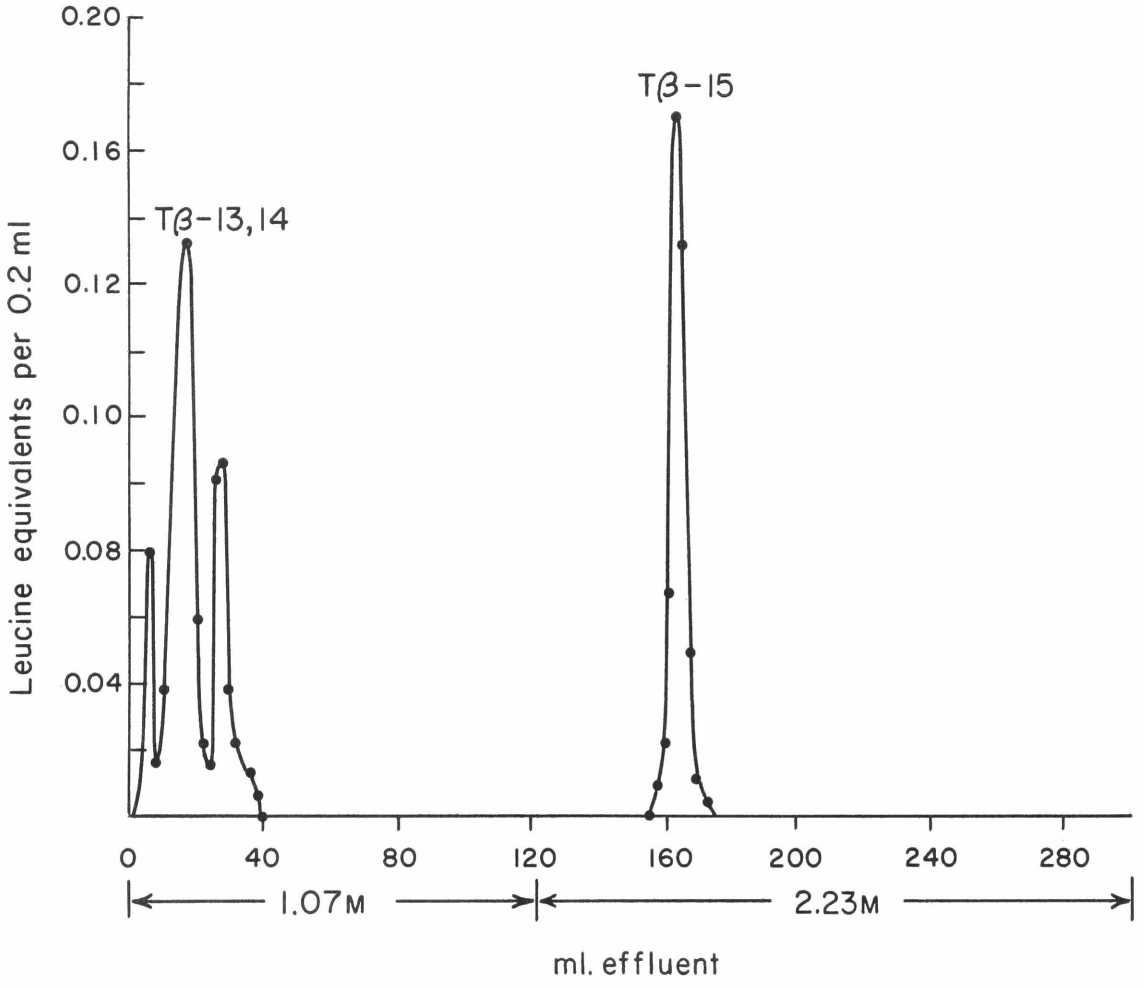


Fig. 7. Chromatography of the dichloroacetic acid-insoluble peptides of the normal β chain (20 mg) after performic acid oxidation on a column of Dowex 50-X2 (0.9 x 15 cm). The flow rate was 30 ml per hour; 2-ml fractions were collected; the column temperature was 50°C. The elution schedule and the molarity of the pyridine acetate buffers are shown at the bottom of the figure.

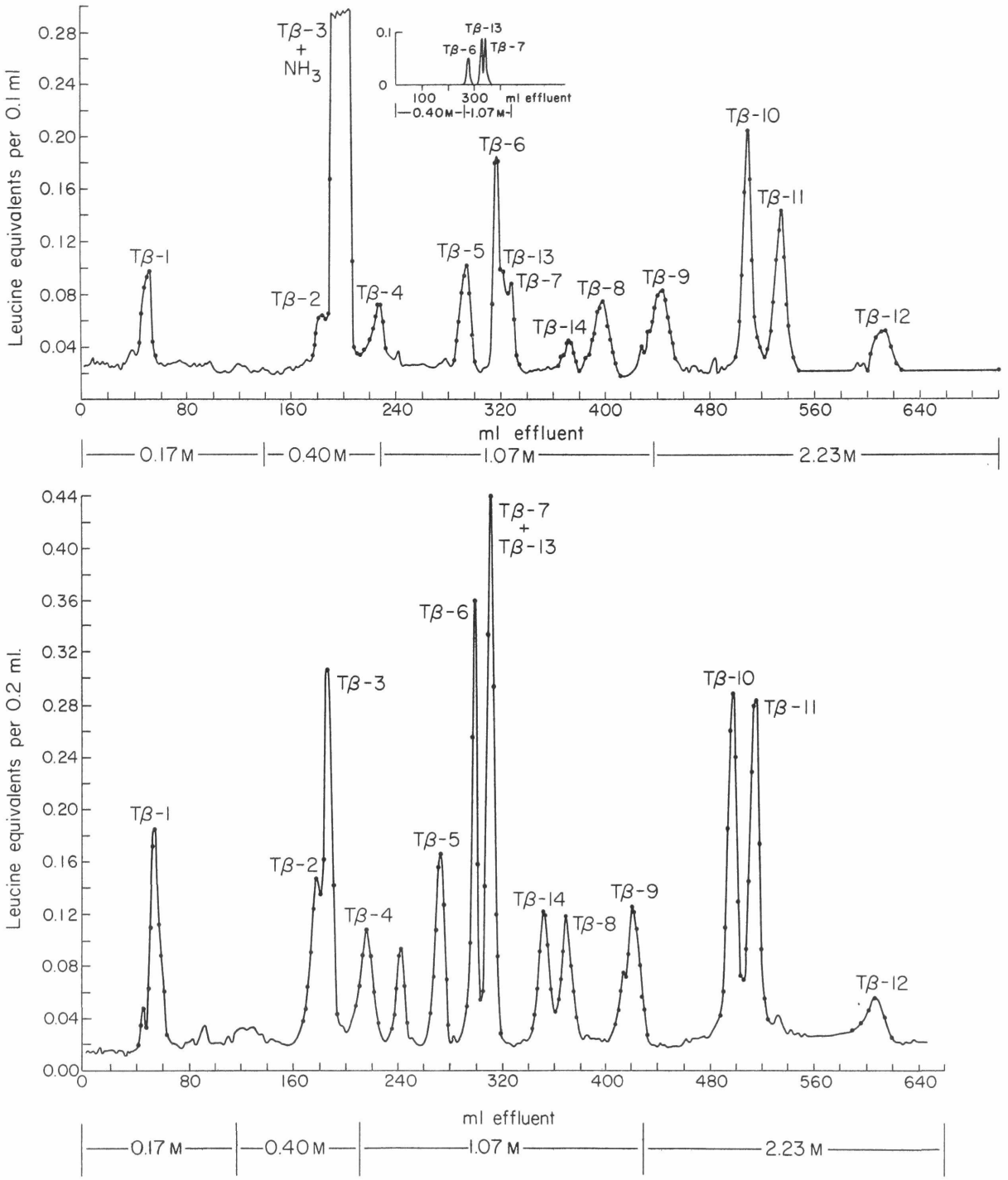


Fig. 8. Chromatography of the dichloroacetic acid-soluble peptides of the β chains derived from CO hemoglobin reacted with INH_2, 250 mg, (top panel) and from CO hemoglobin reacted with NEM, 150 mg, (bottom panel) on a column of Dowex 50-X2 (0.9 x 50 cm). The flow rate was 15 ml per hour; 2-ml fractions were collected; the column temperature was 35°C. The hemoglobin derivatives were those described in Figs. 4 and 5. The inset in the top panel indicates the elution positions of Tβ6, Tβ13, and Tβ7 when these fractions were rerun on the same column using a different elution schedule. The elution schedules and the molarity of the pyridine acetate buffers are shown at the bottom of each chromatogram.

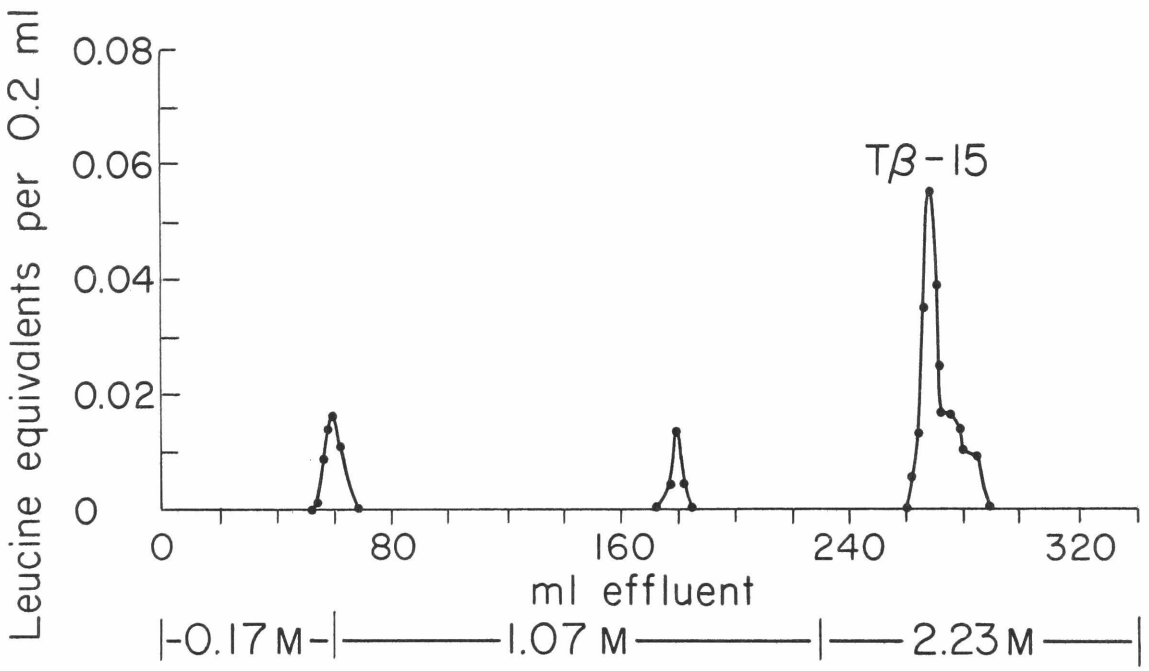


Fig. 9. Chromatography of the dichloroacetic acid-insoluble peptides of the β chain derived from CO hemoglobin reacted with INH_2, 10 mg, on a column of Dowex 50-X2 (0.9 x 15 cm). The flow rate was 30 ml per hour; 2-ml fractions were collected; the column temperature was 50°C. The elution schedules and the molarity of the pyridine acetate buffers are shown at the bottom of the figure. The insoluble material was oxidized with performic acid before chromatography.

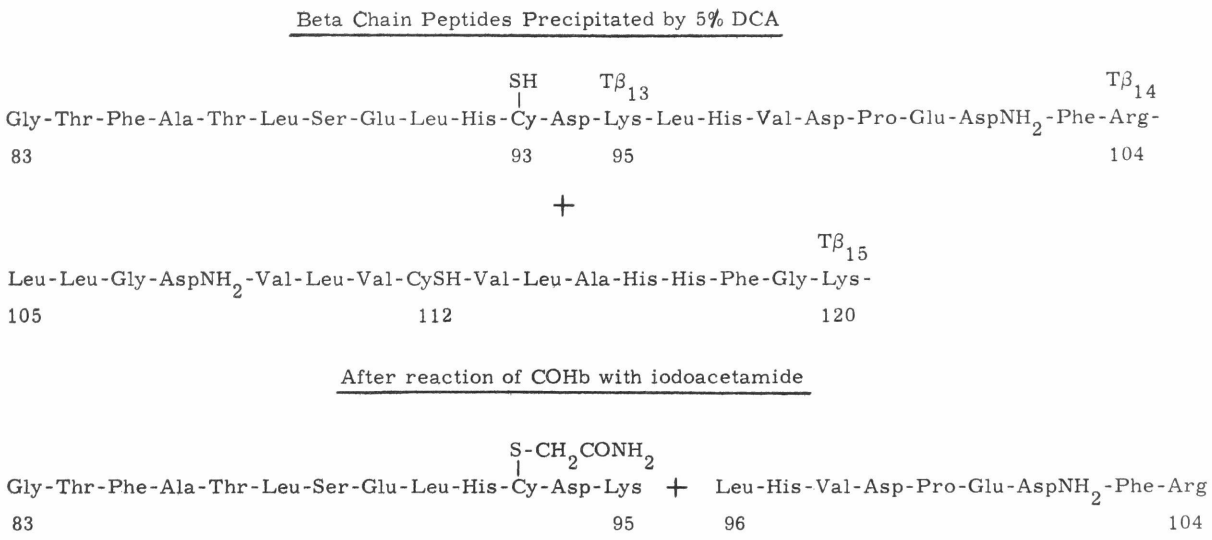


Fig. 10. The effect of alkylation of cysteine residue 93β on the sensitivity of lysine residue 95β to enzymatic hydrolysis by trypsin. DCA stands for dichloroacetic acid; COHb stands for CO hemoglobin.

sterically unapproachable by trypsin. Since this cannot happen after alkylation, trypsin can now hydrolyze the bond between residues 95β and 96β. The resulting peptides, Tβ13 and Tβ14, are soluble in 5% dichloroacetic acid and thus remain in the supernatant.

Table V gives the results of the amino acid analyses performed on the peptides Tβ13 and Tβ14 derived from the β chains of both the INH_2-reacted and NEM-reacted hemoglobins. In the first column of the table, the composition of Tβ13,14 from the normal β chain is shown. The sum of the amino acids in each set of peptides, Tβ13 and Tβ14, corresponds closely to the amino acid composition of Tβ13,14. Of course, instead of cysteic acid, Tβ13 contains S-carboxymethylcysteine or S-succinylcysteine. The yields of Tβ13 and Tβ14 are shown at the bottom of the table. They are close to the maximum yields obtainable by the chromatographic procedure.

Clearly then, cysteine residue 93β contains the SH group that reacts with INH_2 and NEM in the molecule of native adult human hemoglobin. Therefore, cysteine residue 93β will be called the reactive SH group of human hemoglobin.

D. Discussion

1. The function of the reactive SH groups.

The work described in the previous sections has indicated that the oxygenation parameters of hemoglobin are, to various extents, modified by reaction of two of the six SH groups in the molecule with a variety of reagents. It has been shown in the preceding section that these reactive SH groups are those of cysteine residue 93β. From the known amino acid sequence of the β chain (Braunitzer et al., 1961; Konigsberg et al., 1963) and by correlation with the X-ray crystallographic data of Cullis et al. (1962), it turns out that this residue, cysteine 93β, is located right next to the histidine residue which is directly linked to the iron atom of the heme group. It is not surprising that this particular SH group should be involved in stabilizing interactions during the oxygenation of the molecule. Thus, when this SH group is no longer available, these interactions are absent and the oxygenation of the molecule is altered. Nevertheless, it is not at all apparent how this effect is mediated and the entire question of the Bohr effect, the heme-heme interactions, and the oxygen affinity still lacks an adequate explanation.

TABLE V

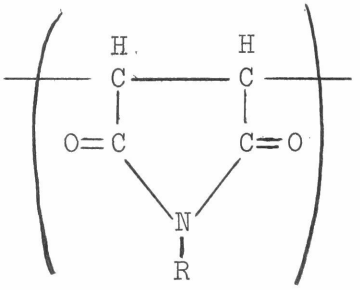
Amino Acid Composition of the Peptides Tβ13 and Tβ14

Amino acid	Amino acid residues per molecule of peptide				
		INH_2 β chain*		NEM β chain*	
	Tβ13,14	Tβ13	Tβ14	Tβ13	Tβ14
Lysine	1	0.98		1.00	
Histidine	2	0.95	1.00	0.98	1.10
Arginine	1		1.00		1.00
Aspartic acid	3	1.00	1.99	1.00	1.98
Threonine	2	1.76		1.71	
Serine	1	0.90		0.75	
Glutamic acid	2	0.98	0.99	1.18	0.97
Proline	1		1.00		1.00
Glycine	1	1.04		1.00	
Alanine	1	1.02		1.00	
Valine	1		1.24		1.11
Leucine	3	1.82	1.28	2.00	1.08
Phenylalanine	2	1.00	0.91	1.00	0.95
Cysteic acid	1				
S-carboxymethylcysteine		0.84			
S-succinylcysteine				0.85	
Ethylamine				0.82	
No. of residues	22	13	9	13	9
Yield (%)	50	75	85	80	93

*INH_2 β chain refers to the β chain of the CO hemoglobin reacted with INH_2; NEM β chain refers to the β chain of the CO hemoglobin reacted with NEM.

No definite evidence for or against any of the theories which have been proposed for these phenomena can be deduced from the data presented here. However, the contention of Wyman and Allen (1951) that these oxygenation effects are caused by a general rearrangement of the molecular structure is supported by the results given here. In Part II, E., the evidence indicating the reversible relationship between the oxygenation parameters and the molecular configuration of hemoglobin have been described. In the preceding section, it was shown that CO hemoglobins whose reactive SH groups have reacted with NEM and INH_2 manifest, through their chromatographic behavior, definite though not identifiable changes in molecular conformation. Thus, the changes in the oxygenation parameters observed with these hemoglobins can be convincingly attributed to changes in conformation. Since, however, the exact relationship between the configuration of the molecule and its ability to combine with oxygen is still not understood, the mechanism of the process remains unexplained.

The theory of R. Benesch and R. E. Benesch (1961), based on the opening of the imide ring of the NEM adduct of cysteine 93β, does not appear likely for two reasons. First, the assumed effect of imidazole in catalyzing the opening of this ring is based on the data of Smyth et al. (1960) who showed that in the presence of imidazole NEM forms a polymeric substance but presented no evidence for the hydrolysis of the imide ring of NEM. That this polymer has a polysuccinimide structure (I) has in fact been shown for



I

both radical-initiated and base-catalyzed polymerization of NEM (Tawney et al., 1961). Second, the peptide Tβ13 derived from the β chain of NEM-reacted hemoglobin had a chromatographic position identical to that of the same peptide derived from the β chain of INH_2-reacted hemoglobin. Thus, the number of charged groups in these two peptides is the same and the imide ring of the S-(N-ethylsuccinimido)-cysteine residue cannot be opened at this stage. Unless the ring closed during the many procedures which preceded

the chromatographic separation of the peptides (an unlikely event), the ring remained closed at all times after the reaction of hemoglobin with NEM.

The theory of Riggs (1961) is in general very closely related to the theory of configurational changes in the molecule.

2. The unreactive SH groups.

The finding that the reactive SH groups in the hemoglobin molecule are at specific sites in the molecule poses the question of why the other SH groups are not reactive and what their function might be.

Greenstein and Edsall (1940) first suggested that there are two types of SH groups in native proteins: a type that did not react with nitroprusside, porphyrindin, and ferricyanide but did react with iodine and iodoacetic acid, and a type that reacted with all these reagents. The many causes for the differential reactivity of SH groups have been reviewed by Cecil and McPhee (1959). Most of these are very difficult to prove. Thus, the presence of thiazoline rings (Linderstrøm-Lang and Jacobsen, 1941) and of thiol ester bonds (Chibnall, 1942-43; Smith, 1958) have been proposed but never really demonstrated.

One of the most accepted theories is that of steric hindrance to the approach of a reagent. This theory has been suggested for hemoglobin by Cullis et al. (1962) on the basis of the three-dimensional structure of the molecule which shows that the unreactive SH groups are located well inside the tetrameric structure. However, steric effects may also be effective in the local environment of the thiol group by hindrance to solvation of ions (Cecil and McPhee, 1959).

Hydrogen bonds between the SH groups and nucleophilic groups have been also implicated (Laskowski and Scheraga, 1954). Cecil (1950) proposed this hypothesis for the decrease in the rate of reaction of glutathione with Ag^+ compared to that of cysteine. On the basis of studies with glutathione, phenylacetyl-L-cysteinyl-glycine, and phenacetyl-L-cysteinyl-D-valine, R. E. Benesch and R. Benesch (1953) suggested that steric effects involving aliphatic side chains are mediated by hydrogen bonds of the SH groups to the peptide bond. This hypothesis was based on the anomalous behavior of the phenylmercuric derivative of phenacetyl-L-cysteinyl-D-valine by polaro-

graphic analysis as compared to this derivative of the other two peptides. With regard to these considerations, it should be noticed that the sequences around the unreactive SH groups of hemoglobin are -histidinyl-cysteinyl-valyl- for the α chain and -valyl-cysteinyl-valyl- for the β chain. It is not justifiable, however, to suggest the Benesch and Benesch hypothesis as an explanation for the unreactivity of the SH groups of hemoglobin, since in this protein the amino acid residues all have the L-configuration and since the unreactive cysteines are located in helical parts of the polypeptide chains. In addition, it has been found in this laboratory (Konigsberg, 1962) that strong urea solutions do not increase the number of SH groups that react with alkylating reagents--thus making improbable the presence of hydrogen bonds.

The effects of charged groups in the vicinity of the SH groups can hinder or enhance the approach of charged reagents. In this context, the inability of iodoacetic acid to react with cysteine 93β might be attributed in part to the aspartic acid residue at position 94β. Since the rate of reaction of iodoacetic acid with hemoglobin is not increased in solutions of high ionic strength (R. E. Benesch and R. Benesch, 1962; Konigsberg, 1962), the coulombic effects cannot be very important. Nor can coulombic interactions be the cause of the unreactivity of the unreactive SH groups because the reagents used, INH_2 and NEM, do not carry any formal charge and also because in strong salt solutions they fail to react with these unreactive SH groups (see Part IV, E. 1. d.).

The side chains of residues which can interact with the SH groups may influence the dissociation of the SH groups by direct coulombic effects (R. E. Benesch and R. Benesch, 1955; Elson and Edsall, 1962). Thus, the pK's of the SH groups of model compounds have been found to vary between 8 and 10 depending on the ionic environment of the SH group. Since the observed rate constant for the alkylation of an SH group is, within limits (see B. 2. above), directly proportional to the dissociation constant of the SH group, variations of 100 fold in the rate of reaction are to be expected. However, these effects should also be decreased by solvents of high ionic strength. Thus, they are probably of little importance in the case of hemoglobin.

The possibilities would seem then to be confined to steric effects due to the conformation of the particular molecular forms of hemoglobin or

to steric effects due to the local environment of the SH groups. At present, it is difficult to choose between the two. However, on the basis of the data to be presented in the next part, it appears that both effects are important but that the major contribution to the unreactivity of the SH groups is from the conformation of the molecule. The relationships between the conformation of a protein and the reactivity of its functional groups have, of course, been well illustrated by Stark et al. (1961) for ribonuclease.

In trying to explain the function of the unreactive SH groups, one is faced with the problem of the function of all the other amino acid residues in the chains. The α chain is composed of 141 amino acids and the β chain of 146 amino acids, but the reason for which nature has specified particular sequences of the amino acids in each chain is not understood. A posteriori, it is possible to state that this sequence specifies the folding of the chains and that this unique folding is necessary for the peculiar property of the heme in hemoglobin. But the mechanism by which the amino acid sequence specifies the folding of the chains is not evident. With regard to the SH groups, Cecil and Snow (1962b) have given some evidence that they are not involved in the heme linkage or in interchain bonds; but the instability of hemoglobin in which all the SH groups have been reacted has been taken to indicate that these groups are important in intrachain stabilizing bonds. Comparison with other heme proteins provides no further understanding. Sperm whale myoglobin contains no SH groups (Edmundson and Hirs, 1962) but is a stable molecule; and lamprey hemoglobin, with a molecular weight of 17,000, contains two SH groups (Allison et al., 1960) and is also a stable molecule. Thus, the function of the SH groups in the molecule of hemoglobin must remain at the stage of hypotheses.

PART IV

The Dissociation of Hemoglobin

A. Introduction

In Part I, it was shown that the major portion of human hemoglobin within certain limits of concentration at neutral pH and in solutions of moderate ionic strength has a molecular weight of about 65,000 and is composed of two α chains and two β chains, $\alpha_2\beta_2$.

From the very first observations, it was noticed that under certain conditions this molecule dissociates reversibly into smaller units. This property has served in the study of the function of hemoglobin and in the development of some of the current concepts of the genetic control of proteins. It furthermore is a clear example of the general phenomenon of interactions between macromolecules in solution. Considerable interest has consequently centered around the mechanism of dissociation.

B. The Dissociation of the Molecule

The first evidence that human hemoglobin is a system in rapid equilibrium was provided by Lamm and Polson (1936) who found that the value of the diffusion coefficient increased from 6.73×10^{-7} cm^2/sec to 7.39×10^{-7} cm^2/sec over a ten-fold concentration range (3.8% to 0.2% CO hemoglobin in 0.1 M NaCl at pH 6.5). Furthermore, when such a solution was allowed to diffuse into distilled water, the coefficient increased to 9.74×10^{-7} cm^2/sec. These investigators also mentioned that K. O. Pedersen (unpublished data) found a similar decrease in the sedimentation coefficient of CO hemoglobin upon dilution. Other investigators (Kegeles and Gutter, 1951; Schumaker and Schachman, 1957; Benhamou et al., 1960; Rossi-Fanelli et al., 1961a) measured the light scattering, sedimentation and diffusion coefficients of aquo-, oxy-, and carbonmonoxyhemoglobin at very low concentrations (see Table II) and found instead no evidence of dissociation in solutions of neutral pH and moderate ionic strength.

Similar disagreement exists with regard to the molecular weight of hemoglobin in strong salt solutions. K. O. Pedersen, quoted by Svedberg and Pedersen (1940, p. 356), found that CO hemoglobin had a decreased sedimentation coefficient in strong salt solutions. Gutfreund (1949) later reported that the osmotic pressure of oxyhemoglobin (3% to 0.5%) in 1 M NaCl was greater than that in 0.2 M phosphate buffer at neutral pH. Subsequently, Benhamou et al. (1960), Rossi-Fanelli et al. (1961a), and R. E.

Benesch et al. (1962) have found that the molecular weight of oxy- and aquohemoglobin in 1 to 2 M NaCl solutions at neutral pH has values ranging from 32,000 to 45,000 (see Table II). However, there is a fundamental inconsistency in these data. Thus, while the first group showed that the values of the light scattering data and diffusion and sedimentation coefficients are definitely concentration dependent, as expected for a system in equilibrium, the latter two groups failed to notice this feature even over a fifty-fold concentration range (1% to 0.02%).

The effects of the dielectric properties of the solvent are also shown in Table II. The results of Gutter et al. (1956) for CO hemoglobin in 4 M urea illustrate that changes in the sedimentation coefficient may be attended by no change in the molecular weight. Mercaptoethanol (1.4 M) instead actually causes partial dissociation. Even here, however, the light scattering data of Benhamou and Weill (1957) are in disagreement (M.W. 54,000 for oxyhemoglobin in 4 M urea). The sedimentation coefficient of oxyhemoglobin in solvents containing sodium dodecylsulfate has been reported to be 2 (Ingram, 1959a).

Only in the case of the pH dependence of dissociation is there general agreement, as shown in Table VI. In the pH range 6 to 10, the molecular weight, for the stated concentrations, has a value of approximately 65,000 while below pH 6 and above pH 10 there is a gradual decrease in this value approaching 35,000 at pH 11.0 and pH 4.0. This is the value expected for a dimer of the chains. The value of the frictional coefficient at low and high pH is close to that at neutral pH indicating little change in the molecular shape. This fact is important with regard to the diffusion experiments to be reported later. Benhamou (1956a), by surface film methods, also found that the molecular weight of oxyhemoglobin decreased from a value of 70,000 at pH 6, to a value of 36,000 at pH 5, and further to a value of 16,000 below pH 4.

From these data, it is concluded that the hemoglobin tetramer dissociates at least into dimers at high and low pH and that it may do so also at low concentrations in both moderate and strong salt solutions. The values for the molecular weights, given in Tables II and VI, are not integral multiples of the values calculated for the individual chains. They are, however, perfectly compatible with those expected for a system of tetramers

and dimers in rapid equilibrium as are also the symmetrical sedimentation boundaries (Field and O'Brien, 1955; Hasserodt and Vinograd, 1959; Rossi-Fanelli et al., 1961a; Benhamou et al., 1960). This last point follows from the treatment of systems in rapid dissociation equilibrium in moving-boundary experiments (Field and Ogston, 1955).

C. The Mechanism of Dissociation

Theoretically, a tetramer of the formula $\alpha_2\beta_2$ can dissociate into dimers either symmetrically $\alpha_2 \rightleftarrows 2\alpha\beta$, asymmetrically $\alpha_2 \rightleftarrows \alpha_2 + \beta_2$, or randomly $2\alpha_2\beta_2 \rightleftarrows 2\alpha\beta + \alpha_2 + \beta_2$.

1. The existing hypotheses.

By a set of very ingenious experiments consisting of mixing hemoglobins with different relative charges (normal CO hemoglobin, several abnormal CO hemoglobins, ferrihemoglobin), exposing them together to an acid pH, and then analyzing the products after readjusting the pH to neutral values, Itano and Singer (1958) and Singer and Itano (1959) concluded that dissociation is asymmetrical. Thus, when they mixed normal CO hemoglobin ($\alpha_2^A\beta_2^A$) and sickle cell hemoglobin ($\alpha_2^A\beta_2^S$), they obtained only the starting hemoglobins (recombination). This result can be explained by asymmetrical dissociation

$$\underset{(0)}{\alpha_2^A\beta_2^A} + \underset{(+2)}{\alpha_2^A\beta_2^S} \rightleftarrows \underset{(0)}{\alpha_2^A} + \underset{(0)}{\beta_2^A} + \underset{(0)}{\alpha_2^A} + \underset{(+2)}{\beta_2^S}$$

but not by symmetrical dissociation

$$\underset{(0)}{2\alpha_2^A\beta_2^A} + \underset{(+2)}{2\alpha_2^A\beta_2^S} \rightleftarrows \underset{(0)}{4\alpha^A\beta^A} + \underset{(+1)}{4\alpha^A\beta^S} \rightleftarrows \underset{(0)}{\alpha_2^A\beta_2^A} + \underset{(+2)}{\alpha_2^A\beta_2^S} + \underset{(+1)}{2\alpha_2^A\beta^A\beta^S}$$

In these and the following equations, the numbers in parentheses under the formulas indicate the charge of the hemoglobin relative to normal hemoglobin; the location of the charge difference is shown by the superscript to the α and β chains, where A indicates normal hemoglobin, S and C sickle cell hemoglobin and hemoglobin C, respectively, and + ferrihemoglobin. When instead these investigators mixed normal CO hemoglobin and ferrihemoglobin C, they obtained the two starting hemoglobins and two new ones (hybridization) as predicted by asymmetrical dissociation:

TABLE VI

The pH Dependence of the Sedimentation Coefficient of Human Hemoglobin

pH	$s_{20,w}$ (c.g.s. units x 10^{13})			M.W.s,d*	M.W.eq*	f/f_o*
	(1)	(2)	(3)	(1)	(3)	
3.50	3.25					
3.96		2.38				
4.01	3.4					
4.30		2.49				
4.60	3.5					
4.79		2.79		42,000		1.19
5.00	3.75					
5.42	4.03					
5.65		3.81				
6.73		4.08				
8.00	4.24					
8.2			4.3			
10.0			4.2			
10.5			3.6			
10.8			2.7			
11.0			2.55		36,000	1.27
11.2			2.55			
11.6			2.0			

(1) Field and O'Brien (1955). CO hemoglobin: 0.71 g %; 0.1 M sodium acetate buffers.

(2) Itano and Singer (1958). CO hemoglobin: 0.7 g %; 0.1 M sodium acetate buffers.

(3) Hasserodt and Vinograd (1959). CO hemoglobin: 1 g %; buffers of ionic strength 0.25.

*M.W.s,d is the molecular weight by sedimentation and diffusion; M.W.eq is the molecular weight by sedimentation equilibrium; f/f_o is the frictional coefficient.

$$\underset{(0)}{\alpha_2^{A}\beta_2^{A}} + \underset{(+8)}{\alpha_2^{A+}\beta_2^{C+}} \rightleftarrows \underset{(0)}{\alpha_2^{A}} + \underset{(0)}{\beta_2^{A}} + \underset{(+2)}{\alpha_2^{A+}} + \underset{(+6)}{\beta_2^{C+}} \rightleftarrows \underset{(0)}{\alpha_2^{A}\beta_2^{A}} + \underset{(+8)}{\alpha_2^{A+}\beta_2^{C+}} + \underset{(+6)}{\alpha_2^{A}\beta_2^{C+}} + \underset{(+2)}{\alpha_2^{A+}\beta_2^{A}}$$

Whereas if dissociation were symmetrical, the result would have been:

$$\underset{(0)}{2\alpha_2^{A}\beta_2^{A}} + \underset{(+8)}{2\alpha_2^{A+}\beta_2^{C+}} \rightleftarrows \underset{(0)}{4\alpha^{A}\beta^{A}} + \underset{(+4)}{4\alpha^{A+}\beta^{C+}} \rightleftarrows \underset{(0)}{\alpha_2^{A}\beta_2^{A}} + \underset{(+8)}{\alpha_2^{A+}\beta_2^{C+}} + \underset{(+4)}{2\alpha^{A}\alpha^{A+}\beta^{A}\beta^{C+}}$$

Subsequently, Robinson and Itano (1960) showed that at alkaline pH the mechanism of dissociation was also asymmetrical. This method of recombination and hybridization has served to identify the altered peptide chain in many abnormal hemoglobins (for review, see Baglioni, 1962). In all cases, the data followed expectations.

However, Vinograd and Hutchinson (1960) pointed out at an early date that all the data could just as easily be explained by symmetrical dissociation if two assumptions were made. These assumptions were: 1) that a further equilibrium between dimers and monomers existed at both alkaline and acid pH's and 2) that only like dimers could recombine into a tetramer. Thus:

$$\underset{(0)}{2\alpha_2^{A}\beta_2^{A}} + \underset{(+8)}{2\alpha_2^{A+}\beta_2^{C+}} \rightleftarrows \underset{(0)}{4\alpha^{A}\beta^{A}} + \underset{(+4)}{4\alpha^{A+}\beta^{C+}} \rightleftarrows \underset{(0)}{4\alpha^{A}} + \underset{(0)}{4\beta^{A}} + \underset{(+1)}{4\alpha^{A+}} + \underset{(+3)}{4\beta^{C+}}$$

$$\downarrow\uparrow$$

$$\underset{(0)}{\alpha_2^{A}\beta_2^{A}} + \underset{(+6)}{\alpha_2^{A}\beta_2^{C+}} + \underset{(+2)}{\alpha_2^{A+}\beta_2^{A}} + \underset{(+8)}{\alpha_2^{A+}\beta_2^{C+}} \rightleftarrows \underset{(0)}{2\alpha^{A}\beta^{A}} + \underset{(+3)}{2\alpha^{A}\beta^{C+}} + \underset{(+1)}{2\alpha^{A+}\beta^{A}} + \underset{(+4)}{2\alpha^{A+}\beta^{C+}}$$

This hypothesis was proposed because it was not possible otherwise to reconcile the slow rate of hybridization (Vinograd and Hutchinson, 1960; Robinson and Itano, 1960; Itano and Robinson, 1960; Jones et al., 1959b; Huehns and Shooter, 1961) with the fast rate of dissociation (Itano and Singer, 1958).

Subsequently, several other phenomena have been discovered which also cannot be explained by the Itano theory of asymmetrical dissociation but are compatible with the Vinograd theory of symmetrical dissociation. A searching discussion on this point has recently been presented (Antonini et al., 1962b). Some of these phenomena are as follows:

a) Charlwood et al. (1960) have pointed out that both fetal ($\alpha_2^{F}\gamma_2^{F}$) and adult ($\alpha_2^{A}\beta_2^{A}$) hemoglobin are denatured in strongly alkaline solutions

in a first-order reaction, but the process is 100 times faster for adult hemoglobin. At the pH of denaturation, both hemoglobins are dissociated into dimers so that a first-order reaction implies that the dimers of adult hemoglobin are being denatured at one rate and those of fetal hemoglobin at another rate. Since the α chains of adult and fetal hemoglobin are identical, $\alpha^F = \alpha^A$, (Jones et al., 1959b; Schroeder et al., 1961), dissociation cannot be asymmetrical.

b) Bovine hemoglobin has been found to dissociate into dimers at both acid and alkaline pH's but does not hybridize with human hemoglobin (Antonini et al., 1962b; Itano and Robinson, 1959).

c) In strong salt solution in which hemoglobin is presumably dissociated into dimers (see Table II), canine and horse hemoglobins do not hybridize with human hemoglobin (Antonini et al., 1962b).

d) Although the dissociation process can be detected below pH 6.0, hybridization is not appreciable until the pH is lowered below pH 5 (Antonini et al., 1962b).

Thus, there were two ways to explain the dissociation data of hemoglobin: 1) The Itano hypothesis of asymmetrical dissociation, which explained most of the data in the literature with the exception of the set of experiments described above. 2) The Hutchinson and Vinograd hypothesis of symmetrical dissociation, which could explain the phenomena above but involved two hypotheses for which there was no proof. In any case, the data seem to exclude the existence of the random mechanism of dissociation or of other mechanisms such as $2\alpha_2\beta_2 \rightleftarrows 4\alpha + \beta_4$.

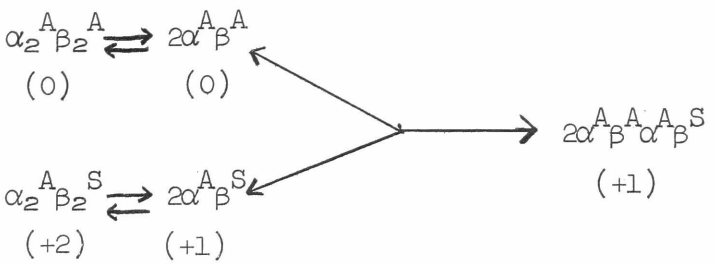
2. A revised hypothesis.

The concentration dependence of the diffusion coefficient (Lamm and Polson, 1936; see B. of this part) is a good indication that even at neutral pH and in solutions of moderate ionic strength hemoglobin is not a static molecule but in dynamic equilibrium with its subunits. This situation immediately requires that the dissociation be symmetrical, at least at neutral pH, if the lack of hybridization at neutral pH is to be meaningful (Singer and Itano, 1959). This in turn implies that the $\alpha\beta$ subunit is more stable than either the α_2 or the β_2 subunits. Circumstantial evidence has in fact been obtained on this point. Thus, while in solutions of neutral pH

and of moderate ionic strength hemoglobin H (see Part II, E. 2.), composed of β chains only, has a molecular weight of 68,000 (β_4), the experimentally produced hemoglobin α^A (Huehns et al., 1961), containing only α chains, has a molecular weight of 17,000 (α). It follows that under conditions favoring dissociation evidence for the reaction $\alpha_2 \rightleftarrows 2\alpha$ should also be observed if the dissociation were asymmetrical ($\alpha_2\beta_2 \rightleftarrows \alpha_2 + \beta_2$).

These considerations lead directly to the conclusion that the Hutchinson and Vinograd hypothesis of symmetrical dissociation, $\alpha_2\beta_2 \rightleftarrows 2\alpha\beta \rightleftarrows 2\alpha + 2\beta$, is the most likely. If, however, the existence of an equilibrium between dimers and monomers at the limits of pH and of an equilibrium between tetramers and dimers at neutral pH could be definitely demonstrated, the assumptions required by the aforementioned investigators would not be needed. The equilibrium between dimers and tetramers at neutral pH implies that any hybrid molecule composed of unlike dimers ($\alpha^A\beta^A\alpha^A\beta^S$) would predominantly separate into two tetrameric components ($\alpha_2{}^A\beta_2{}^A + \alpha_2{}^A\beta_2{}^S$) during the process of separation if the rate of attainment of equilibrium were appreciably faster than the rate of the separation process.

The last statement is based on a consideration of the recent analyses of the behavior of two substances interacting reversibly in moving-boundary experiments (Longsworth and MacInnes, 1942; Longsworth, 1959; Gilbert and Jenkins, 1959) and during countercurrent distribution (Bethune and Kegeles, 1961). The calculations by the latter investigators postulating that the system $A + B \rightleftarrows C$, where C has a partition coefficient between those for A and B and where the association constant has a value of 10^2 (unit volume/mass x phase volume), will separate almost completely into pure A and B components in as few as fifty transfers bear directly on the hemoglobin problem. The close relationship between countercurrent distribution and ion exchange chromatography and between the system $A + B \rightleftarrows C$ given above and any hemoglobin system of the type



is evident. It is not surprising, therefore, that components made up of hybrid molecules of the type $\alpha^A\beta^A\alpha^A\beta^S$ are not seen after separation procedures. Support for the contention that hemoglobin in effect behaves in this fashion comes from the data of Pauling et al. (1949) and of Wells and Itano (1951). These investigators found that after separation of a mixture of normal and sickle cell hemoglobin by Tiselius electrophoresis the measured concentrations of sickle cell hemoglobin did not correspond to the actual concentrations. In fact they had to construct calibration curves in order to measure the actual concentrations. Longsworth and MacInnes (1942) showed that this behavior obtains in the case of interacting systems. Furthermore, the elevation of the baseline between the pure components on ion exchange chromatography (Schnek and Schroeder, 1961; Huisman et al., 1958; Hill et al., 1962) is in agreement with the proposed hypothesis.

On the basis of these considerations, the most likely model for the dissociation of hemoglobin is the following:

$$\alpha_2\beta_2 \overset{K_1}{\rightleftarrows} 2\alpha\beta \overset{K_2}{\rightleftarrows} 2\alpha + 2\beta$$

where K_1 and K_2 represent the dissociation constants. The requirements of this model, based on the existing data, are as follows:

1) K_1 is small but measurable at neutral pH and at moderate ionic strength.
2) K_1 increases as the pH varies from the isoelectric point of hemoglobin.
3) K_2 is very small at neutral pH; it increases at the extremes of pH.
4) K_1 increases with increasing ionic strength at neutral pH, but K_2 does not.
5) The values and pH dependence of K_1 and K_2 vary with the type of hemoglobin and with the state of the iron atom in the molecule.

Clearly, the validity of this model rests on the ability to demonstrate the postulated equilibria and to obtain values for the dissociation constants

under the various conditions. This is the object of the remaining part of this dissertation.

D. Experimental

1. General methods.

The experimental procedures have largely been described in Part III. In these experiments, the chromatograms of the CO hemoglobins were analyzed by measuring the optical density of the fractions not only at 415 mμ but also alternatively at 540 mμ.

The alkylations of CO hemoglobin at pH 7.15 were performed in the phosphate buffer, 0.2 M Na^+ (previously described). The reactions at pH 4.7 were done in a buffer 0.2 M in sodium acetate and 0.2 M in acetic acid (see Table VII) which also was 0.2 M Na^+. Where indicated, the buffers were also 2.0 M NaCl. The hemoglobin concentration was 6×10^{-4} M in all cases. The concentration of INH_2 and NEM varied in the different experiments; and, consequently, their concentrations are shown in the tables of results. The reactions were performed in a CO atmosphere at 25° for 1 hour, 6 hours, or 17 hours; and then the hemoglobin solutions were treated as previously described.

2. Method of restricted diffusion.

The restricted diffusion (thin film dialysis) technique has been described by Craig and King (1962). The membrane was a 20/32 Visking cellophane casing, which was treated for 6 minutes with 64% $ZnCl_2$. After the treatment, the membrane was washed with 0.1 N HCl for 24 hours, then with distilled water and with 0.1 N acetic acid. When not in use, it was kept at 5° in 1 N acetic acid. One end of the membrane was slipped over an open glass tube, while the other end was tied off at a distance of 10 cm from the edge of the glass tube. A mandril, inserted through a rubber stopper, fitted inside the membrane so that 0.4 ml of solution was spread over the entire surface of the membrane in a film 0.1 to 0.2 mm in thickness. The membrane was then placed in a test tube which contained 14 ml of pure solvent (the outside solution). This was sufficient to cover the entire surface of the membrane. In order to perform the dialysis experiments in a CO atmosphere, the entire apparatus was placed inside a filtering flask. The stopper around the mandril fitted snugly in the neck of the flask and kept the apparatus upright. The flask was then repeatedly evacuated and

flushed with CO through the side arm. It was necessary to do the experiments in a strictly CO atmosphere in order to prevent adsorption of the hemoglobin to the membrane.

All the experiments were done at 25° and without stirring the outside solution (Craig and King, 1962). At various times after starting, the flask was opened and the outside solution removed with a syringe. After adding fresh solvent, the flask was again flushed with CO. The absorbancy of the outside solution was determined at 415 mU on a Beckman DU Spectrophotometer; and at the end of the experiment, the absorbancy of the solution remaining inside the membrane was also determined after dilution to 14 cc. The half escape times were obtained by plotting the per cent of solute remaining inside the membrane as a function of time on semilogarithmic graph paper. In each experiment, the optical density of an aliquot of the starting solution was determined in order to check the recovery of solute from the membrane. Only when the recovery was 95% or greater were the runs considered satisfactory.

The buffers used in the dialysis experiments were made with Baker's analyzed reagent; their compositions are shown in Table VII. They were 0.2 M Na^+ and had an ionic strength between 0.2 and 0.3 except for a few cases in which potassium acetate was used instead of sodium acetate (see Fig. 14). However, the rates of diffusion were found to be identical in potassium and sodium acetate buffers. The solutes used to calibrate the membrane were the following: horse heart ferricytochrome C from the Sigma Chemical Company; sperm whale myoglobin, a crystallized salt-free preparation from the Mann Research Laboratories; chymotrypsinogen, a crystallized salt-free preparation from the Worthington Biochemical Corporation; pepsinogen from the Worthington Biochemical Corporation (obtained from Dr. G. E. Perlmann); and bovine plasma albumin, a crystallized preparation from the Armour Laboratories.

E. Results

1. The relationship between the reactivity of the SH groups and the dissociation of hemoglobin.

In Part III, it has been shown that the reactive SH groups of human hemoglobin ($\alpha_2\beta_2$), in solutions of neutral pH and moderate ionic strength, are those of cysteine residues 93β. The other SH group on each

TABLE VII

The Composition of the Sodium Acetate and Sodium Phosphate Buffers

pH	$NaH_2PO_3 \cdot H_2O$ g/l	Na_2HPO_4 g/l	$Na_3PO_4 \cdot 12\ H_2O$ g/l	pH	Sodium acetate g/l	Acetic acid ml/l
4.38	27.7	-	-	3.20	27.2	(1)
4.90	26.2	0.7	-	4.40	27.2	(1)
6.10	22.1	-	4.95	4.71	27.2	11.4
7.15	11.0	-	15.2	4.98	27.2	4.5
8.20	8.1	-	17.8	5.95	27.2	0.4
9.10	-	14.2	-	7.00	27.2	-
10.10	-	14.2	1.0	8.00	27.2	(2)
10.85	5.5	-	23.0	9.60	27.2	(2)
11.54	2.75	-	23.0			

(1) Adjusted with 0.1 N HCl.

(2) Adjusted with 0.1 N NaOH.

β chain, that of cysteine residue 112β, and the SH group on each α chain, that of cysteine residue 104α, do not react under these conditions. This lack of reactivity, discussed in Part III, was attributed predominantly to the polymeric state of the hemoglobin molecule.

Under certain conditions, the unreactive SH groups can be made to react even without employing denaturing agents. Thus, R. Benesch and R. E. Benesch (1962) state that at low pH all the SH groups of human hemoglobin react with PCMB in 3 hours, while Cecil and Snow (1962a) found that even at neutral pH mercuric chloride and phenylmercuric hydroxide eventually did react with all the SH groups. These hemoglobin derivatives were, however, very unstable and precipitated out of solution on standing. Furthermore, since it was found (R. E. Benesch et al., 1961) that in hemoglobin H (β_4) all eight SH groups were reactive at neutral pH, it was suggested that the unreactivity of the SH groups in the normal molecule was due to interactions between the α and β chains. When these data are taken together, they suggest that the ability of all the SH groups in the chains to react might be taken as an indication of the presence of monomers.

The foregoing considerations, together with the possibility of producing hemoglobins with different chromatographic properties simply by reaction of the SH groups, (as described in Part III) immediately suggested a way of investigating the mechanism of dissociation of hemoglobin.

a. The equilibrium between tetramers and dimers at pH 6.4.- When CO hemoglobin is exposed at neutral pH simultaneously to NEM and INH_2, present in a ratio equal to that of their specific rate constants for the alkylation of CO hemoglobin, the reactive SH groups have an equal chance to react with either reagent. The resulting solution of CO hemoglobin should contain three species: a CO hemoglobin in which both reactive SH groups have reacted with NEM, $\alpha_2\beta_2^*$; one in which both SH groups have reacted with INH_2, $\alpha_2\beta_2^\dagger$; and one in which one SH group has reacted with NEM and one with INH_2, $\alpha_2\beta^*\beta^\dagger$. These derivatives should be present in a ratio of 1:1:2. The possibility of obtaining only the two pure species, $\alpha_2\beta_2^*$ and $\alpha_2\beta_2^\dagger$, cannot be completely excluded but is not probable.

When such a reaction mixture is analyzed by chromatography, as described in Part III, two situations are to be expected. In the absence of an equilibrium between tetramers and symmetrical dimers at the pH of the chromato-

graphic separation, pH 6.42, three components should be obtained: one at the position of NEM-reacted CO hemoglobin, $\alpha_2\beta_2$*; one at the position of INH_2-reacted CO hemoglobin, $\alpha_2\beta_2^{\dagger}$; and one at an intermediate position, $\alpha_2\beta^*\beta^{\dagger}$. If instead such an equilibrium exists, it follows from the revised hypothesis that only two peaks should be obtained representing the two pure species, $\alpha_2\beta_2$* and $\alpha_2\beta_2^{\dagger}$.

The chromatographic patterns and the results of the analyses of the cysteine derivatives of CO hemoglobins resulting from the reaction of CO hemoglobin with NEM and INH_2 at 25° for 1 hour are shown in Fig. 11 and Table VIII. Panels A, B, and C of Fig. 11 show the chromatographic patterns obtained for normal CO hemoglobin, for the CO hemoglobin reacted with NEM alone, and for the CO hemoglobin reacted with INH_2 alone, respectively. The first peak to emerge from the column contains the minor CO hemoglobin components of the red cell hemolysate and is of little interest here. The positions of the CO hemoglobins reacted with NEM and INH_2 alone, panels B and C, are the characteristic ones for these derivatives (see Part III). The patterns in panels D and E of Fig. 11 show that when CO hemoglobin is reacted simultaneously with the two reagents, present in a ratio close to that of their specific rate constants, only two peaks are obtained: one at the position of the CO hemoglobin reacted with NEM and one at the position of CO hemoglobin reacted with INH_2. The concentration of CO hemoglobin, the concentration of the reagents, the ratio of these quantities, the fraction of total CO hemoglobin under each peak, and the results from the analyses of the cysteine derivatives of the CO hemoglobins before and after chromatography are shown in the successive columns from left to right in Table VIII. The experiments A to E correspond to those shown in panels A to E in Fig. 11. It is clear from cases D and E that there is in fact complete separation of the doubly reacted CO hemoglobin into two pure species: $\alpha_2\beta_2$* and $\alpha_2\beta_2^{\dagger}$. The relative amounts of the two species are in agreement with the relative amounts of S-carboxymethylcysteine and S-succinylcysteine determined from the amino acid analyses of the unpurified hemoglobin.

These data provide direct evidence for the postulated equilibrium between tetramers and dimers

$$\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$$

at pH 6.42.

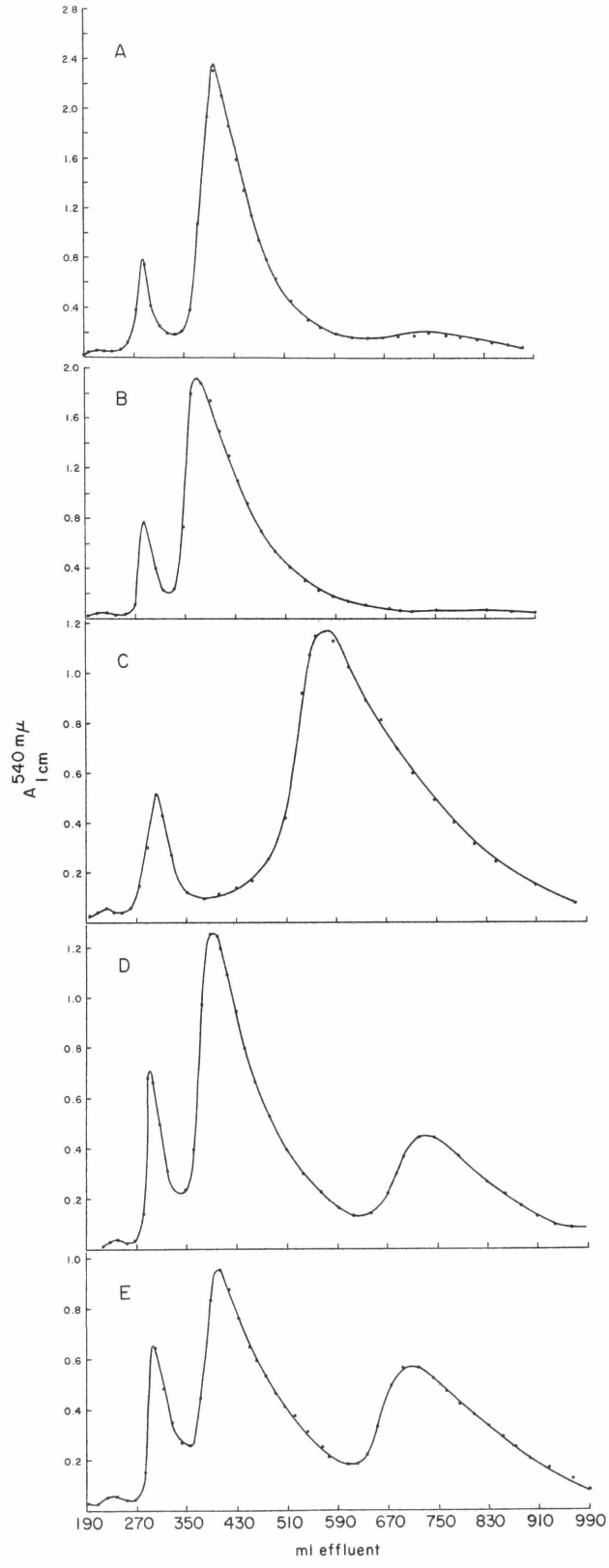


Fig. 11. Chromatography on Bio-Rex 70 of CO hemoglobins (350-400 mg). A: normal CO hemoglobin; B: CO hemoglobin reacted with NEM alone; C: CO hemoglobin reacted with INH_2 alone; D and E: CO hemoglobin reacted with INH_2 and NEM simultaneously. The reaction conditions were: CO hemoglobin 6×10^{-4} M; 1 hour at 25°C; the concentrations of INH_2 and NEM are shown in Table VIII.

TABLE VIII

Cysteine Derivatives from the Amino Acid Analyses of Doubly Reacted CO Hemoglobins

Experiment	NEM ($M \times 10^3$)	INH_2 ($M \times 10^3$)	$\frac{INH_2}{NEM}$	Fraction of Hb*	Residues per molecule of protein†		
					S-carboxymethyl-cysteine	S-succinyl-cysteine	Ethyl-amine
A							
Before chromatography					-	-	-
Peak 2	-	-	-	0.90	-	-	-
B							
Before chromatography					-	0.80	0.80
Peak 2	1.8	-	-	0.89	-	0.85	0.86
C							
Before chromatography					1.00	-	-
Peak 2	-	36	-	0.92	1.01	-	-
D							
Before chromatography					0.41	0.52	0.50
Peak 2				0.53	0.01	0.86	0.88
Peak 3	1.8	18	10	0.37	1.05	-	-
E							
Before chromatography					0.51	0.42	0.44
Peak 2				0.43	0.02	0.90	0.87
Peak 3	1.8	36	20	0.47	1.02	-	-

*Fraction of Hb refers to the fraction of all the CO hemoglobin recovered from the column, under the designated peak.

†The values are reported for an $\alpha\beta$ dimer.

Conditions: CO hemoglobin 6×10^{-4} M; 1 hour at pH 7.15 and 25°.

b. The equilibrium between tetramers and dimers at pH 4.7.- When the reaction of CO hemoglobin with INH_2 is carried out at pH 4.7, several results may be expected, depending on the mechanism of dissociation of the CO hemoglobin and considering the fact that there will be a slow rate of reaction. If the dissociation is asymmetrical, $\alpha_2\beta_2 \rightleftarrows \alpha_2 + \beta_2$, the β_2 subunit may resemble hemoglobin H (β_4); and all four SH groups of the β chains should be reactive though slowly. If instead the β_2 dimers behave as they do in the intact tetramer, $\alpha_2\beta_2$, only the two cysteine residues 93β will be available for reaction. In this case if the reaction is not carried to completion, three species will result: a normal dimer, β_2; a dimer in which only one SH group has reacted, $\beta\beta^\dagger$; and a dimer in which both SH groups have reacted, $\beta_2^\dagger$ (present in a ratio of 1:x:1, respectively). The value of x will depend on the rates of the successive alkylation reactions:

$$\beta_2 + INH_2 \xrightarrow{k_1} \beta\beta^\dagger + I^- + H^+$$

$$\beta\beta^\dagger + INH_2 \xrightarrow{k_2} \beta_2^\dagger + I^- + H^+$$

where k_1 and k_2 are the rate constants for the successive reactions. Thus, three components should be obtained on chromatography at pH 6.42 after recombination of the chains: namely, the species $\alpha_2\beta_2$, $\alpha_2\beta\beta^\dagger$, and $\alpha_2\beta_2^\dagger$.

On the other hand, if the dissociation is symmetrical, $\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$, the αβ unit would have only the cysteine residue 93β available for reaction. When the reaction is not carried to completion, here also three species would be obtained by recombination of the subunits at pH 6.42: $\alpha_2\beta_2$, $\alpha_2\beta\beta^\dagger$, and $\alpha_2\beta_2^\dagger$; but they would separate into the two pure species $\alpha_2\beta_2$ and $\alpha_2\beta_2^\dagger$ by by chromatography as demonstrated in the preceding subsection.

Table IX shows the results from the amino acid analysis performed on the globin and on the separated α and β chains of CO hemoglobins reacted for 6 and 17 hours with INH_2 at pH 4.7 and then for 1 hour with NEM at pH 7.15. The fact that the sum of the residues of S-carboxymethylcysteine and S-succinylcysteine is close to one and that the S-carboxymethylcysteine is found in the β chain indicates that only residue (cysteine) 93β is reactive under these conditions. This result excludes the possibility that in the β_2 subunit all four SH groups are equally reactive.

TABLE IX

Cysteine Derivatives from the Amino Acid Analyses of CO Hemoglobins Reacted at pH 4.7 with INH_2, Then at pH 7.15 with NEM

CO hemoglobin derivative	Residues per molecule of protein			
	S-carboxymethyl-cysteine	S-succinyl-cysteine	Ethyl-amine	Sum
CO hemoglobin reacted with INH_2 for 6 hrs. at pH 4.7, then with NEM at pH 7.15 for 1 hr.				
globin[1]	0.68	0.40	0.38	1.08
CO hemoglobin reacted with INH_2 for 16 hrs. at pH 4.7, then with NEM at pH 7.15 for 1 hr.				
globin[1]	1.05	-	-	1.05
α chain	-	-	-	-
β chain	1.02	-	-	1.02

[1]The globin values are reported for an $\alpha\beta$ dimer.

Conditions: CO hemoglobin 6×10^{-4} M; INH_2 3.6×10^{-2} M; NEM 1.8×10^{-3} M; 25°.

Fig. 12 shows the chromatographic patterns on the left and the separation of the chains by countercurrent distribution on the right for normal CO hemoglobin (top panel), CO hemoglobin reacted at pH 7.15 for 1 hour with INH_2 (middle panel), and hemoglobin reacted at pH 4.7 for 6 hours with INH_2 (lower panel). In the latter case, the countercurrent distribution pattern (shown only for peak 3) is similar to that for the hemoglobin reacted at pH 7.15 shown in the middle panel. The results from the amino acid analyses performed on the CO hemoglobin reacted at pH 4.7 before and after chromatography and on the separated chains are shown in Table X. The results shown in the lower panel of Fig. 12 demonstrate that only two major peaks are obtained for the partially alkylated CO hemoglobin; one at the position of normal CO hemoglobin and one at the position of totally alkylated CO hemoglobin. The latter contains one residue of S-carboxymethylcysteine per $\alpha\beta$ unit, the former none.

This result provides convincing evidence for the hypothesis that the dissociation of hemoglobin is symmetrical at acid pH:

$$\alpha_2\beta_2 \rightleftarrows 2\alpha\beta$$

c. The equilibrium between dimers and monomers at pH 4.7.- The results presented above are strongly in favor of the hypothesis of symmetrical dissociation of hemoglobin. Thus, the only way to explain the hybridization data is to accept the Vinograd and Hutchinson hypothesis of an equilibrium between dimers and monomers at low and high pH's: $\alpha\beta \rightleftarrows \alpha + \beta$. Necessarily, the concentrations of the monomers must be low (10% or less of the total hemoglobin concentration) in order to be consistent with the observed molecular weights at these pH's and with the rate of hybridization.

In the monomers, all the SH groups should be reactive if truly the differential reactivity of these groups is controlled by the interactions between the α and β chains. The presence of the monomers should thus be easily detected by exposing hemoglobin at pH 4.7 to NEM, since this compound reacts with SH groups at a rate much faster than that of INH_2 at this pH.

Fig. 13 shows the chromatographic patterns on the left and the separation of the chains by countercurrent distribution on the right for normal CO hemoglobin (top panel), CO hemoglobin reacted at pH 7.15 for 1 hour with NEM (middle panel), and CO hemoglobin reacted at pH 4.7 for 6 hours with

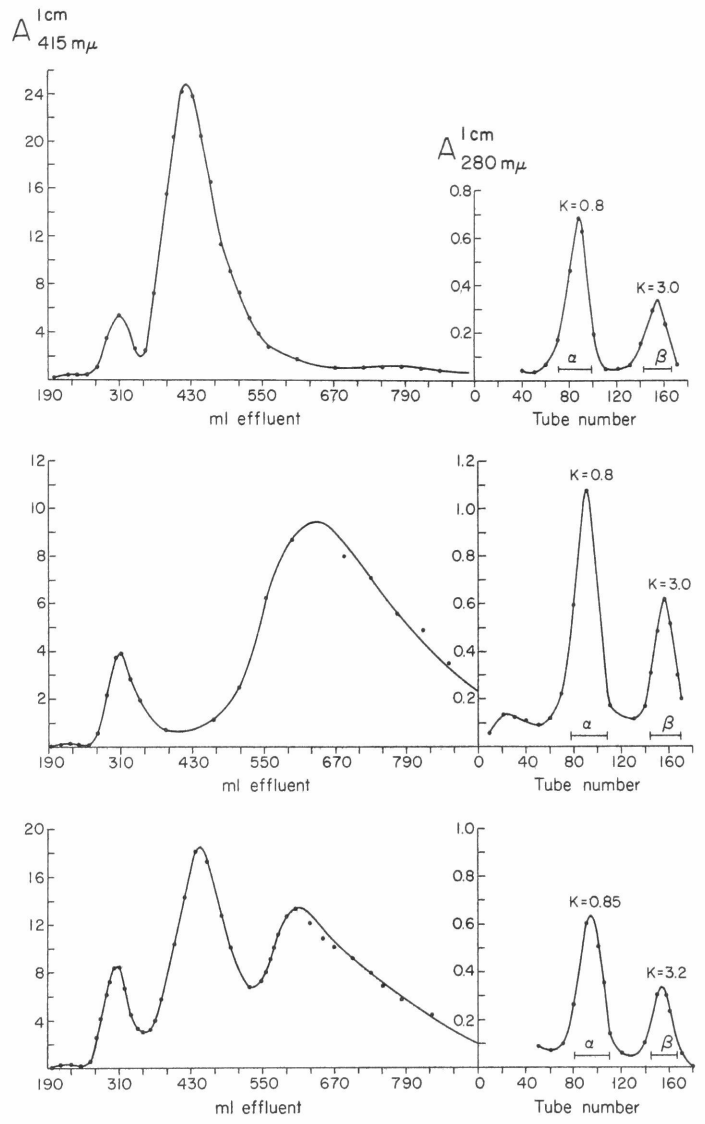


Fig. 12. Chromatography on Bio-Rex 70 (left side) and countercurrent distribution (right side) of normal CO hemoglobin, 350 mg, (top panel), CO hemoglobin reacted with INH_2 at pH 7.15, 350 mg, (middle panel), and CO hemoglobin reacted with INH_2 at pH 4.7, 700 mg, (bottom panel). The reaction conditions were: CO hemoglobin 6×10^{-4} M; INH_2 3.6×10^{-2} M; 1 hour at 25°C and pH 7.15; 6 hours at 25°C and pH 4.7. Countercurrent distribution of peak 2 for the top and middle panels and of peak 3 for the bottom panel: 200 transfers, 3 ml each phase; system: sec-butanol, 9; 0.5 M acetic acid, 10; 10% (v/v) dichloroacetic acid, 1. Only the concentrations in the lower phase are shown.

TABLE X

Cysteine Derivatives from the Amino Acid Analyses of CO Hemoglobin Reacted at pH 4.7 with INH_2

CO hemoglobin derivative	Residues per molecule of protein S-carboxymethylcysteine
Before chromatography	
globin[1]	0.65
Peak 2	
globin[1]	-
α chain	-
β chain	-
Peak 3	
globin[1]	1.02
α chain	-
β chain	0.98

[1]The globin values are reported for an αβ dimer.

Conditions: CO hemoglobin 6×10^{-4} M; INH_2 3.6×10^{-2}; 6 hours at 25°.

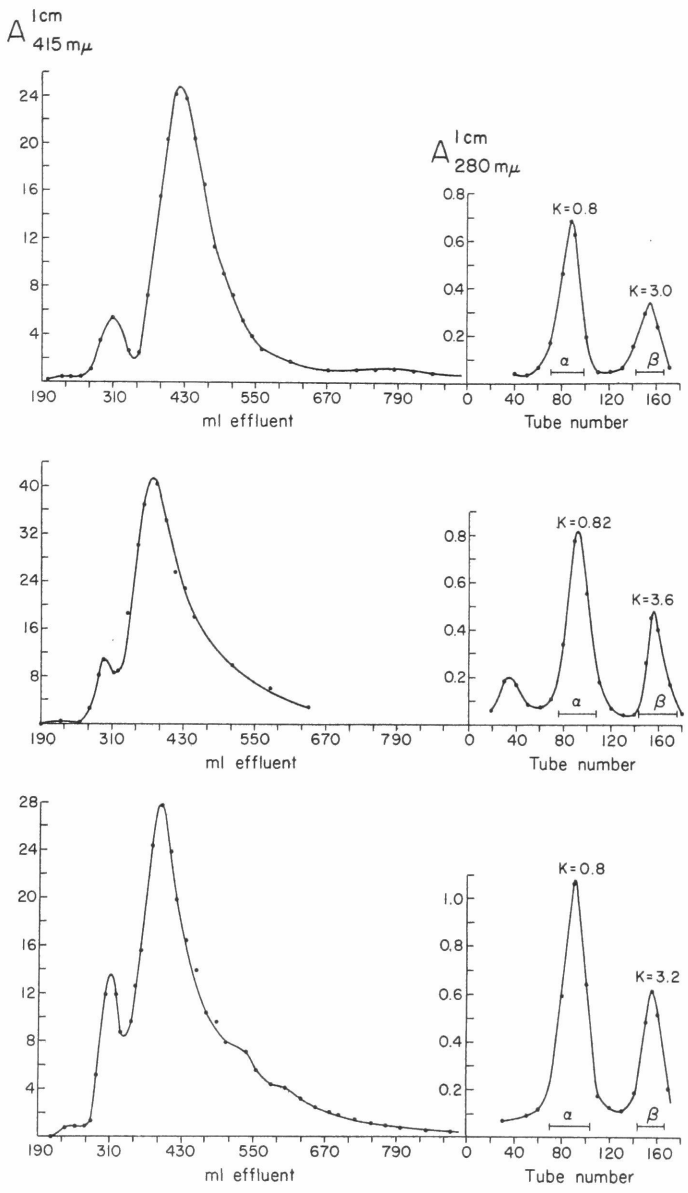


Fig. 13. Chromatography on Bio-Rex 70 (left side) and countercurrent distribution (right side) of normal CO hemoglobin, 350 mg, (top panel), CO hemoglobin reacted with NEM at pH 7.15, 700 mg, (middle panel), and CO hemoglobin reacted with NEM at pH 4.7, 400 mg, (bottom panel). The reaction conditions were: CO hemoglobin 6×10^{-4} M; NEM 1.8×10^{-3} M at pH 7.15, 1 hour at 25°C; NEM 3.6×10^{-2} M at pH 4.7, 6 hours at 25°C. Countercurrent distribution of peak 2: 200 transfers, 3 ml each phase; system: sec-butanol, 9; 0.5 M acetic acid, 10; 10% (v/v) dichloroacetic acid, 1. Only the concentrations in the lower phase are shown.

NEM (lower panel). In the latter case, there is some evidence of inhomogeneity of the main chromatographic peak, attributable to the instability of this CO hemoglobin derivative. The countercurrent distribution pattern for the pH 4.7 hemoglobin is similar to that for the hemoglobin reacted at pH 7.15.

Table XI shows the results from the amino acid analyses performed on CO hemoglobins reacted for 6 hours and for 17 hours at pH 4.7 with NEM and on their separated α and β chains. These data show that there are two rates of reaction: the fast rate involves one SH group, that of cysteine residue 93β; and the slow one relates to the unreactive cysteine residues on the α and β chains. After 17 hours, nearly all the cysteine residues have reacted. However, this hemoglobin derivative is very unstable and precipitated out of solution on standing at 5° for 1 week.

This result is consistent with the equilibrium between dimers and monomers at pH 4.7:

$$\alpha\beta \rightleftarrows \alpha + \beta$$

d. The dissociation equilibria in strong salt solutions.- Hybridization does not take place in strong salt solutions of neutral pH (Antonini et al., 1962b). It follows from the arguments developed so far that an appreciable concentration of α and β chain monomers should not exist. Therefore, providing the conformation of the various subunits is not drastically altered by solutions of high ionic strength, only the cysteine residue 93β should be able to react with either NEM or INH_2 under these conditions at neutral pH. At acid pH, however, it was expected that the same situation would obtain as in the absence of strong salt solutions.

Table XII shows the results from the amino acid analyses performed on CO hemoglobins reacted with INH_2 and NEM for 6 hours at pH 4.7 and pH 7.15 in 2 M NaCl and on the separated chains. In all cases, at both pH's, only cysteine residue 93β has reacted, indicating that monomers are not present under these conditions.

This result agrees well with the known lack of hybridization in strong salt solutions at neutral pH. It is, however, striking that the dissociation to monomers which occurs at acid pH is suppressed by solutions of high ionic strength.

TABLE XI

Cysteine Derivatives from the Amino Acid Analyses of CO Hemoglobins Reacted with NEM at pH 4.7

CO hemoglobin derivative	Residues per molecule of protein	
	S-succinylcysteine	Ethylamine
CO hemoglobin reacted for 6 hrs.		
globin[1]	1.82	1.95
α chain	0.45	0.50
β chain	1.33	1.48
CO hemoglobin reacted for 17 hrs.		
globin[1]	2.53	2.71
α chain	0.82	0.95
β chain	1.67	1.72

[1]The globin values are reported for an αβ dimer. Only the results of the analyses done before chromatography of the CO hemoglobins are shown.

Conditions: CO hemoglobin 6×10^{-4} M; NEM 3.6×10^{-2} M; 25°.

TABLE XII

Cysteine Derivatives from the Amino Acid Analyses of CO Hemoglobins Reacted in 2 M NaCl

CO hemoglobin derivative	Residues per molecule of protein		
	S-carboxymethylcysteine	S-succinylcysteine	Ethylamine
INH_2-reacted CO hemoglobin			
A pH 7.15			
globin[1]	0.95		
α chain	-		
β chain	0.91		
B pH 4.7			
globin[1]	0.51		
α chain	-		
β chain	0.55		
NEM-reacted CO hemoglobin			
A pH 7.15			
globin[1]		0.90	-
α chain		-	-
β chain		0.81	-
B pH 4.7			
globin[1]		0.89	1.17
α chain		-	-
β chain		0.85	1.05

[1]The globin values are reported for an αβ dimer.

Conditions: CO hemoglobin 6×10^{-4} M; INH_2 3.6×10^{-2} M; NEM 3.6×10^{-2} M; 6 hours at 25°.

2. The restricted diffusion of hemoglobin.

Craig and King (1962) have shown that the diffusion of a pure solute through a cellophane membrane is a first-order process in which the rate of diffusion depends upon the permeability of the membrane and the type of solvent. This behavior has been observed with a variety of solutes and has been used to study the purity and the conformational changes of the solute as a function of the ionic strength, pH, and type of solvent. The advantage of this technique stems from the fact that membranes of graded pore sizes can be obtained by altering the membrane by stretching, inflating, acetylation, or treatment with $ZnCl_2$ (Craig and King, 1962). Thus, membranes can be prepared which will allow the desired solute to escape with a half escape time between 6 and 12 hours. In this range, the diffusion technique can discriminate between closely related solutes having small differences in size and shape. In any case, the differentiation of a solute from another twice its size is very striking. The membrane is calibrated by measuring its permeability with several solutes of known molecular sizes and then used to measure the molecular size of an unknown solute.

In the case of a solute which dissociates reversibly into subunits, this type of diffusion (contrary to that of a process operating under equilibrium conditions) appears to emphasize the effect of the smallest subunits present in the system; since their rate of diffusion will be much faster than that of the associated solute. Thus, by choosing a membrane of the right pore size, such that the associated solute would be almost totally excluded but the subunits would diffuse rapidly, definite evidence for the presence of subunits can be obtained. The analysis of the resulting diffusion curves is of course limited, since it involves the solution of non-linear differential equations. Only in limiting cases can values for the equilibrium constants of the association-dissociation reactions be obtained.

Fig. 14 shows the type of curves obtained for the diffusion of chymotrypsinogen and of CO hemoglobin in several solvents. (KAc stands for potassium acetate.) The ordinate shows the percentage of the solute remaining inside the membrane, and the time is shown on the abscissa. In the case of CO hemoglobin, the diffusion curves do not deviate far from first-order kinetics at least to the point where 50% of the solute has escaped from the inside of the membrane; since this covers only a two-fold change in

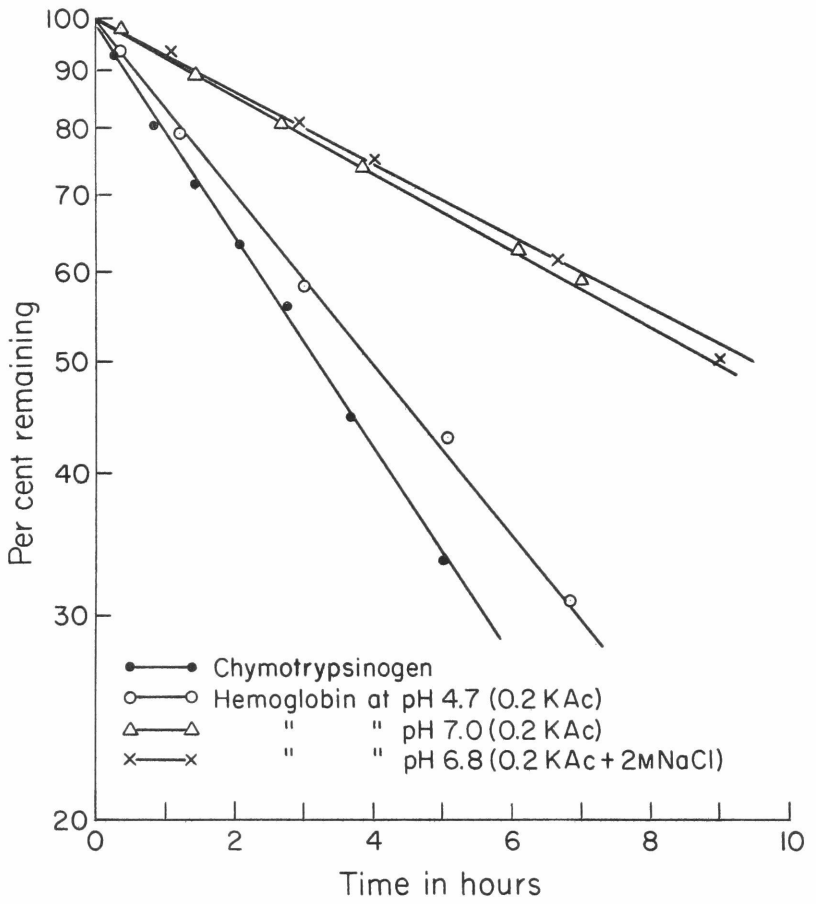


Fig. 14. The rates of diffusion of chymotrypsinogen and of CO hemoglobin.

concentration. This behavior obtains in all the diffusion studies on CO hemoglobin; and, therefore, the data are reported in half escape times. For a first-order reaction, the half escape time of the process is inversely related to the specific rate constant.

Table XIII shows the molecular weights and the half escape times of the solutes used to calibrate the membrane and the effect of solvents on the half escape times. Under the conditions used, the ionic strength or pH of the solvents has little effect on the rate of diffusion of these solutes.

Table XIV shows the half escape times for CO hemoglobin at a concentration of 0.3 to 0.4% as a function of pH in sodium acetate and sodium phosphate buffers, both 0.2 M Na^+ and of ionic strength 0.2 to 0.3. The highest half escape time for CO hemoglobin in the phosphate buffer is 8.0 hours, which by comparison with the data in Table XIII corresponds to that for a solute of molecular size 40,000. Thus, CO hemoglobin at this concentration is diffusing at a rate faster than would be expected for pure tetramer. As the pH is decreased below pH 6 or increased above pH 10, the half escape time decreases progressively to the value of 4 hours, which is that to be expected for a solute of molecular size 30,000. Thus, the hemoglobin tetramer is completely dissociated into dimers at these limits of pH. The pH profile shown in Fig. 15 is very similar to that obtained for the sedimentation coefficients (Hasserodt and Vinograd, 1959; Field and O'Brien, 1955). The dip which is seen at pH 8 was unexpected but consistently reproducible. It probably represents some type of conformational change in the molecular architecture of the tetramer or the dimers. In sodium acetate buffers, however, the situation is quite different from that in phosphate buffer. At pH 4.7 and below, there is evidence of further dissociation to monomers; and at pH above 7, there is evidence of a greater shift of the equilibrium in favor of the tetramer or of a striking conformational shift.

The difference in behavior of CO hemoglobin in these two buffers, at low and high pH, is difficult to explain, except as a manifestation of a specific ion effect on the conformation. Similar effects have, of course, been noticed on the oxygenation properties of hemoglobin and have been in fact attributed to the degree of association of the subunits (Sidwell et al., 1938; Altschul and Hogness, 1939).

Since the molecule of CO hemoglobin in solution shows definite evidence

TABLE XIII

The Half Escape Times of the Solutes Used to Calibrate the Membrane

Solute	M.W.	Initial conc. g %	Half escape times in hours		
			0.2 M sodium acetate, pH 7	0.2 M sodium acetate, 2 M NaCl, pH 7	0.2 M sodium acetate, 0.2 M acetic acid, pH 4.7
Ferricyto-chrome C	12,000(1)	0.4	0.95	1.1	-
Myoglobin	17,816(2)	0.4	1.25	1.4	1.6
Chymotrypsinogen	25,100(3)	0.7	3.2	3.8	3.0
Pepsinogen	40,000(4)	0.5	8.5	-	-
Albumin (bovine plasma)	66,000(5)	0.8	18-20	18-20	> 22

(1) Margoliash et al., 1961.

(2) Edmundson and Hirs, 1962.

(3) Desnuelle and Rovery, 1961.

(4) Perlmann, 1962.

(5) Edsall, 1953.

TABLE XIV

The pH Dependence of the Membrane Diffusion of CO Hemoglobin[1]

pH	Sodium acetate buffers[2] Half escape time in hours	pH	Sodium phosphate buffers[2] Half escape time in hours
3.20	2.5		
4.40	3.5	4.38	4.8
4.71	3.8		
4.98	4.0	4.90	5.6
5.95	7.5	6.10	7.5
7.00	7.5	7.15	7.8
8.00	9.5	8.20	6.2
9.60	10.5	9.10	7.5
		10.10	7.5
		10.85	3.9
		11.54	3.7

[1]Concentration: 0.3-0.4 g %.

[2]See Table VII for composition.

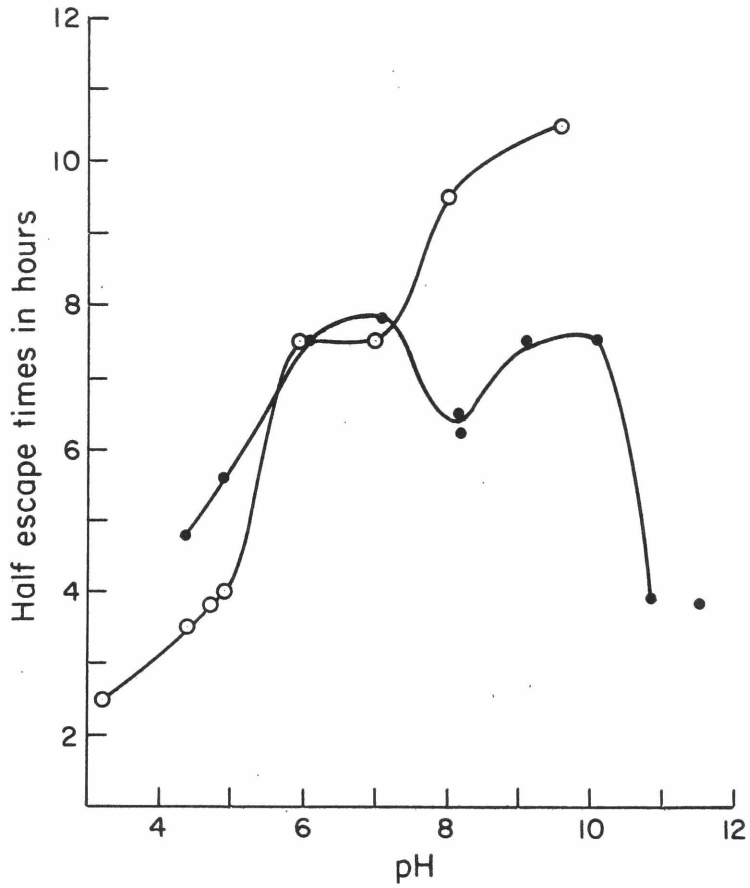


Fig. 15. The pH dependence of the half escape times of CO hemoglobin. o——o , 0.3-0.4% CO hemoglobin in sodium acetate buffers; ●——● , 0.3-0.4% CO hemoglobin in sodium phosphate buffers. Both solvents were 0.2 M in Na^+ and had an ionic strength of 0.2-0.3.

of dissociation at all pH's, the law of mass action demands that the rates of diffusion be concentration dependent. The two-fold concentration range covered in a single escape pattern might not be sufficient. Therefore, a wider concentration range was investigated.

Table XV shows the half escape times for CO hemoglobin in the sodium phosphate and the sodium acetate buffers at pH's 7 and 4.7 and in these buffers also 2.0 M in NaCl. The concentration range of the CO hemoglobin is 3.78 to 0.03%. In all cases, the rate of diffusion is dependent on the hemoglobin concentration. In the sodium phosphate buffer of neutral pH and moderate ionic strength, the observed half escape time of about 2.5 hours at a hemoglobin concentration of 0.03% is a clear indication of the presence of monomers. When very low hemoglobin concentrations are used with the sodium acetate buffer of acid pH and moderate ionic strength, the half escape time approaches that found with myoglobin.

The most striking results are those obtained with the solvents 2.0 M in NaCl. Here, the rates of diffusion are slower than those found with solvents of moderate ionic strength and are independent of pH. At the lowest hemoglobin concentration, the rates of diffusion approach that for a pure dimer without further decrease. Thus, the molecule of CO hemoglobin appears not be be dissociated beyond the dimer stage in strong salt solution.

Table XVI shows the values obtained for the dissociation constants K_1 and K_2 for CO hemoglobin in the various solvents. Necessarily, these values for the constants K_1 and K_2 are only of limited accuracy, because many assumptions were made in order to simplify the problem of their derivation. However, they are most likely of the right order of magnitude (for the derivation, see Appendix).

E. Discussion

The method of thin film dialysis, which incidentally has been used to separate the α and γ chains of fetal globin (Matsuda et al., 1961), presents several distinct advantages. Not only does it allow measurements to be made at low solute concentrations, but it emphasizes the effect of the smallest molecules present in the system. Thus, in the case of a dissociating solute, it can detect even very small concentrations of the subunits which would go undetected by other methods. Furthermore, in contrast to diffusion in gels in which the shape of the boundary leads to the detection of a

TABLE XV

The Concentration Dependence of the Membrane Diffusion of CO Hemoglobin

Initial conc. g %	Half escape time in hours			
	0.2 M sodium phosphate pH 7.15	0.2 M sodium phosphate, 2 M NaCl pH 7.15	0.2 M sodium acetate 0.2 M acetic acid pH 4.7	0.2 M sodium acetate 0.2 M acetic acid 2 M NaCl pH 4.7
3.78	8.4	9.5	6.5	
0.77		9.3	4.3	
0.42	7.8			
0.32		8.5	3.8	9.2
0.15	6.25			8.4
0.11		6.8		
0.09	4.5	5.8	3.0	6.2
0.035	2.75	5.2	1.75	

TABLE XVI

The Values of the Dissociation Constants K_1 and K_2.

Constant (M)	0.2 M sodium phosphate pH 7.15	0.2 M sodium phosphate, 2 M NaCl pH 7.15	0.2 M sodium acetate 0.2 M acetic acid pH 4.7	0.2 M sodium acetate 0.2 M acetic acid 2 M NaCl pH 4.7
K_1	1×10^{-5}	1.36×10^{-5}	1×10^{-4}	1×10^{-5}
K_2	1×10^{-6}	$< 10^{-6}$	2×10^{-6}	$< 10^{-6}$

dissociating solute (Schantz and Lauffer, 1962), in membrane diffusion the shape of the boundary in the membrane is in most cases of little consequence due to the thinness of the membrane. For this reason, the rate of diffusion alone, measured by the appearance of solute on the outside of the membrane, is sufficient to indicate the size of the diffusing solute.

The diffusion data described above are in agreement with several results in the literature. Thus, the concentration dependence of the sedimentation coefficient (Pedersen, in Lamm and Polson, 1936) and of the diffusion coefficient (Lamm and Polson, 1936) of CO hemoglobin in solutions of neutral pH and moderate ionic strength have been reported. The concentration dependence of the molecular weight by light scattering and of the diffusion and sedimentation coefficients of oxyhemoglobin in strong salt solutions (Benhamou et al., 1960) are of more recent origin. In all cases, the effects of concentration became apparent below a hemoglobin concentration of 0.2% and are in agreement with the results given here. On the other hand, there are several conflicting data bearing on this matter. Several investigators (Kegeles and Gutter, 1951; Schumaker and Schachman, 1957; Rossi-Fanelli et al., 1961a; Benhamou et al., 1960) have found that the sedimentation coefficient of both CO hemoglobin and oxyhemoglobin increased rather than decreased even at very low hemoglobin concentration (0.005%) in solutions of neutral pH and moderate ionic strength. Most perplexing are the results of Rossi-Fanelli et al. (1961a) and of R. E. Benesch et al.(1962) who found no concentration dependence for the molecular weights of oxy- and aquohemoglobin in strong salt solutions by ultracentrifugal and light scattering methods, in spite of the fact that the reported values (45,000) themselves indicated partial dissociation of the molecule. The reasons for these disagreements are not easily explained, but they may be related to the difficulties attending the physical measurements at very low solute concentrations and in strong salt solutions.

The results presented here provide convincing evidence that the revised model of dissociation

$$\alpha_2\beta_2 \overset{K_1}{\rightleftharpoons} 2\alpha\beta \overset{K_2}{\rightleftharpoons} 2\alpha + 2\beta$$

represents the actual mechanism. The values of the dissociation constants K_1 and K_2 reach a minimum in the pH range 7 to 9 and increase with increasing

and decreasing pH in solutions of moderate ionic strength. In strong salt solutions, the constant K_1 is not altered, but the constant K_2 is decreased and both constants are insensitive to pH changes from acid to neutral pH. Although the data apply only to hemoglobin in one form (CO hemoglobin), it is extremely likely that the behavior of the other forms of human hemoglobin (aquo-, oxy-, ferrihemoglobin) is similar but not necessarily identical, for the values of the constants K_1 and K_2 can certainly be expected to vary with the various forms.

The pH dependence of the constants K_1 and K_2 might suggest which groups are involved in holding the subunits together. However, the nature of the forces involved in molecular interactions is extremely complicated. These interactions probably result from combinations of electrostatic forces, hydrogen bonds, Van der Waals forces, and hydrophobic bonds. Thus, while the effects of pH on the interactions are most easily explained in terms of electrostatic forces, there is no way to exclude that in fact only the conformation of the chains is altered by pH and that the interchain bonds are hydrophobic ones. If, however, electrostatic forces were the main ones concerned in interchain bonds, then the equilibrium between the tetrameric structure and the dimeric one would appear to be governed by imidazole groups and that between dimer and monomer by carboxyl and by α- and ε -amino groups.

This revised model for the mechanism of dissociation of hemoglobin provides an unequivocal means of explaining the recombination and hybridization data in the literature. Thus, the difference in the rates of hybridization of various human (Jones et al., 1959b; Huehns and Shooter, 1961) and animal (Itano and Robinson, 1959) hemoglobins, the lack of hybridization in strong salt solutions at neutral pH (Antonini et al., 1962b), the strict pH dependence for hybridization (Antonini et al., 1962b) are all related to the values of the dissociation constants K_1 and K_2 and to their dependence on pH and ionic strength. The difference in the alkaline denaturation rates of adult and fetal hemoglobins is most easily explained by assuming that it is the monomers of the chains that denature and that in the reaction

$$\alpha\beta \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \alpha + \beta \xrightarrow{k_3} \text{denatured } (\alpha + \beta)$$

$$\alpha\gamma \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \alpha + \gamma \xrightarrow{k_3} \text{denatured } (\alpha + \gamma)$$

k_1 is greater than k_4. Furthermore, according to this model, hybridization should not take place in strong salt solutions even at acid pH. It has been recently shown (Herner and Riggs, 1963) that this is indeed the case.

With regard to the oxygenation of hemoglobin in strong salt solutions, the present data, which show that CO hemoglobin does not dissociate into subunits under these conditions except at very low concentration, are in better agreement with the observed values of n in the Hill equation (Rossi-Fanelli et al., 1961b) than the data of Rossi-Fanelli et al. (1961a) and of R. E. Benesch et al. (1962).

Nevertheless, there are some data which are not explained by this revised model. The separation of a partially oxidized solution of CO hemoglobin into five components, representing all the intermediate species, by electrophoresis (Itano and Robinson, 1956) and by chromatography (Morrison, 1957; Huisman et al., 1958) is not compatible with the revised hypothesis which predicts that only three species should be obtained: the one in which all the iron atoms are in the ferrous state, the one in which two are in the ferrous and two in the ferric state, and the one with all the iron atoms in the ferric state. This separation of the five components is not indisputable, however, because the resolution of the boundaries on electrophoresis is poor, the spectra of the isolated components show anomalies (Huisman et al., 1958), and because of the known exchange of hemes between hemoproteins (Rossi-Fanelli and Antonini, 1960; Banerjee, 1962) and the exchange of electrons in oxidation-reduction systems (Sutin, 1962). More difficult to explain is the chromatographic isolation by Schroeder et al. (1962b) of a fetal hemoglobin, F_I, in which only one γ chain is acetylated at the NH_2-terminal end. It remains to be seen, however, whether the other γ chain does not also have one less positive charge at the pH of the separation procedure.

In any case, a principal point of the present observations is that hemoglobin in all cases should be considered as a solute in rapid association-dissociation equilibrium.

PART V

General Discussion

Forty years ago, one scarcely knew the molecular weight of a single protein. Today, the chemist has at his disposal a vast knowledge concerning many proteins by which he can identify each one in terms of its composition and sequence of amino acids and its gross and detailed size and shape. Nevertheless, the relationship between these features and the function of the molecule is still poorly understood.

One of the reasons for this elusive quality of these macromolecules is related to the fact that our knowledge is necessarily confined to certain fixed states of the molecules. Thus, one may study the primary structure of a protein but then not know how this specifies the tertiary structure; or one may study the tertiary structure of certain functional end states but not know how this varies in the process of going from one state to the other. The specific function of proteins may well be related to their ability to undergo even small conformational changes during the performance of the function. In any case, it does not appear justifiable to consider a protein molecule, even composed of a single polypeptide chain, as a fixed unit with no conformational degrees of freedom. This problem is accentuated in macromolecules like hemoglobin which are made up of non-covalently linked subunits.

The work in this dissertation is concerned with the conformational changes of the hemoglobin molecule attending chemical alteration of the sulfhydryl groups and as a function of the solvent conditions. It was demonstrated that under all conditions hemoglobin in solution is to be considered as a system whose polypeptide chains are in dynamic equilibrium.

This feature of hemoglobin, although realized, has received scant emphasis, especially in studies of its function under physiological conditions. The view that the normal functioning molecule is always composed of four polypeptide chains has been strengthened by the many observations on the molecular weight of hemoglobin and by the three-dimensional structure obtained from the X-ray diffraction patterns of crystals. However, no theory has successfully explained the peculiar oxygenation properties of hemoglobin using the tetrameric model of the molecule (the Adair hypothesis).

With this regard, the implications of the dynamic properties of the hemoglobin system are of considerable interest. It may be considered that all the forms of hemoglobin (tetramers, dimers, monomers) react with oxygen

but that the affinity is greatest for the monomers and least for the tetramers. Since during the process of oxygenation the concentration of the aquohemoglobin crosses the entire concentration range, the relative amounts of tetramer, dimer, and monomer will change at each step. The sigmoid oxygenation curve and the heme-heme interactions would, therefore, depend on the values of the tetramer-dimer-monomer dissociation constants for the aquohemoglobin, on those for the fully oxygenated hemoglobin and for the various intermediate forms, and on the values of the association constants for the reactions of all the various forms with oxygen. The oxygenation would then proceed by a funnelling of the tetramers of aquohemoglobin to the monomers, which have the highest affinity with oxygen and which then reassociate to tetramers. Any chemical alteration of the primary structure of the chains, reflected in changes in the tertiary structure, would be manifested by changes in the values of the tetramer-dimer-monomer dissociation constants and thus be expected to alter the oxygenation parameters.

This mechanism implies that in the tetramer the heme groups are truly sterically hindered by interactions between the chains, in contrast to what appears from the X-ray crystallographic data. It also requires a marked transient configurational change in nearly all the molecules during oxygenation and that the oxygen affinity of hemoglobin be concentration dependent. There is some evidence which is very suggestive with regard to these points. Thus, while oxyhemoglobin in solution will rapidly deoxygenate as the partial pressure of oxygen is decreased, the value of the rate constant for this reaction being approximately 20 sec^{-1} at 15° (Roughton et al., 1949), the oxyhemoglobin crystal will lose oxygen only very slowly (Granick, 1942). Admittedly, this phenomenon may depend on the decreased competition for oxygen between the "frozen" hemoglobin molecules or on the decreased rates of diffusion of oxygen and water in the crystal. In any case, it is a striking demonstration of the difference between hemoglobin in solution and in the crystalline form. Configurational changes during the process of oxygenation are clearly indicated by the studies of the dielectric increment and dispersion and of the viscosity (see Part II, E. 1.). Finally, Roughton et al. (1955) have shown that the oxygen affinity of hemoglobin is greater for dilute hemoglobin solutions (0.3%) than for concentrated ones (3%).

Clearly, however, before proposing this hypothesis as the actual mechanisms of oxygenation, the values for the tetramer-dimer-monomer dissociation constants at every step during the oxygenation will have to be obtained. This will not be an easy task.

The behavior of hemoglobin as a system in dynamic equilibrium is not a unique feature of mammalian hemoglobin or of hemoproteins alone. Thus, lamprey hemoglobin (Briehl, 1963), Gastrophilus erythrocruorin (Keilin and Wang, 1946), and Spirographis chlorocruorin (Fox, 1932) exhibit oxygenation parameters compatible with the hypothesis that the molecules are in equilibrium with their subunits. The example furnished by the Spirographis chlorocruorin, which is a molecule containing 190 iron atoms but with an oxygenation curve very similar to human hemoglobin, is especially striking. The changes in the molecular weight of hemoglobin during anuran metamorphosis (Trader et al., 1963) are a further example of these interacting systems. The enzyme, glutamic dehydrogenase, which has variable enzymatic activity depending on cofactor-mediated aggregation of the subunits is a closely related system (Frieden, 1963). In effect, the entire problem of enzymatic action and control of enzymatic action by product inhibition is similar to the hemoglobin problem, for in the final analysis it is very likely related to conformational changes in the protein molecule.

From these considerations, there arises a fascinating picture for the hemoglobin molecule. Starting from a myoglobin-like molecule, which existed as the monomer, the metabolic needs of vertebrates up to man specified, by selection, the alteration of the original hemoprotein into two different polypeptide chains. These chains had the right configuration to associate with each other into a pair and each pair with another pair. This combination of four chains is not static, however, but is continuously being broken down to individual chains and being built up again. Although the chains have a greater affinity for oxygen as monomers than as members of a tetrameric group, they exist most of the time as tetramers in order to satisfy the demands of the tissues they supply. Thus, by a complex set of rapidly reversible equilibrium reactions, hemoglobin fulfills its task.

PART VI

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Appendix

The observation that the membrane diffusion of hemoglobin, a rapidly dissociating solute, follows first-order kinetics can be reconciled with theoretical expectations in the following manner.

CO hemoglobin in solution may be considered to be a system of monomers-dimers-tetramers in equilibrium. Let (H) represent the concentration of hemoglobin (M.W. 64,500) in moles per liter; η and ρ the fraction of hemoglobin (M.W. 64,500) present as the dimer and the monomers, respectively. Then:

$$\underset{(H)(1-\eta-\rho)}{\alpha_2\beta_2} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(H)2\eta}{2\alpha\beta} \tag{1}$$

$$\underset{(H)2\eta}{\alpha\beta} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \underset{(H)2\rho}{\alpha} + \underset{(H)2\rho}{\beta} \tag{2}$$

$$K_1 = \frac{k_1}{k_2} = (H)\frac{4\eta^2}{(1-\eta-\rho)} \tag{3}$$

$$K_2 = \frac{k_3}{k_4} = (H)\frac{4\rho^2}{2\eta} \tag{4}$$

where K_1 and K_2 are the dissociation constants.

The process of diffusion through the membrane can be represented, for the described boundary conditions, by the irreversible reactions:

$$\begin{aligned} A_4 &\xrightarrow{k_t} B_4 \\ A_2 &\xrightarrow{k_d} B_2 \\ A &\xrightarrow{k_m} B \end{aligned} \tag{5}$$

where k_t, k_d, and k_m are the rate constants for the diffusion of pure tetramer, dimer, and monomer, respectively; the B_n and A_n represent the species outside and inside the membrane, respectively.

The differential equations describing the diffusion are:

$$\frac{d(B_4)}{dt} = k_t(A_4)$$

$$\frac{d(B_2)}{dt} = k_d(A_2) \qquad (6)$$

$$\frac{d(B)}{dt} = k_m(A)$$

$$\frac{d(A_4)}{dt} = -k_1(A_4) + k_2(A_2)^2 - k_t(A_4)$$

$$\frac{d(A_2)}{dt} = k_1(A_4) - k_2(A_2)^2 - k_3(A_2) + k_4(A)^2 - k_d(A_2) \qquad (7)$$

$$\frac{d(A)}{dt} = k_3(A_2) - k_4(A)^2 - k_m(A)$$

In equations (6) and (8) the concentrations of the species on the outside of the membrane are expressed by $(B_n) = (B_n)' \, V_o/V_i$, where $(B_n)'$ is the measured concentration, and V_o and V_i are the volumes of solution outside and inside the membrane, respectively. It is an arduous task to attempt solutions, even approximate, of these differential equations. If, however, during any experiment (H) varies only between (H_o) and $\frac{1}{2}(H_o)$, where

$$(H_o) = (H) + (B_4) + \frac{(B_2)}{2} + \frac{(B)}{4} \qquad (8)$$

and

$$(H) = (A_4) + \frac{(A_2)}{2} + \frac{(A)}{4} \qquad (9)$$

the terms η and ρ will vary only slightly from their values at $(H) = \frac{(H_o) + \frac{1}{2}(H_o)}{2} = \frac{3}{4}(H_o)$, providing $(H) > K_1$ and $(H) > 4K_2$. This relationship is shown in Fig. 16. The concentration and fraction of monomer are plotted

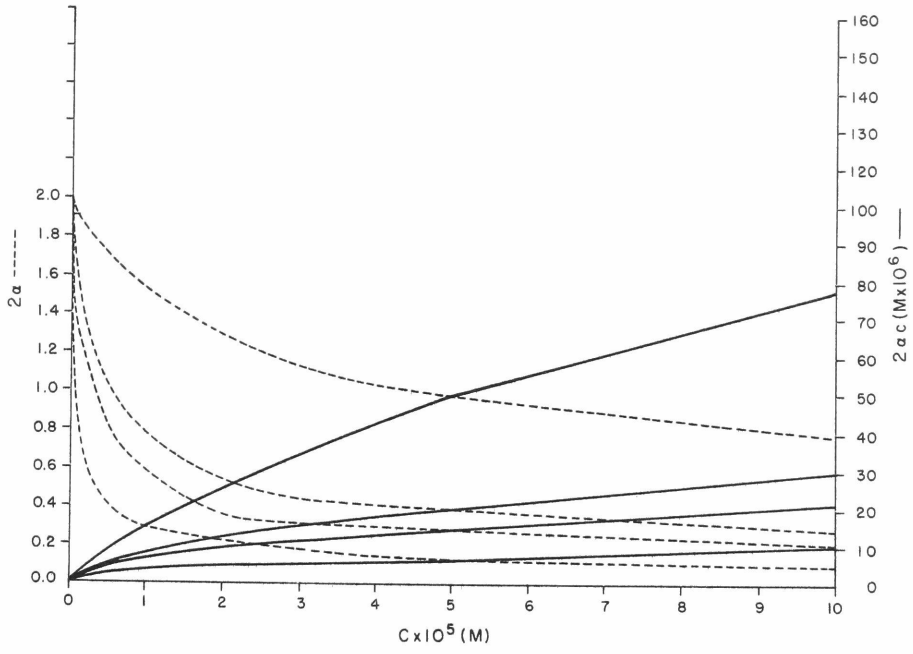

Fig. 16. The relationship between the concentration and fraction of monomer and the total concentration of solute for the case of a monomer-dimer system:

$$\underset{C(1-\alpha)}{A} \underset{}{\overset{K}{\rightleftharpoons}} \underset{2\alpha C}{2B}$$

C is the total solute concentration in moles per liter; α is the fraction of solute (dimer) present as the monomer; and K is the dissociation constant, $K = 4\alpha^2 C/(1-\alpha)$. The curves from top to bottom, both ----- 2α and ——— $2\alpha C$, are for the following values of the dissociation constant: $K = 10^{-4}$ M, $K = 10^{-5}$ M, $K = 5 \times 10^{-6}$ M, and $K = 10^{-6}$ M, respectively.

against total solute concentration for different values of the dissociation constant in the case of a simple monomer-dimer system.

The values of η and ς may, therefore, be taken as constant during any experiment. From (1) and (2), it follows that (A_4), (A_2), and (A) become directly proportional to (H) only:

$$\begin{aligned} (A_4) &= (H)(1-\eta-\varsigma) \\ (A_2) &= (H)(2\eta) \\ (A) &= (H)(4\varsigma) \end{aligned} \tag{10}$$

Solving for η and ς from (3) and (4) above:

$$\eta = \frac{K_1}{8(H)}\left(-1+\sqrt{1+\frac{16(H)}{K_1}}\right) \tag{11}$$

$$\varsigma = \frac{1}{4(H)}\left[K_1K_2\left(-1+\sqrt{1+\frac{16(H)}{K_1}}\right)\right]^{\frac{1}{2}} \tag{12}$$

for values of (H) such that $(H) > 4K_2$.

Replacing (H) by $\frac{3}{4}(H_o)$ in (11) and (12) and substituting (11) and (12) into (10):

$$\begin{aligned} (A_4) &= (H)\left[1-\frac{K_1\varepsilon}{6(H_o)}-\frac{1}{3(H_o)}(K_1K_2\varepsilon)^{\frac{1}{2}}\right] \\ (A_2) &= (H)\left[\frac{K_1\varepsilon}{3(H_o)}\right] \\ (A) &= (H)\left[\frac{4}{3(H_o)}(K_1K_2\varepsilon)^{\frac{1}{2}}\right] \end{aligned} \tag{13}$$

where $\varepsilon = \left(-1+\sqrt{1+\frac{12(H_o)}{K_1}}\right)$

It follows from (8) that

$$-\frac{d(H)}{dt} = \frac{d(B_4)}{dt} + \frac{1}{2}\frac{d(B_2)}{dt} + \frac{1}{4}\frac{d(B)}{dt} \tag{14}$$

Substituting (6) into (14):

$$- \frac{d(H)}{dt} = k_t (A_4) + \frac{1}{2} k_d (A_2) + \frac{1}{4} k_m (A) \qquad (15)$$

and then (13) into (15):

$$- \frac{d(H)}{dt} = k_t (H) \left[1 - \frac{K_1 \varepsilon}{6(H_o)} - \frac{1}{3(H_o)} (K_1 K_2 \varepsilon)^{\frac{1}{2}} \right] + k_d (H) \left[\frac{K_1 \varepsilon}{6(H_o)} \right] +$$

$$+ k_m (H) \left[\frac{1}{3(H_o)} (K_1 K_2 \varepsilon)^{\frac{1}{2}} \right]$$

Simplifying:

$$- \frac{d(H)}{dt} = (H) \left[k_t + (k_d - k_t) \frac{K_1 \varepsilon}{6(H_o)} + (k_m - k_t) \frac{(K_1 K_2 \varepsilon)^{\frac{1}{2}}}{3(H_o)} \right] \qquad (16)$$

or

$$- \frac{d(H)}{dt} = (H) \left\{ k_t + \frac{1}{(H_o)} \left[(k_d - k_t) \frac{K_1 \varepsilon}{6} + (k_m - k_t) \frac{(K_1 K_2 \varepsilon)^{\frac{1}{2}}}{3} \right] \right\} \qquad (17)$$

It was shown above that the process of diffusion at any given hemoglobin concentration follows first-order kinetics but the half escape times decrease with decreasing initial hemoglobin concentration. Therefore, the process can be described by:

$$- \frac{d(H)}{dt} = k_{obs} (H) \qquad (18)$$

where k_{obs} is a function of the initial hemoglobin concentration. From (17) and (18), it follows that:

$$k_{obs} = k_t + \frac{1}{(H_o)} \left[(k_d - k_t) \frac{K_1 \varepsilon}{6} + (k_m - k_t) \frac{(K_1 K_2 \varepsilon)^{\frac{1}{2}}}{3} \right] \qquad (19)$$

Over the hemoglobin concentration range 50 x 10^{-5} M to 0.5 x 10^{-5} M, the value of the expression in brackets varies at a much slower rate than (H_o), so that (19) becomes

$$k_{obs} = k_t + \frac{1}{(H_o)} g \qquad (20)$$

where g represents the expression in brackets of (19) and may be considered to be a constant.

The experimental data should, therefore, agree with this relationship.

Fig. 17 shows that the plot of $\frac{C}{t\ 1/2}$ versus C, where C is the initial hemoblobin concentration in g % and t 1/2 the half escape time, is a straight line. This relationship is shown for the case of CO hemoglobin in phosphate buffer at pH 7.15 and for the case of CO hemoglobin in the same buffer also 2 M in NaCl.

Therefore:

$$t_{1/2} = \frac{C}{\gamma C + \delta}$$

where γ and δ are constants; converting the concentration to moles per liter:

$$t\ 1/2 = \frac{(H_o)}{m(H_o) + p} \qquad (21)$$

where m and p are also constants.

But for a first-order process:

$$k_{obs} = \frac{\ln 2}{t\ 1/2} \qquad (22)$$

so that substituting (21) into (22):

$$k_{obs} = \ln 2 \left(m + \frac{p}{(H_o)} \right) \qquad (23)$$

This expression is of the same form as (20), which was obtained analytically.

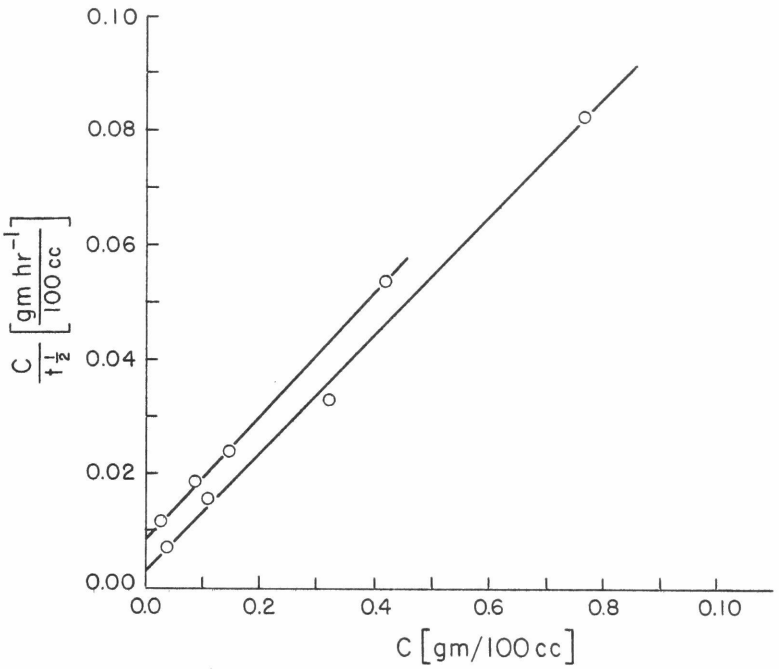

Fig. 17. The concentration dependence of the half escape times for the dialysis of CO hemoglobin. Upper curve: CO hemoglobin in 0.2 M phosphate buffer, pH 7.15; lower curve: CO hemoglobin in 0.2 M phosphate buffer and 2 M NaCl, pH 7.15. C is the initial CO hemoglobin concentration in grams % and t 1/2 is the half escape time.

The latter, therefore, may be taken to describe the data for this dissociating system.

Values for the dissociation constants K_1 and K_2 were obtained as follows:

1) From an approximate solution of (6) and (7) for the case of hemoglobin in the phosphate buffer containing 2 M NaCl, pH 7.15, in which hemoglobin behaves only as a tetramer-dimer system, a value of $K_1 = 1.37 \times 10^{-5}$ M was obtained.

2) k_{obs} was plotted again $\frac{K_1 \Sigma}{6(H_o)}$ for the given value of K_1 and a straight line was obtained. This shows that equation (19) is substantially correct. From (19) it follows that the intercept on the ordinate gives the value of k_t and the slope the value of $(k_d - k_t)$. Thus, k_t and k_d were determined as 0.054 $hr.^{-1}$ and 0.171 $hr.^{-1}$, respectively.

3) k_m was given arbitrarily the same value as the rate constant for the diffusion of myoglobin: $k_m = 0.580$ $hr.^{-1}$.

4) Assuming that these values of k_t, k_d, and k_m do not vary much with solvent conditions (as is indicated by Table XIII), values for K_1 and K_2 were picked, for each set of solvent conditions, so that the calculated values of the RHS of (19) matched as closely as possible k_{obs}.

The results are shown in Table XVI.

End