boundaries within the crust. Observed P-wave velocities are compatible with downward increase in the proportion of diorite, quartz diorite, and calcic granodiorite relative to quartz monzonite and granite in the upper crust, with amphibolite or gabbro-basalt in the lower crust, and with periodotite in the upper mantle. The synclinorium was formed in Paleozoic and Mesozoic strata during early and middle Mesozoic time in a geosyncline marginal to the continent. Granitic magmas are believed to have formed in the lower half of the crust at depths of 25 to 45 kilometers or more, primarily as a result of high radiogenic heat production in the thickened prism of crustal rocks. Magma was generated at different times in different places as the locus of downfolding shifted. It rose into the upper crust because it was less dense than rock of the same composition or residual refractory rocks. Refractory rocks and crystals that were not melted and early crystallized mafic minerals that settled from the rising magma thickened the lower crust. Wall and roof rocks settled around, and perhaps through, the rising magma and provided space for its continued rise. Erosion followed each magmatic episode, and 10 to 12 kilometers of rock may have been eroded away since the Jurassic and 7 to 10 kilometers since the early Late Cretaceous.

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Hemoglobin and its Reaction with Ligands

The alpha-beta dimer appears to be the important unit in hemoglobin function.

Eraldo Antonini

For almost a century hemoglobin has been the object of intense study by workers in the natural sciences. There is widespread interest in this protein not only because it has a prominent role in respiration, but also because it exemplifies many fundamental aspects of protein behavior.

Hemoglobin is contained in the red blood cells of all the vertebrates, and it accounts for more than 95 percent of the total proteins in these cells. Its physiological function is that of carrier of the respiratory gases (oxygen and carbon dioxide) between the tissues and the outer environment. The transport of oxygen depends on the specific property of the iron within the prosthetic group, which undergoes a reversible reaction with molecular oxygen; carbon dioxide, on the other hand, is bound, as such or in ionic form, by the amino acid side chains of the protein. In this article I describe a few aspects of the reaction of hemoglobin with ligands, with particular emphasis on the interrelation between the functional behavior and the structural properties of the protein.

Structure of Hemoglobin

Hemoglobin from red blood cells of mammals and most vertebrates, as far as is known, exists under many conditions as a tetrameric molecule of molecular weight 65,000; the molecule consists of four subunits, identical in pairs (*1–5*). The two different polypeptide chains have been called α- and β-chains so that the full molecule is indicated as $\alpha_2\beta_2$. Each polypeptide chain is bound to a prosthetic group, the heme, which is an iron II complex of protoporphyrin IX (*6*); in the native protein the ferrous iron binds reversibly molecular oxygen, carbon monoxide, nitric oxide, and other nongaseous ligands, such as alkylisocyanides and nitrosoaromatic compounds,

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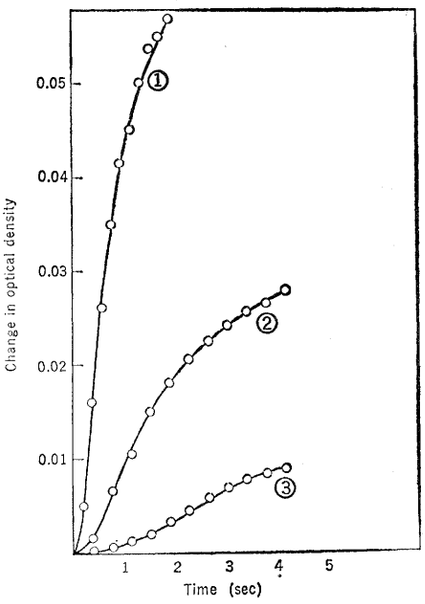

Fig. 1. Kinetics of the reaction between α- and β-chains of human hemoglobin (*14*). The reaction is followed at 430 mμ where the deoxygenated chains have a lower extinction coefficient than deoxygenated hemoglobin (*p*H 7, 30°C). Concentration of each type of chain (in heme): curve 1, $1.50 \times 10^{-6}M$; curve 2, $0.75 \times 10^{-6}M$; curve 3, $0.37 \times 10^{-6}M$.

in a ratio of 1 to 1. The amino acid sequence of the α- and β-chains of a number of hemoglobins is now known, as a result of work of research groups in Germany and the United States (*1, 7*).

The overall architecture of the hemoglobin molecule, the arrangement of the polypeptide chains, and the position of the heme groups have been elucidated by the work of Perutz and his co-workers (*8*). Each chain is roughly spherical, with an inner structure similar to that of myoglobin. Myoglobin is the pigment which is contained in the red muscles of animals; like hemoglobin, it reversibly binds molecular oxygen and consists of a single polypeptide chain, of molecular weight about 17,000, bound to a heme group (*9*). Myoglobin may be taken to represent, with respect to both structural and functional properties, a simpler type of respiratory protein from which hemoglobin has evolved.

Both x-ray studies and optical rotation data show that about 75 percent of the hemoglobin and myoglobin chains are in the α-helical configuration (*8–10*). Together the four hemoglobin chains form an irregular tetrahedron, with close contacts between unequal chains and looser contacts between each pair. The heme groups are located almost at the corners of the tetrahedron, on the surface of the molecule, but they are embedded in the polypeptide chains which fold around them.

Equilibria Involving Hemoglobin Components

The various parts of the tetrameric hemoglobin molecule—the four polypeptide chains and the four heme groups—are not bound by covalent bonds. The full protein may be considered in a state of reversible equilibrium with its components, but the dissociation may not be evident under many conditions, owing to the high value of the association constants. Thus, the dissociation of the prosthetic group from the protein part is finite, at least in ferrihemoglobin, even at neutral *p*H; depolymerization of the $\alpha_2\beta_2$-tetramer into dimers first, and even into monomers, may occur under conditions in which the protein is "native" (*2, 5*). The presence of monomers accounts for the formation of "hybrid" molecules when mixtures of hemoglobins from different species are exposed to dissociating conditions (*11*).

It is therefore possible to completely dissociate hemoglobin, the heme from the protein—the α- from the β-chains—and to separate and isolate the various components; the dissociation and isolation may be carried out under very mild conditions, and thus irreversible change may be avoided (*2, 12, 13*). When this has been done, the original protein can be reformed very rapidly by simply mixing the isolated components in stoichiometric amounts (*13, 14*). Thus the reassembling of the full hemoglobin molecule from its components is a spontaneous, fast, and specific process (Fig. 1). This conclusion has obvious implications for the problem of synthesis of complex molecules.

The isolated parts—the single chains, the globin, and the heme—have very different properties from those which they show when they are assembled in the full molecule. Thus hemoglobin may be visualized as a system in which the polypeptide chains, the heme, and the iron ligands undergo reversible modifications in kind as a result of specific binding and interactions among all the components. A striking and important phenomenon in this respect is the effect that the binding of oxygen or other ligands has on the conformation of the protein as a whole. This is brought out by the comparison of the properties of ligand-bound and nonligand-bound hemoglobin, a comparison which reveals marked changes in parts of the molecule not directly involved in the binding reaction.

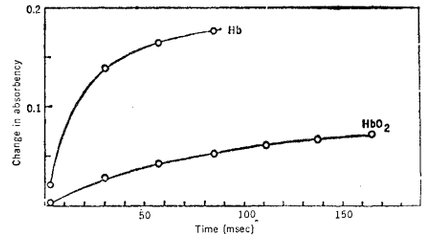

Fig. 2. The reaction of oxygenated and deoxygenated hemoglobin with bromothymol blue (*20*); 0.1*M* phosphate, *p*H 7.38, 21°C. The hemoglobin concentration was $0.36 \times 10^{-4}M$ in heme; the bromothymol blue concentration was $0.21 \times 10^{-4}M$. Dashed lines give asymptotes. Absorbancies were measured at 620 mμ.

The first clear-cut evidence for structural changes on oxygenation of hemoglobin was obtained by Haurowitz (*15*). When oxygen was allowed to diffuse into a suspension of crystals of deoxyhemoglobin, the crystals broke, indicating that crystals of oxy- and deoxyhemoglobin are not isomorphous. The x-ray analysis of oxy- and deoxyhemoglobin crystals has shown that the relative position of the subunits is different in the two derivatives (*16*). On deoxygenation, the β-chains move away from each other and out from the center of the tetramer. The distance between the iron atoms in the β-chains increases on deoxygenation. The x-ray analysis thus gives direct evidence for the conformational change, and there is little doubt that those changes seen in the crystal occur also in solutions of tetrameric hemoglobin. However, apart from these crystallographic changes, deoxy- and ligand-bound hemoglobin differ in a number of other properties which may be studied in solution, and which may be taken to reflect differences in the conformation of the two derivatives. These are as follows:

1) Reactivity of specific side groups of the polypeptide chains. The "reactive" sulfhydryl (SH) groups in position 93 of the chains in human hemoglobin react much faster with SH-

reagents when the protein is in the oxygenated, than when it is in the deoxygenated form (*17*). On the binding of oxygen, ionizable groups change their affinity for protons in the *p*H range from 5 to 9, and these changes in *pK* represent the physiologically important Bohr effect (*18*). On the other hand, the amino groups in oxy- and deoxyhemoglobin have a different tendency to form carbamino compounds with carbon dioxide (*19*).

2) Affinity and rates of the reaction of the hemoglobin as a whole with small molecules. The best example is given by the reaction of hemoglobin with the indicator dye bromothymol blue. The affinity and rates of reactions with this dye are much greater for deoxyhemoglobin than for ligand-bound derivatives (*20*) (Fig. 2).

3) Rates of digestion by proteolytic enzymes. The rate of digestion of hemoglobin by carboxypeptidase A, which removes the terminal tyrosine-histidine on the β-chains, and by carboxypeptidase B, which removes the terminal arginine from the α-chains, is greater with oxy- than with deoxyhemoglobin, the difference being greater with carboxypeptidase A than with B (*21*).

4) Reaction with antibodies. Oxy- and deoxyhemoglobin differ in their immunochemical reactions measured by complement fixation with serums from immune animals (*22*).

5) Reaction with haptoglobin. Haptoglobin is a protein found in serums and other liquids of mammals which binds specifically to hemoglobin or globin. Deoxygenated hemoglobin is not bound at all by haptoglobin, although the reaction appears to be irreversible with ligand-bound hemoglobin (*23, 23a*).

6) Association-dissociation into subunits. Dissociation into subunits may be regarded in some respects as a conformational change. It is generally accepted that the dissociation occurs in steps, the tetramer first dissociating into symmetrical $\alpha\beta$-dimers and the dimers then dissociating into monomers (*2, 3*). Ligand-bound and deoxyhemoglobin differ in the tendency to dissociate into subunits, especially when the products of dissociation are single-chain molecules (*2, 14, 24*).

7) Kinetic effects. There are a number of effects in the kinetics of the reactions of hemoglobin with ligands, which have their most probable explanation in conformational changes associated with ligand binding (*25*). The clearest effect is seen on flash photolysis of carbon monoxide hemoglobin near *p*H 9 where, just after photodissociation, the hemoglobin again reacts with the carbon monoxide but at a much higher rate than does normal hemoglobin; this occurs, presumably, because the sudden photodissociation leaves hemoglobin in the conformation corresponding to the ligand-bound derivative (*26*). The decay of the "quickly reacting hemoglobin" into "slowly reacting hemoglobin," which occurs at *p*H 9 and 1°C with a half-time of a few milliseconds, would then represent the return to the "normal" deoxyhemoglobin conformation.

8) Optical rotation properties. Oxy- and deoxyhemoglobin differ in optical rotation at 233 mμ, the difference being about 8 percent in the value of the mean residue rotation R^1 (*27*).

All the above-mentioned effects are correlated in the sense that when one is abolished as a result of some modification of the protein, generally all the others vanish too. They depend on the presence of both α- and β-chains. Many of the aforesaid differences between ligand-bound and deoxyhemoglobin persist under conditions where both hemoglobin derivatives dissociate into dimer molecules of composition $\alpha\beta$- or when the α- and β-chains, as in "hybrid" hemoglobins, are from proteins of different species (*21, 27, 28*); on the other hand, they are not present in isolated hemoglobin chains and myoglobin (*20, 29*). From kinetic studies it may be deduced that the intramolecular conformational changes occur rapidly in relation to the rate of uptake of the ligand (*25, 30*).

Features of the Reaction of Hemoglobin with Ligands

On exposure to molecular oxygen, ferrous hemoglobin binds the gas in a ratio of one oxygen molecule per heme iron unit. Simple ferrous heme compounds undergo oxidation and not oxygenation in the presence of oxygen, and it is the attachment to the specific protein that confers to the heme iron the possibility of a reversible reaction with molecular oxygen. In this respect an important role is ascribed to the hydrophobic environment provided by the protein side chains surrounding the heme group (*9, 31*). The reaction with oxygen or other ligands is associated with large changes in the spectral and magnetochemical properties, which, as far as has been verified, are proportional to the amount of ligand bound to the heme iron (*32*) (see also Fig. 3).

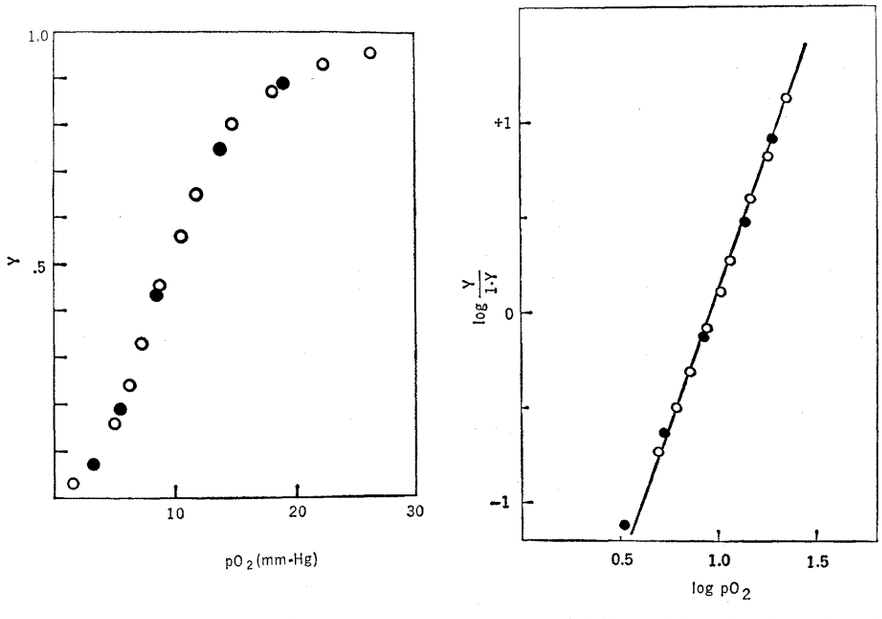

Fig. 3. (Left) Oxygen dissociation curve of human hemoglobin; (right) the data plotted according to the Hill equation. Open circles are points obtained by gasometric determinations (from unpublished work by Lyster and Roughton); closed circles are points obtained by spectrophotometric determinations (19°C; 0.6*M* phosphate buffer, *p*H 7.0; hemoglobin concentration 1 to 4 percent or 0.7 to 2.5 $\times$ $10^{-3}M$).

The linear relation between spectrum changes and the amount of carbon monoxide bound has now been directly established for human hemoglobin (*32a*).

The binding of oxygen by hemoglobin is reversible and may be represented by the oxygen-dissociation curve which gives the fraction of hemoglobin combined with oxygen (y) as a function of oxygen pressure (pO_2).

Since the early work of Bohr, it has been known that the reaction of hemoglobin with oxygen is complex and that the oxygen-dissociation curve cannot be described by a simple mass law equation for the reaction

$$Hb + O_2 \rightleftharpoons HbO_2$$

The oxygen-dissociation curve for whole blood or dilute hemoglobin solution is sigmoid in shape, and the affinity for oxygen increases with increasing saturation (Fig. 3). A dissociation curve with this shape is much more suitable than a hyperbolic dissociation curve for the physiological role of the protein in the transport of oxygen. Another characteristic feature of the oxygen-dissociation curve is that its position along the pO_2 axis (the oxygen affinity), and even its shape, are dependent on the composition of the medium in which the protein is dissolved. Thus, the pH, salt concentration, and the presence of carbon dioxide and other molecules and ions affect the affinity of the protein for oxygen; the effect of pH is the most familiar one, and it is known as the Bohr-Krogh-Hasselbalch effect (*33*). Apart from any mechanism of model, the sigmoid character of the equilibrium curve implies that the affinity of the oxygen-binding sites changes as a result of the presence of the ligand on the other sites; in other words, that there are interactions between the oxygen-binding sites. These are the heme-heme interaction, or "homotropic" interactions (*3, 34*). In the same way, the effect of pH, salt concentration, and other such factors implies interactions between the oxygen-binding sites and other groups in the molecule which bind hydrogen ions or other ligands.

The shape and position of the equilibrium curve can be described empirically by the Hill equation (*35*).

$$\frac{y}{1-y} = K\,(pO_2)^n$$

or

$$\log \frac{y}{1-y} = n \log pO_2 + \log K$$

where y is the fractional saturation with the ligand, pO_2 is the partial pressure of oxygen, and K and n are constants. Although the Hill equation has no direct physical meaning, it is a useful way of describing the dissociation curve with only two parameters; the exponent n may be taken as an index of the interaction between sites, while the value of $P(\frac{1}{2})$, the oxygen pressure corresponding to half-saturation, may be taken as a measure of oxygen affinity. A value of $n > 1$ indicates "positive" heme-heme interactions; $n = 1$, equivalent sites with no interaction; $n < 1$, nonequivalent sites or "negative" heme-heme interactions.

Mammalian hemoglobins under conditions similar to those described in Fig. 3 have a value of $n \simeq 2.9$. Functional interactions of the type familiar to students of the hemoglobin reactions have now been recognized in many other proteins, especially enzymes, and are thought to play a role in biochemical regulatory mechanisms (*36*). This might explain why studies of the behavior of hemoglobin are useful for understanding problems of protein function in general.

From the immense body of data on the equilibrium of hemoglobin with ligands, the following generalizations can be made. In the case of different ligands of the ferrous form (that is, oxygen, carbon monoxide, isocyanides, nitrosoaromatic compounds), the shape of the equilibrium curves and the Bohr effect are very nearly the same in spite of large differences in the affinity of the various ligands for hemoglobin (*2–5*). To some extent this is true also for the oxidation-reduction equilibrium (*37, 38*). However, the heme-heme interaction, the Bohr effect, and other such are lacking in the reactions of ferric hemoglobin with ligands (*39*). Thus, irrespective of the type of ligand, the interaction effects are present in the reaction of ferrous, but not of ferric, hemoglobin with ligands. Any modification of the molecule which produces a change in the equilibrium curve for one ligand produces a parallel change in the others, and the relative affinity of the protein for the two ligands remains unchanged (*5*).

The heme-heme interaction, the oxygen affinity, and the Bohr effect are dependent on the integrity of the protein. A number of modifications of hemoglobin which do not affect its oxygen-combining capacity may produce changes in the heme-heme interaction and in the Bohr effect. The changes are always, or nearly so, in the direction of a decrease of the heme-heme interactions and the Bohr effects; often, especially for the more severe treatments, both the heme-heme interaction and the Bohr effect are abolished. However, in some other cases the heme-heme interaction and the Bohr effect may be modified to a different extent (*2, 5*).

The shape of the dissociation curve depends on the salt concentration of the medium. The curve is less sigmoid at low ionic strength; its shape may depend on pH, but apparently not on temperature (*2, 5, 18*). Usually, but not always, a decrease in heme-heme interaction is associated with an increased affinity for the ligand. The shape of the ligand-equilibrium curve, the Bohr effect, and the ligand affinities are very similar for all the mammalian hemoglobins, but these characteristics may be vastly different in hemoglobins from other animals (*40*).

Kinetics of the Reaction of Hemoglobin with Ligands

The velocity of the reaction of hemoglobin with ligands is in most cases very high, and the kinetics must be studied by special methods for measuring rapid reactions in solutions, techniques such as rapid mixing, flash photolysis, and relaxation (temperature jump). The kinetics of hemoglobin reactions have now been under active investigation for more than 40 years, starting with the classical pioneering work of Hartridge and Roughton (*25, 41*). Results of early experiments, when the resolution was not perfect, indicated that hemoglobin kinetics might approximate second-order reactions in the combination with ligands and first-order reaction in the dissociation of ligands. However, the work of Gibson and Roughton has revealed a number of striking kinetic effects which reflect the homo- and heterotropic interactions, so evident in the equilibria (*25*). In contrast, myoglobin exhibits a simpler kinetic behavior (*5, 25, 42*).

The basic kinetic features are apparently the same for the reaction with different ligands (*43*), although the rates of combination and dissociation may differ by several orders of magnitude.

At pH 7 and 20°C, the initial ap-

parent second- (on) and first-order (off) velocity constants for the reaction with oxygen are about 10^6 mole^{-1} sec^{-1} and 40 sec^{-1}, respectively; the same constants for the reaction with carbon monoxide are 10^5 mole^{-1} sec^{-1} and 0.06 sec^{-1}.

Under conditions in which the rate of the forward reaction is high in comparison with the dissociation reaction, as in the presence of a large excess of ligand, the time course of the reactions follows approximately a second-order process, the half-time being inversely proportional to the concentration of the reagents (*25*, *30*). However, the shape of the progress curves does not correspond to a second-order reaction, but shows a definite increase in the apparent second-order rate constant as the reaction proceeds (*25*). This may be taken as kinetic evidence for heme-heme interaction. The rate of combination with ligand is very little affected by *p*H, at least in mammalian hemoglobins.

The dissociation of oxygen may be measured directly by mixing oxyhemoglobin with dithionite. The reaction is rapid and corresponds very nearly to first-order kinetics (*25*, *44*). For mammalian hemoglobins, the rate of reaction is strongly dependent on *p*H, and therefore the equilibrium Bohr effect arises mainly from changes in the dissociation velocity constant with *p*H.

For ligands other than oxygen the rate of dissociation can be measured in displacement reactions of the type

$$\mathrm{Hb \cdot X + Y \longrightarrow Hb \cdot Y + X}$$

where X and Y are two different ligands. These displacement reactions, carried out under conditions where deoxyhemoglobin is never present in significant amounts, follow the simple time course of a first-order process, but, as is evident in the case of dissociation of oxygen, the rate of ligand dissociation measured in this way is different from that directly measured (*25*).

Under conditions where the process is contributed both by the forward and backward reactions, the kinetics are more complex than as outlined above, and the approach to equilibrium may appear diphasic (*25*). Under these conditions the results are similar to those obtained in temperature-jump experiments, where (at least) two relaxations are seen under many conditions (*45*).

Compounds of ferrous homoproteins with ligands are dissociated by light, the quantum yield being high (near 1) for the carbonyl (CO) compounds (*46*). If a liganded hemoglobin or myoglobin derivative is exposed to the brief intense light of an electronic flash, the compound is dissociated, and the subsequent recombination in the dark may be followed. With myoglobin, the kinetics of combination with ligand after flash photolysis are the same as in rapid mixing experiments, but with hemoglobin they are not (*25*, *26*, *47*). With hemoglobin, under many conditions, the recombination with ligand after flash photolysis is characterized by a rapid phase because of the material reacting with ligand much faster than hemoglobin does in mixing experiments (Fig. 4). The rapidly reacting material is especially evident under some conditions, namely very dilute hemoglobin solutions, very brief photolytic flashes, or partial photodissociation of the ligand (*26*). Although the quickly reacting material might originate as a result of different effects, it appears to be in one way or another related to the conformational transitions from ligand-bound, to deoxyhemoglobin which may occur slowly in comparison with the photodissociation process (*24*, *47*).

In spite of the beautiful work carried out by a number of investigators on the kinetics of hemoglobin reactions, it has not yet been possible to accommodate all the kinetic results into an unequivocal reaction scheme. However, recent work has clarified many aspects of the kinetic behavior of hemoglobin so far unexplained, and experiments under conditions where the protein is dissociated have shown that the kinetics of the hemoglobin reactions might be analyzed in terms of reaction schemes taking into account only the two sites in the $\alpha\beta$-dimer (*47*). On the other hand, a number of perplexing features of hemoglobin kinetics may be explained on the assumption of a reversible dissociation into single-chain molecules occurring in dilute solutions of ligand-bound, but not of deoxyhemoglobin (*24*, *47*, *47a*).

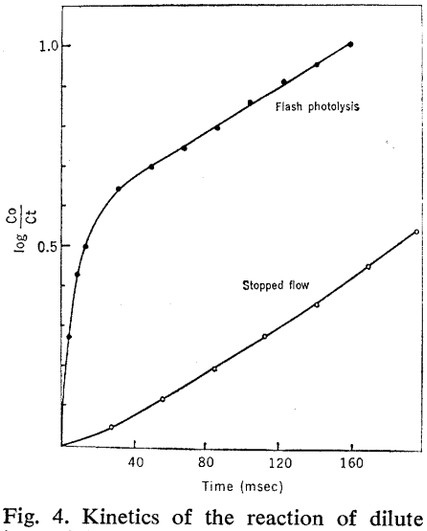

Fig. 4. Kinetics of the reaction of dilute hemoglobin solutions with carbon monoxide in rapid mixing and flash photolysis experiments (*47*). There is a rapid initial phase in the flash photolysis experiments and an increase in rate with progress of reaction in the stopped flow experiments (0.1*M* phosphate buffer, *p*H 7.0, 20°C; carbon monoxide, $5 \times 10^{-4}M$; hemoglobin, $2 \times 10^{-6}M$ in heme).

Structure-Function Relations in Hemoglobin

The main structural features of hemoglobin, clearly differentiating it from similar less complex hemoproteins like myoglobin, are that the molecule contains more than one heme-carrying polypeptide chain and that the binding of ligands is associated with a "large" change in the conformation of the protein.

The relation between the state of polymerization of hemoglobin and its functional behavior may be studied experimentally, in that the tetrameric molecule may dissociate into subunits of lower molecular weight under conditions where the protein maintains its oxygen-binding capacity. The association-dissociation processes are rapidly reversible equilibria, and the relation between state of aggregation of hemoglobin and oxygen equilibrium may be considered from two points of view, namely, the effect of dissociation into subunits on the oxygen equilibrium and the effect of oxygenation on the association-dissociation equilibria. These effects are related and are thermodynamically linked to each other (*3*).

Dissociation into dimers occurs when oxy- and deoxyhemoglobin are dissolved in concentrated salt solutions, in acetate buffers below *p*H 6, and at alkaline *p*H (*2*). When hemoglobin dissociates into dimers under all these conditions, there is no change in the oxygen equilibrium of the protein, contrary to what would have been expected on the basis of the classical model, which accounted for intramolecular interactions between all four hemes in the tetramer (*48*). In con-

centrated salt solutions the value of n in the Hill equation remains unchanged at 2.8 to 3 or even increases slightly (Fig. 5). Apart from the apparent paradox that the value of n is higher than expected from the average number of heme units per molecule, these results indicate that the subunit resulting from dissociation maintains all the characteristic reactivity with ligands. There is clear evidence that the tetramer, under the conditions stated above, dissociates into $\alpha\beta$-dimers (*2*); therefore, it should be admitted that the $\alpha\beta$-dimer represents the functional unit in hemoglobin, whether it exists as an isolated molecule or as part of a tetramer. Even if the interaction energy were infinite between the two oxygen binding sites in the $\alpha\beta$-dimer, the maximum value of n would be 2. The fact that the value of n reaches 3, or more, requires additional effects over and above intramolecular interactions within the $\alpha\beta$-dimer (*2*, *3*, *5*). Also, in the kinetics of the reactions of hemoglobin with ligands, there is no change when hemoglobin dissociates into dimers, and this indicates that the characteristic kinetic features of hemoglobin may be ascribed only to the $\alpha\beta$-dimer (*47*).

When dissociation goes beyond the $\alpha\beta$-dimer, or when it involves molecular species containing single chains, there is a drastic change in the functional behavior of the system, and the degree of dissociation becomes markedly dependent on the degree of saturation with ligand (*14*). This is related to the functional behavior of the isolated hemoglobin chains (*49*, *50*). Regardless of their state of aggregation, isolated α- and β-chains show high affinity for ligands, hyperbolic equilibrium curves, and absence of the Bohr effect. The kinetics of the reaction with ligands of the isolated chains are vastly different from that of hemoglobin, especially in the combination rate, which increases by almost two orders of magnitude (*50–52*). These changes in functional behavior are reversible; when the α- and β-chains are mixed they rapidly associate, and the system then functions again like normal hemoglobin (*50*) (Fig. 6).

The oxygen equilibrium of mammalian hemoglobin shows small but significant changes with changes in hemoglobin concentration; these changes, however, do not appear to be correlated with the dissociation of the tetramer into $\alpha\beta$-dimers (Fig. 7).

Ligand Binding and Conformational Changes

Apart from any detailed mechanism, it is more or less obvious that the functional interactions in hemoglobin are not "direct effects," since they involve binding sites which are far apart in the molecule. This is true both for the heme-heme interaction and for the Bohr effect and other "heterotropic" interactions.

The interaction effects are correlated with the change in conformation that accompanies ligand binding to the heme iron, since disappearance of the conformation change is always associated with disappearance of the interactions and vice versa. This is illustrated by the behavior of hemoglobin digested by carboxypeptidase A, which removes the carboxy-terminal sequence tyr-his from the β-chain of human hemoglobin (*53*). This hemoglobin (modified only in the β-chain) shows a hyperbolic ligand equilibrium curve, very high ligand affinity, and large reduction in the Bohr effect. Conversely, the ligand-bound and deoxy derivatives of this hemoglobin do not show the difference in properties (ascribed to the conformational change) evident in the case of normal hemoglobin. The two derivatives are isomorphous in the crystal (*54*), they are digested at the same rate by carboxypeptidase B, and they react in the same way with dyes and SH-reagents (*20*, *21*).

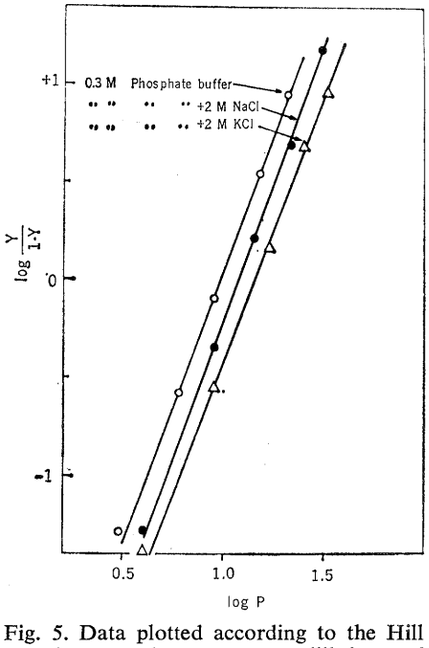

Fig. 5. Data plotted according to the Hill equation on the oxygen equilibrium of human hemoglobin at low and very high salt concentrations. The results show that dissociation of the $\alpha_2\beta_2$-tetramer into $\alpha\beta$-dimers (in strong salt) has no effect on the heme-heme interaction, as measured by the value of n, the slope of the Hill-equation curve (20°C; hemoglobin concentration $3 \times 10^{-4} M$ in heme; *p*H 7.1).

That conformational integrity of the protein is required for its normal function is also shown by the fact that almost any modification of the protein, even in parts of the polypeptide chains far from the heme group, produces changes in the ligand equilibria and kinetics, changes which are generally associated with the loss of the homo- and heterotropic interactions. Under these conditions the behavior of the whole system approaches that of the isolated hemoglobin chains (*2*, *5*).

Several models have been proposed (*55*, *56*) for the mechanism by which the conformation exerts its control on the reactivity of the heme iron. It is noteworthy that the isolated chains and all the modified forms of hemoglobin which no longer show cooperative effects have an altered spectrum in the deoxygenated state (*23a*, *26*, *50*, *51*).

The Bohr-Krogh-Hasselbalch effect was initially discovered as the effect of carbon dioxide on the oxygen-affinity of hemoglobin (*33*); however, the effect of carbon dioxide is largely due to changes in the *p*H of the medium and the Bohr effect is now considered primarily as the effect of *p*H on the oxygen affinity of hemoglobin (*18*). The effect is a physiologically important regulatory mechanism of the respiratory function of blood and is also a prototype of *p*H effects on the biological activity of proteins in general.

Since hemoglobin may be considered in true thermodynamic equilibrium with both hydrogen ions and oxygen, the Bohr effect implies that the acid-base properties, that is, the proton-binding capacity of hemoglobin, changes when the protein binds oxygen. The quantitative relation between the difference in proton-binding capacity between oxy- and deoxyhemoglobin and the change in oxygen affinity may be expressed by (*3*, *18*)

$$d \log p_m / (d\ p\mathrm{H}) = \Delta \bar{H}^+$$

where $\Delta(\bar{H})^+$ is the difference in protons per heme by oxy- and deoxyhemoglobin, p_m is the median oxygen pressure (*3*), which, in the case of "symmetry" of the oxygen-dissociation curve with *p*H may be identified with $P_{1/2}$. This equation is of general application only if binding of other ions of the

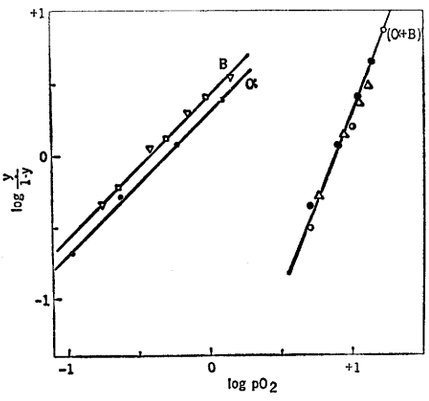

Fig. 6. Data on the oxygen equilibrium of isolated α- and β-chains and of hemoglobin obtained by recombination of the chains. The isolated chains show no heme-heme interaction and much higher oxygen affinity than the reassembled hemoglobin. 0.1M phosphate buffer, pH 7; 20°C. Concentrations of protein were about $2 \times 10^{-4}M$ in heme. Different symbols correspond to different preparations (*50a*).

medium is negligible in comparison with binding of hydrogen ions.

The Bohr effect at different temperatures for the oxygen equilibrium of human hemoglobin is shown in Fig. 8. The oxygen affinity is at a minimum around pH 6 to 6.5 and increases on both sides so that it is customary to speak of an alkaline or "normal" and an acid or "reverse" Bohr effect. The Bohr effect is present in the reaction of ferrohemoglobin with other ligands and in the oxidation-reduction equilibrium (*38*), but is not present, or is different, in the binding of ligands to ferric hemoglobin.

In mammalian hemoglobins the effect of pH is to alter the position but not the shape of the oxygen-dissociation curve; this is not the case in hemoglobins from other species, notably fish hemoglobins. Detailed measurements of the Bohr effect under the same conditions as those of Fig. 8 have shown that the Bohr effect is very similar in different mammalian hemoglobins, contrary to old views that there is a relation between the size of the animal and the Bohr effect (*57*). Nonetheless, the Bohr effect might be different in hemoglobins from other animal classes; it may be absent, it may be present in different pH regions, or it may be accompanied by large changes in the shape of the dissociation curve (*58*).

The Bohr effect may be interpreted as arising from changes in the pK of a few specific groups in the polypeptide chains, not necessarily near in space to the heme group. Thus the main questions regarding the Bohr effect are the identification of the groups responsible for it and the mechanism by which they change pK when the heme iron binds the ligand.

The minimum number of groups involved in the Bohr effect may be estimated by the maximum change in the proton-binding capacity, or from the maximum change in log $P(½)$. From these values for human and other mammalian hemoglobins this number is found to be one per heme for the alkaline Bohr effect. On this basis, a model has been adopted on the assumption that there are two oxygen-linked acid groups per heme, one responsible for the acid, the other for the alkaline Bohr effect. On oxygenation, the pK of the alkaline group increases, that of the acid group decreases (*3*, *18*, *58*).

The chemical nature of these groups may be identified from the values of the pK's and the value for the heats of ionization, if these values are similar to those that the same groups would have in simple compounds. For mammalian hemoglobins the data indicate that the groups responsible for the Bohr effect are an imidazole or an α-amino group on the alkaline side, and a carboxyl group on the acid side (*59*). However, from chemical evidence the α-amino groups can be ruled out since guanidination of these groups does not modify the Bohr effect (*60*).

The curves of the Bohr effect change in position and shape with temperature (*59*, *61*). The temperature dependence of the Bohr effect may be explained on the assumption that the involved groups have the same heat of ionization in oxy- and deoxyhemoglobin, though different for the two groups; accordingly, the Bohr effect would arise mainly from entropy effects (*59*). However, another interpretation of the temperature effect has been given and the matter is not yet settled (*61*).

Thus the Bohr effect arises from interactions between the hemes and the ionizable groups, these interactions are mediated by the conformation changes associated with ligand binding, and require the presence of both α- and β-chains, even if each chain in the full molecule contributes equally. Thus the Bohr effect is absent in the isolated hemoglobin α- and β-chains (*49*, *50*), but is present in intermediates where one of the chains is frozen in the ligand-bound form (*62*).

Theories and Models of Cooperative Effects in Hemoglobin

Various theories and models have been proposed to explain the sigmoid dissociation curve for the oxygen-hemoglobin reaction. Although most of the models fit the behavior of the protein, at least under certain conditions, there are still difficulties in accounting for all the various features

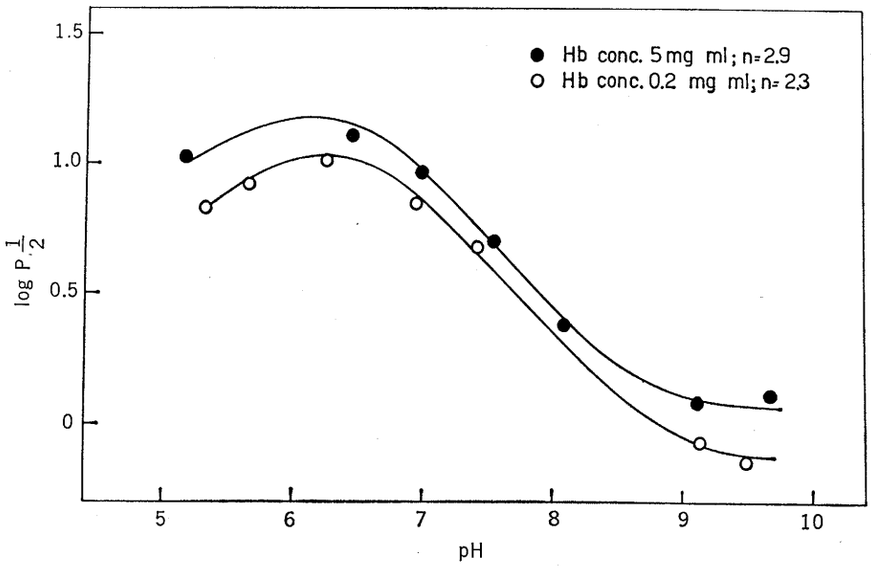

Fig. 7. Effect of protein concentration on the oxygen equilibrium of human hemoglobin. The value of n in the Hill equation and the value of $P(½)$ decrease slightly with dilution of the protein. The ionic strength of the acetate, phosphate, and borate buffers was 0.1 to 0.4; 20°C.

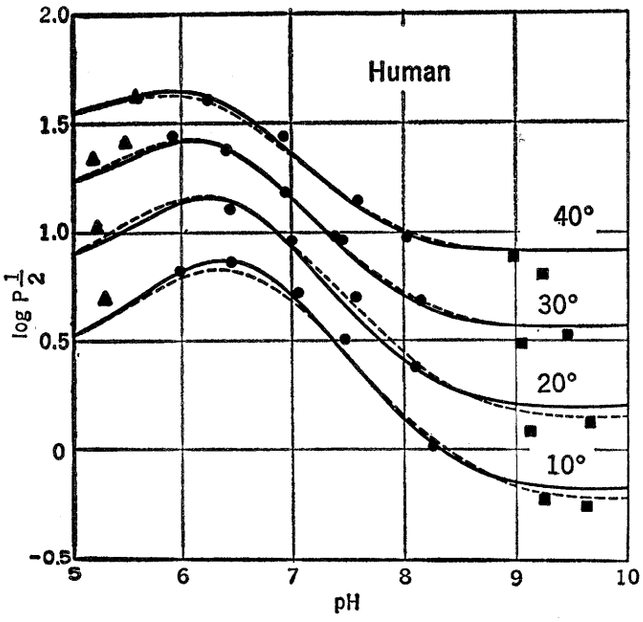

Fig. 8. Bohr effect in human hemoglobin at different temperatures (*59*). Points show experimental values of log $P(½)$ from oxygen equilibrium curves in phosphate (solid circles), acetate (solid triangles), and borate (solid squares) buffers. Dashed lines correspond to values of $d \log P(½)/d$ pH calculated from values of $\Delta\bar{H}^+$ (see equation in the text) measured by differential titrations. Full curves have been calculated with the following model: the alkaline Bohr effect is due to one oxygen-linked acid group per heme of pK 6.45 in oxy- and of pK 7.85 in deoxyhemoglobin at 20°C; the heat of ionization in oxy- and deoxyhemoglobin ΔH is +9000 calories. The acid Bohr effect is due to another oxygen-linked acid group per heme with pK 6.26 in oxy- and pK 5.46 in deoxyhemoglobin at 20°C and ΔH equal to −1.500 calories in oxy- and deoxyhemoglobin.

of the equilibria and kinetics of the reaction of hemoglobin with ligands. The interpretation of the heme-heme interaction in hemoglobin has again acquired general interest, in view of the recognition that cooperative effects and interactions between binding sites are present in several other proteins and enzymes.

According to the Haldane theory (*63*), oxyhemoglobin and deoxyhemoglobin are in association-dissociation equilibrium with monomers. Only the monomers combine with the ligand, and the sigmoid equilibrium curve results from differences in the degree of polymerization of ligand-bound and deoxygenated hemoglobin. This theory seemed to be ruled out by Adair's finding that the molecular weight of oxy- and deoxyhemoglobin was the same and corresponded to a tetramer; however, it has recently been proposed again, in a slightly different form, to explain the behavior of hemoglobin under dissociating conditions, or that of lamprey hemoglobin.

In Hill's theory (*35*), it is assumed that hemoglobin, both in the oxygenated and deoxygenated form, has an average degree of polymerization, relative to monomers that contain an iron atom equal to n. The reaction with oxygen was then thought to be a true n-order reaction, thus leading to the familiar equation.

But the Hill equation no longer has a physical basis, since it has been shown that (i) the reaction is not a true n-order reaction, since n approaches 1 at low saturation, and kinetically the rate of reaction is proportional to the first power of ligand concentration (*41*); (ii) the Adair direct molecular weight measurements showed no direct correlation between the value of n in the Hill equation and the number of sites in hemoglobin (*34*). However, the equation is still in use as a practical way to describe the oxygen-dissociation curve of hemoglobin, since the value of n in the middle part may be taken as a measure of the sigmoidicity of the oxygen-dissociation curve, and hence of the heme-heme interaction (*3*, *18*).

Adair (*34*) discovered that mammalian hemoglobin exists as a tetramer both in the oxygenated and deoxygenated form under conditions where the oxygen-dissociation curves are usually obtained. Hence it may be postulated that oxygenation occurs in four successive steps. The sigmoid shape of the curve is explained if one allows that the affinity of the deoxygenated hemes changes on binding of oxygen by other hemes in the same molecule. The Adair theory is of quite general validity, and in its original form does not imply any particular relation between the four equilibrium constants.

After the Adair hypothesis had been proposed the problem of the oxygen equilibrium of hemoglobin was confined to that of the interactions between the four hemes in the hemoglobin tetramer. Further studies up to the 1960's aimed (i) to find more restricted models adequate for describing the oxygen-dissociation curves, but containing fewer than four independent constants, and at the same time (ii) to understand the physical mechanisms underlying the heme-heme interaction. In the first group of studies is included the Pauling equation (*64*), the analysis of Coryell (*65*), and especially that of Wyman (*18*). With regard to the physical basis of heme-heme interactions St. George and Pauling (*66*) proposed the hypothesis of "buried hemes", and Wyman and Allen (*67*) were the first to point out the possible dependence of the functional behavior of hemoglobin on the conformation of the protein. In the same period, Gibson (*25*) and Roughton *et al.* (*68*), using highly ingenious and exact experimental methods, attempted to determine directly the values of the equilibrium and kinetic constants corresponding to the four-stage Adair scheme.

Recent Models and Theories

New developments originated from the detailed knowledge of the structure of hemoglobin—mainly from the x-ray work—from the notion of conformational changes associated with ligand binding and from the recognition that, under many conditions, the hemoglobin tetramer is in a state of reversible association-dissociation equilibrium with its subunits.

Most of the recent theories and models for the cooperative effects in hemoglobin are centered about the conformational ("allosteric") transitions

(*56*, *69*), association-dissociation (*70*) into subunits, or both (*71*). Long-range forces between the hemes have also been invoked (*71a*).

It would take too long to describe the details of the various models, but it must be pointed out that, while most of them give an adequate fit of the equilibrium data, none has been subjected to stringent tests and no comprehensive scheme for the kinetics has yet been proposed.

This does not mean that no progress has been made in understanding the functional behavior of hemoglobin; as mentioned, correlation of functional behavior with structural properties has been achieved in many cases. However, there may be conditions under which the behavior of mammalian hemoglobin cannot be explained on the basis of intramolecular interactions alone or on the basis of a ligand-linked association-dissociation process. There seems to be some evidence for assuming either the presence of intermolecular interactions or of effects due to conformational relaxations which may produce sigmoid binding curves independently of the number of sites per molecule (*72*).

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