*THE INFLUENCE OF REVERSIBLE OXYGEN BINDING ON THE INTERACTION BETWEEN HEMOGLOBIN SUBMITS**

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The conformational change which might result from the combination of biologically active proteins with smaller molecules and the manner in which this could account for the functional properties of the macromolecule is a matter of great interest. In the case of enzymes, several workers have recently concluded that combination with substrate or coenzyme can lead to major changes in the conformation of the protein.[1-4] However, the oxygenation of hemoglobin is perhaps the oldest and most clear-cut example of such a reversible transformation.

Some of the differences between oxygenated and deoxygenated hemoglobin† clearly involve the hemoglobin molecule as a whole. Thus, in the case of many species, there are pronounced differences in the crystal structure and solubility.[5-7] The titration curves of the two forms of the protein are also not identical[8] and the Bohr Effect is, of course, a consequence of this alteration in acid strength with oxygen binding. Oxygenation has a marked influence on the reactivity of the -SH groups in position 93 in the β chain of human hemoglobin toward certain reagents. Iodoacetamide, for example, reacts readily in neutral solution with oxy- but not at all with deoxyhemoglobin.[9] Haurowitz and his collaborators[10] followed the kinetics of the alkali denaturation of oxy- and deoxyhemoglobin of several mammalian species and found that in each case the heat of activation was smaller and the entropy of activation more negative for deoxy- than for oxyhemoglobin. They interpreted their findings in terms of a configurational change of the protein in the oxygenation reaction. In view of these results, it is perhaps not surprising that Ottesen and Schroeder[11] found recently that subtilisin attacks deoxyhemoglobin more readily than oxyhemoglobin.

The three-dimensional structure of oxyhemoglobin (horse) is now known in some detail.[12, 13] The same information for deoxyhemoglobin is not yet available. Nevertheless, it seems possible to make certain specific predictions about the differences between the two forms of the protein.

The folding of the individual chains is unlikely to be drastically affected by deoxygenation, since essentially the same chain-fold has been found in every myoglobin or hemoglobin which has so far been examined (sperm whale metmyoglobin,[14] seal metmyoglobin,[15] horse oxyhemoglobin;[12, 13] cf. also Perutz's discussion[16]). This assumption derives further support from the UV rotatory dispersion measurements of Briehl,[17] who concluded that deoxygenation produces no significant change in the secondary structure of the globin chains.

The major difference in structure between the oxygenated and deoxygenated form of the protein must therefore be sought in the arrangement of the chains in relation to one another. Furthermore, the sigmoid shape of the oxygen dissociation curve and the concept of "heme-heme interaction," as well as other evidence, led Haurowitz to suggest 25 years ago[18] that in deoxyhemoglobin the chains are so arranged as to bring the hemes into close proximity to each other inside the molecule. Oxy-

genation would then destroy this interaction and thus cause the hemes to move apart. The finding of Perutz *et al.*[12, 13] that the hemes are far apart and on the outside of the molecule in oxyhemoglobin confirms at least one aspect of this hypothesis. The idea that the hemes are inside the molecule in deoxyhemoglobin is supported by the fact that those -SH groups which are located in the immediate vicinity of the hemes become "masked" upon deoxygenation.[9, 19]

The work of Rossi-Fanelli and his collaborators[20, 21] carries the argument one step further. Their evidence indicates that the hemes themselves participate in interchain bonding in deoxyhemoglobin, since modification of the vinyl side chains of the hemes leads to considerable reduction in heme-heme interaction.

There is as yet no *direct* evidence that deoxyhemoglobin contains interchain bonds which are broken on oxygenation. One obvious method of investigating such bonding is a comparison of the dissociation of oxy- and deoxyhemoglobin into subunits. Molecular weight determination by light scattering and sedimentation led Rossi-Fanelli *et al.*[22] to the conclusion that both oxy- and deoxy-hemoglobin were split into half molecules at salt concentrations of 2 *M* or greater. At the same time, they reported[23] that under identical conditions the exponent n in Hill's equation‡ was 3, i.e., that the half-molecules of deoxyhemoglobin behaved functionally as though they contained at least 3 hemes per mole. Since these results are obviously irreconcilable, we have reinvestigated this dissociation.

Results.—The results of the molecular weight determinations are shown in Table 1. They confirm the conclusion of Rossi-Fanelli *et al.*[22] that oxyhemoglobin is

TABLE 1

WEIGHT AVERAGE MOLECULAR WEIGHTS OF HEMOGLOBIN

Protein concentration	M_w Deoxyhemoglobin	M_w Oxyhemoglobin
A: 0.05 *M* Phosphate buffer pH 7.0		
0.4%	66.500 ± 2.000	66.500 ± 1.600
1.2%	65.500 ± 1.600	64.700 ± 2.000
B: 0.05 *M* Phosphate buffer pH 7.0; 2.0 *M* NaCl		
0.25%	46.200 ± 1.100	36.600 ± 1.000
0.4%	42.800 ± 1.300	33.000 ± 1.600
0.8%	42.600 ± 0.600	36.800 ± 1.400

Each value of the molecular weight represents the average of 5 separate values calculated from photographs taken at 16-min intervals during the sedimentation equilibrium run.

split into half-molecules in 2 *M* sodium chloride. In the case of deoxyhemoglobin, on the other hand, this dissociation is seen to be incomplete. It should be stressed that sedimentation runs on oxy- and deoxyhemoglobin under the same conditions showed only a single boundary. An analogous situation was described for partially dissociated carbon monoxide hemoglobin in acid solution by Field and O'Brien[24] and interpreted in terms of a rapidly reversible equilibrium between dimers and tetramers.[25] A puzzling feature of our results, however, is that the weight average molecular weight appears to be independent of the hemoglobin concentration. We are at a loss to offer an explanation for this observation.

The main experimental difference between the present work and that of Rossi-Fanelli *et al.*[22] is that these authors used dithionite for the preparation of deoxyhemoglobin, whereas we have avoided the use of any chemical reducing agent (cf. *Experimental*). Dithionite causes degradation of hemoglobin with accompanying spectral changes.[26, 27] Dalziel and O'Brien[27] obtained evidence that these changes

are brought about by an oxidation product of dithionite. Therefore this compound is likely to cause peroxidative degradation of hemoglobin, especially in the presence of oxygen.

Even when nitrogen was used to displace oxygen, it was found essential to deoxygenate the hemoglobin completely before mixing it with the salt solution in order to avoid formation of methemoglobin (Fig. 1, curve *B*). This oxidation occurs very rapidly when hemoglobin is exposed to low oxygen tensions in concentrated salt solutions (Fig. 1, curve *C*). By contrast, fully oxygenated hemoglobin is resistant to autooxidation under the same conditions (Fig. 1, curve *A*). Such a protective effect of oxygenation against oxidation is well known for ferrous complexes, including hemoglobin.[28, 29]

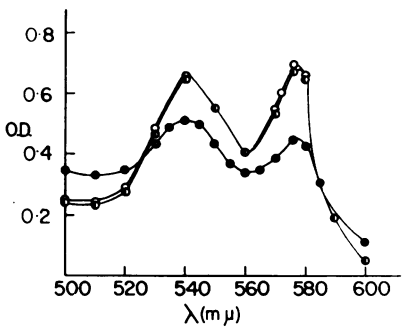

FIG. 1.—●——● Curve *A*: 0.077 per cent oxyhemoglobin in 2.0 *M* NaCl/0.05 *M* phosphate buffer pH 7.0. O——O Curve *B*: Deoxygenated hemoglobin was mixed with an equal volume of 4 *M* NaCl/0.1 *M* phosphate buffer pH 7.0 as described in the text. After $2^1/_2$ hr, this solution was diluted with oxygenated 2 *M* NaCl/0.05 *M* phosphate buffer pH 7.0 to give an oxyhemoglobin concentration of 0.077 per cent. ●——● Curve *C*: As in Curve *B* except that the NaCl solution was added *before* deoxygenation. It should be stressed that the total hemoglobin concentration, measured as methemoglobin cyanide, is identical for the three curves.

Discussion.—Electrostatic forces probably play the major role in holding the chains of the hemoglobin molecule together. It is therefore only when these are disrupted, as, for example, in media of high ionic strength, that the difference in bonding between oxy- and deoxyhemoglobin could be detected. Since it has recently been shown that no exchange of hemoglobin chains takes place in 2 *M* sodium chloride,[30] dissociation must be symmetrical under these conditions. It is therefore reasonable to assume that the oxygenation-labile bonds which we have found in deoxyhemoglobin function as a link between the $\alpha\beta$ subunits.

Finally, mention should be made of a recent discovery which further supports the view that deoxyhemoglobin contains interchain bonds that are broken on oxygenation. It was found that the extent of hybridization between deoxy hemoglobin I and deoxy hemoglobin C is much smaller than with the corresponding oxygenated hemoglobins under the same conditions, i.e., 4 hours at pH 4.7 and 0°C. This dramatic reduction in the rate of hybridization with the deoxy forms must signify the existence of different bonding *between* α and β chains in the deoxygenated molecule. This work, in collaboration with Dr. H. M. Ranney, is still in progress and will be reported in detail in a future publication.

Experimental.—Preparation of hemoglobin stock solutions: Hemoglobin was isolated from the blood of a normal human male as described previously.[9] Neutral citrate was used as the anticoagulant. Hemoglobin concentration was determined spectrophotometrically at 540 mμ after conversion to methemoglobin cyanide.

Oxyhemoglobin in sodium chloride: A hemoglobin solution of twice the desired final concentration was mixed with an equal volume of a solution which was 4 *M* in NaCl and 0.1 *M* in phosphate buffer pH 7.0.

Deoxyhemoglobin in sodium chloride: The preparation and transfer of these solutions is illustrated in Figure 2. Seven ml of a solution 4 *M* in NaCl and 0.1 *M* in phosphate buffer pH 7.0 were placed in the 50 ml filter flask A and an equal volume of oxyhemoglobin solution of twice the

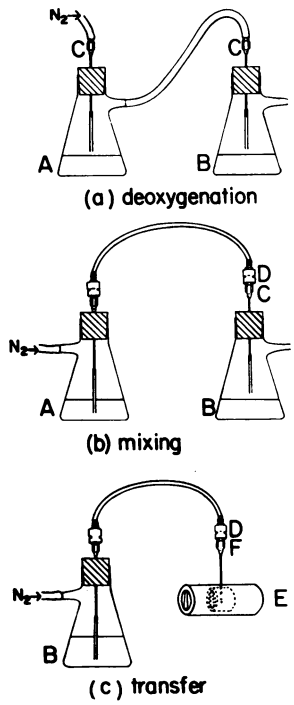

FIG. 2.—*A* and *B*, 50 ml filter flasks. *C*, 2 inch 18 G needle fitted with a short length of PE 160 polyethylene tubing. *D*, No. 608/L Luer-Lok adapter. *E*, Assembled ultracentrifuge cell. *F*, 1 inch 23 G needle.

desired final concentration in flask B. The flasks were connected as shown in Figure 2(*a*) and placed on a shaker. Prepurified nitrogen which had been saturated with water was passed over the solutions with gentle shaking for about 1 hr to deoxygenate both solutions completely. The flasks were then connected as shown in Figure 2(*b*) and the salt solution pushed over into the hemoglobin solution with a gentle pressure of nitrogen. Shaking of the hemoglobin solution was continued during this operation. Finally, the solution was transferred to the ultracentrifuge cell E, again by a pressure of nitrogen as shown in Figure 2(*c*). The deoxyhemoglobin solution itself was used to remove the oxygen from the centerpiece by filling it as shown and removing the solution with a syringe four times. After the final filling operation, the cell was sealed rapidly. In order to check for complete deoxygenation, the spectrum of the solution was recorded between 600 and 800 mμ on a Cary Model 11 spectrophotometer. It was found that with suitable alignment the sealed ultracentrifuge cell could be used directly in the carriage of this instrument. A similar cell filled with salt solution was used for the blank. For quantitative comparison, the ratio of $O.D._{650}/O.D._{730}$ which is 2.3 for deoxyhemoglobin and 1.0 for oxyhemoglobin, was used.

In order to test for the formation of methemoglobin, an aliquot of the solution which had been left in the sealed cell for $2^1/_2$ hr was rapidly reoxygenated by dilution with 4 volumes of buffered 2 *M* NaCl saturated with air. The spectrum between 500 and 600 mμ was then recorded and compared with that of the original oxyhemoglobin solution which had been similarly diluted. The spectrum of a solution which was deoxygenated in the presence of NaCl but was otherwise treated identically was also determined (Fig. 1).

Molecular weight determination: Molecular weights were determined with the Spinco Model E Analytical Ultracentrifuge by the Archibald method essentially as described by Schachman.[31] Only the upper (gas-liquid) boundary was used for measurements because of the difficulty of deoxygenating an organic liquid at the bottom of the cell. Base lines with the buffered salt solution were determined under the same conditions as the equilibrium run in each case and the results corrected accordingly. A Wratten No. 29 red filter was placed over the light source and Kodak 1 N Spectroscopic plates were used. The initial concentration c_0 was determined from a calibration line of hemoglobin concentration (expressed as $O.D._{540}$ after conversion to methemoglobin cyanide) against c_0. The latter was measured at several hemoglobin concentrations using a valve type synthetic boundary cell. The ultracentrifuge patterns were measured with a Gaertner two-dimensional microcomparator. For the equilibrium runs at the lower hemoglobin concentrations a cell with a 30-mm centerpiece was used. The partial specific volume was taken as 0.748.[32] Grossman and Tanford[33] have shown that the change in partial specific volume of hemoglobin with salt concentration is negligible up to 5 *M* NaCl and, in view of the good agreement between the molecular weight of oxy- and deoxyhemoglobin in dilute buffer, it seems justified to assume that this parameter is not influenced significantly by deoxygenation.

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† Fully oxygenated hemoglobin (HbO_8) will be referred to as oxyhemoglobin, fully deoxygenated hemoglobin (Hb) as deoxyhemoglobin. The latter term was chosen in order to avoid "reduced hemoglobin," which implies a change in the valence of the iron.

‡ Per cent oxygenation = $K(pO_2)^n/[1 + K(pO_2)^n]$.

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