Oxidation–Reduction Equilibrium of Human Hemoglobin in Concentrated Sodium Chloride Solutions. Consistency with the ($\alpha\beta$) Dimer Model*

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ABSTRACT: This paper reports a study of the oxidation–reduction equilibrium of human hemoglobin in 2 and 5 M sodium chloride, as a function of pH. The shape of the oxidation–reduction equilibrium curve at *high* ionic strength is pH dependent, the value of n increasing from 1.4 at pH 6.5 to 2 at pH 8. Over approximately the same pH range the value of n for the redox equilibrium at *low* ionic strength (0.2) changes from about 1.2 to about 2.5 (Antonini, E., Wyman, J., Brunori, M., Taylor, J. F., Rossi-Fanelli, A., and Caputo, A. (1964), *J. Biol. Chem. 239*, 907). Therefore at alkaline pH (8–9) there is a decrease in the apparent value of n produced by increasing the ionic strength from 0.2 to 2.2. These results are consistent with the model which proposes that the ($\alpha\beta$) dimer is the functionally important unit in the redox equilibrium of human hemoglobin. The oxidation Bohr effect as a whole is reduced in 2 and 5 M sodium chloride, as compared with that at low ionic strength. However, the "residual" oxidation Bohr effect is still present and unmodified. The constancy of the residual oxidation Bohr effect under different conditions suggests that we are dealing with a direct effect, such as would result from a local conformational change in the neighborhood of the heme. A comparative analysis of the primary structure of myoglobin and hemoglobin, and of the three-dimensional structure of myoglobin, permits a tentative though admittedly speculative assignment of the amino acid side chains involved in the residual oxidation Bohr effect.

The oxidation–reduction equilibrium in hemoglobin is analogous to the equilibrium of hemoglobin with gaseous and nongaseous ligands. This similarity, which was first pointed out by Wyman and Ingalls (1941), has been confirmed and generalized in the course of a systematic study on the oxidation–reduction equilibrium of hemoproteins (Brunori *et al.*, 1964, 1967). Therefore the same basic molecular mechanisms are presumably operative in the redox equilibrium and in the equilibrium of the ferrous form with different ligands, and many general conclusions can be transferred from one case to another.

A main difference between electron transfer and oxygen binding is that the shape of the redox equilibrium curve is pH dependent, the value of n in the electrode equation decreasing from about 2.5 at pH 9 to about 1.2 at pH 6 (Antonini *et al.*, 1964). On the basis of several experimental facts, an interpretation of this behavior based on a difference in the intrinsic oxidation–reduction potential of the two unlike chains in an ($\alpha\beta$) dimer has been proposed (Brunori *et al.*, 1965, 1967).

This interpretation of the redox equilibrium is based on the idea that the dimer is the important functional unit, just as proposed in the case of the equilibrium and kinetics of ferrous hemoglobin with gaseous ligands (Antonini, 1967; Guidotti, 1967; Schuster and Brunori, 1966; Wyman, 1967). The validity of this proposal can be tested by measuring the oxidation–reduction equilibrium of hemoglobin under conditions in which the molecule is dissociated into dimers, as in strong salts (Rossi-Fanelli *et al.*, 1961). Under these conditions one might expect to find a decrease in the value of n at high pH, because its maximum possible value for a dimer is 2. Since the product of the dissociation in high salt is the ($\alpha\beta$) dimer (Rossi-Fanelli *et al.*, 1964), however, the value of n should still be pH dependent, if the interpretation for the dependence of n on pH is correct (Brunori *et al.*, 1967).

This paper reports measurements on the oxidation–reduction equilibrium of human hemoglobin in 2 and 5 M NaCl. The results obtained are fully consistent with the expectations outlined above. Moreover the new data provide the occasion for a further analysis of the oxidation Bohr effect, some aspects of which have not been fully considered before.

Experimental Section

Human hemoglobin was prepared by the toluene method (Taylor and Hastings, 1939). The ferric derivative was obtained using either the nitrite method or the oxidation of the purified hemoglobin by ferricyanide.

Oxidation–reduction equilibria were measured potentiometrically using the *dye titration* method or the *mixture* method, as reported in detail previously (Antonini *et al.*, 1964).

In order to allow a direct comparison of the results at *high* and *low* ionic strength, experiments by the *mixture* method

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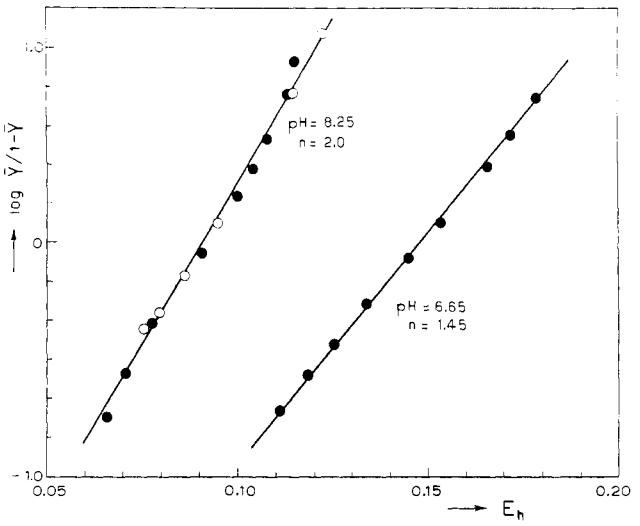

FIGURE 1: Oxidation-reduction equilibrium curves for human hemoglobin, as obtained by the "mixture method" at 30°. Results at two pH values: pH 8.25, borate buffer plus 2 M NaCl; protein concentration 9 mg/ml; pH 6.65, potassium phosphate buffer plus 2 M NaCl; protein concentration 9 mg/ml. (●) Titration of ferrous hemoglobin with (nitrite) ferric hemoglobin. (○) Titration of (nitrite) ferric hemoglobin with ferrous hemoglobin.

were performed using exclusively nitrite-ferric Hb (see also Figure 3 of Antonini *et al.*, 1964).

Protein concentration was between 4 and 10 mg/ml in the experiments by the *mixture* method, and 34 mg/ml in those by the *dye titration* method.

Nominal pH values were those measured directly with the glass electrode without correction.

Results

Shape of the Oxidation-Reduction Equilibrium Curve. At high ionic strength the dye titration method yields smooth titration curves similar to those already reported at low ionic strength, but which are in general even more asymmetrical than those obtained in the same buffers without added sodium chloride. In both cases n varies markedly over the whole range of the titration curve. In one experiment values of n ranging from about 1 to about 3 were obtained from a single plot of log $(Y/(1 - Y))$ *vs.* E.

On the other hand experiments under the same conditions by the mixture method yielded symmetrical equilibrium curves, similar to those obtained before at low ionic strength (0.1–0.2) by this method (Antonini *et al.*, 1964). Figure 1 shows the results of two titrations in 2 M NaCl (+0.1 M phosphate) obtained by the mixture method; in each case the plot is linear between about 15 and 90% oxidation, indicating that the equilibrium curve is symmetrical. This was found to be true at all pH values examined. The values of n considered in this paper were all obtained by this method.

Dependence of n upon pH. Figure 2 reports the value of n between pH 5.95 and 9.4 measured in 2 M NaCl, and also for one experiment in 5 M NaCl at pH 7.8. The results previously obtained at low ionic strength by the same method are also given for comparison (Antonini *et al.*, 1964).

It will be seen that n increases from about 1.4 at pH 5.95 to

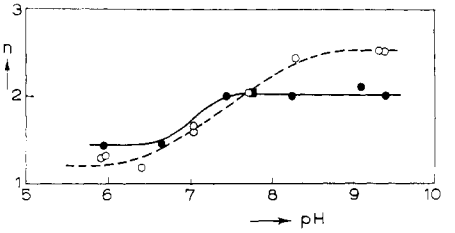

FIGURE 2: Dependence upon pH of the value of n in the redox equilibrium of human hemoglobin. Protein concentration from 4 to 10 mg/ml in the different cases. (○) Results in phosphate and borate buffers at ionic strength 0.1–0.2 (Antonini *et al.*, 1964). (●) Results in the same buffers plus 2 M NaCl (ionic strength 2.1–2.2). (■) Results in the same buffers plus 5 M NaCl (ionic strength 5.1–5.2).

nearly 2 at pH 9.4. The single value of n determined in 5 M NaCl at a nominal pH of 7.8 is also close to 2. The situation is similar to that at low ionic strength, though the effect is smaller in magnitude. The transition from lower to higher values of n occurs over approximately the same pH range at low and high ionic strength.

Oxidation Bohr Effect. The dependence of the oxidation-reduction potential at 50% oxidation ($E_{1/2}$) on pH, in 2 and 5 M NaCl and at 30°, is reported in Figure 3. The results obtained by both experimental methods (*i.e.*, the "dye titration" and the "mixture" methods) are included in the figure, since both methods have been found to give essentially the same values for $E_{1/2}$ as in previous investigations (Antonini *et al.*, 1964; Brunori *et al.*, 1964) at low ionic strength.

It will be seen from Figure 3 that the largest effect of ionic strength on the oxidation reduction is at pH less than 7.5. This is just the opposite of what is found in the case of the oxygen equilibrium, where the greatest effect is at alkaline pH (Antonini *et al.*, 1962). The continuous decrease in $E_{1/2}$ at alkaline pH, amounting to about 60 mV/pH, is presumably due to dissociation of the heme-linked water molecules. The apparent pK' for this transition at high ionic strength was not measured, but from previous work by George and Hanania (1953) on horse Hb, a pK of 8.2–8.3 was estimated for 2.2 M ionic strength.

Discussion

One interesting aspect of a study of the oxidation-reduction equilibrium at high ionic strength stems from the fact that under these conditions tetrameric hemoglobin is dissociated into subunits of lower molecular weight (Rossi-Fanelli *et al.*, 1964). This fact offers a means of investigating the relative role of dimers as compared with tetramers in determining the cooperative effects in the hemoglobin-ligand equilibrium. Of course the whole problem is intimately related to the effect of the ligand on the dissociation of the hemoglobin molecule at high ionic strength. Ligand bound ferrohemoglobin is known to be dissociated in 2 M NaCl even at the protein concentrations used in these experiments, *e.g.*, 4–10 mg/ml (Rossi-Fanelli *et al.*, 1961). Ferric hemoglobin is known to dissociate to about the same extent as oxy- or carbon monoxyhemoglobin (Rossi-Fanelli *et al.*, 1964), although some differences between ferric hemoglobin and oxyhemoglobin have been reported (Merrett, 1966; Guidotti, 1967). The case of deoxyhemoglobin is somewhat less clear; how-

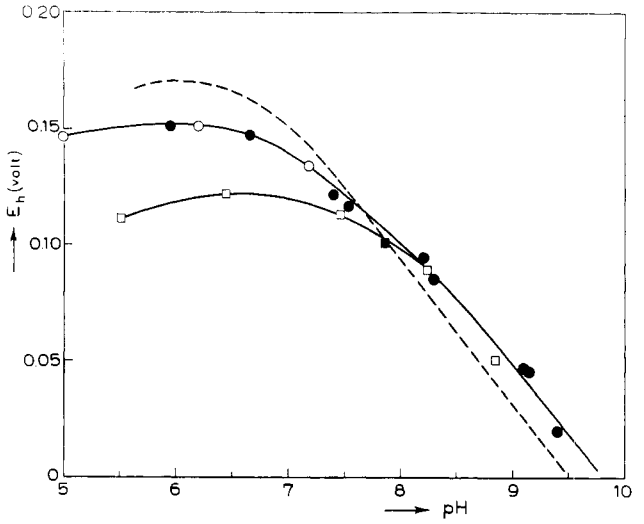

FIGURE 3: Oxidation Bohr effect for human hemoglobin, at 30°. (○, ●) Results at ionic strength 2.1–2.2. (□, ■) Results at ionic strength 5.1–5.2. Open symbols indicate the data obtained by the "*dye titration* method"; closed symbols indicate the data obtained by the "*mixture* method." The results for human hemoglobin at low ionic strength (0.1–0.2) are also reported for comparison (dashed line) (from Antonini *et al.*, 1964).

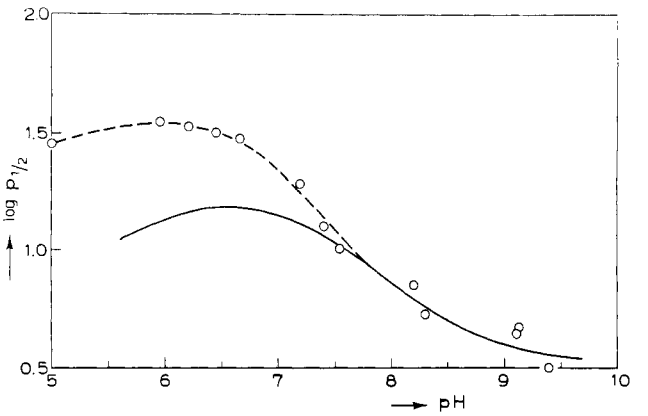

FIGURE 4: Comparison of the oxygen Bohr effect with the oxidation Bohr effect corrected for the ionization of the water molecule. Circles: $E_{1/2} \times 16.65$. Solid line: oxygen Bohr effect in 2 M NaCl (from Antonini *et al.*, 1962).

ever, in spite of some conflicting evidence, it is generally believed that it also is dissociated, although to a lesser extent (Rossi-Fanelli *et al.*, 1964; Guidotti, 1967; Benesch *et al.*, 1962; Briehl, 1964).

The experiments on the oxidation–reduction equilibrium reported in this paper show that at pH >8 there is a decrease in n from 2.5 to 2.0 on increasing the ionic strength from 0.2 to 2.2. This is qualitatively what might be expected on thermodynamic grounds as a result of a significant dissociation of tetrameric hemoglobin into dimers. It should be noted that if this interpretation is correct we should expect a concomitant variation of $E_{1/2}$; however, especially in the case of the redox equilibrium, it is difficult to isolate the effect of dimer dissociation on n and $E_{1/2}$ from other possible effects arising from changes in solvent composition. In spite of this difficulty in providing a quantitative picture of the system, the fact that n is still as high as 2, notwithstanding the dissociation, would suggest that the major contribution to the interaction arises within the ($\alpha\beta$) dimer, although this is not the only possible interpretation. Thus the dimer would appear to be the important unit in the oxidation–reduction equilibrium, just as proposed in the case of the oxygen equilibrium (Antonini, 1967; Guidotti, 1967; Wyman, 1967). The dependence of n on pH, though small, is significant; it might be interpreted on the basis of a difference in the intrinsic oxidation–reduction potential of the two chains (α and β) in a dimer, as in the case of earlier results (Brunori *et al.*, 1964, 1967).

The oxidation Bohr effect above pH 6, both at high and low ionic strength, can be interpreted as a contribution of several oxidation-linked acid groups: (i) the heme-linked water molecule, (ii) the same group responsible for the alkaline Bohr effect for oxygen, (iii) and a third group, in order to account for the so-called "residual oxidation Bohr effect." The presence of this additional oxidation-linked ionizable group became clearly evident on comparing the oxygen Bohr effect with the oxidation Bohr effect after subtracting the contribution due to the ionization of the water molecule (Brunori *et al.*, 1964). In the case of the experiments at high ionic strength reported in this paper, such a comparison is shown in Figure 4. It is apparent that the oxidation Bohr effect is the larger of the two. The significance of this difference, which has been called the residual Bohr effect, becomes especially evident when we examine the data for all the cases which have been studied. An analysis of the available data (see Figure 4 and Brunori *et al.*, 1964) suggests certain conclusions concerning the nature of the effect and identification of the group responsible for it.

In normal human hemoglobin at low and high ionic strength, as well as in several modified hemoglobins and in horse and sperm whale myoglobins, the residual Bohr effect is always present; moreover its magnitude is relatively constant, within limits (20–30 mV), and seems to be independent of the magnitude of the oxygen Bohr effect. These facts suggest that we are dealing with a direct effect, such as would result from a local conformation change in the neighborhood of the heme. This conclusion is reinforced by the presence of the residual Bohr effect in myoglobin. The analysis of the differential titration data (Brunori *et al.*, 1965) and of the redox equilibrium of the hemoglobin–haptoglobin complex (Brunori *et al.*, 1968) indicates that this group is located on only one of the two hemoglobin chains. On the basis of the results obtained by Banerjee and Cassoly (1966) on the redox equilibrium of the isolated subunits, it is obvious that this group must belong to the β chains. Therefore we are led to search for an ionizable group with a pK' in the range 6–8 which is present in myoglobin and in the β chains, but absent from the α chains. This group obviously cannot be the SH group in position β_{93} because this residue is lacking in myoglobin, and because the residual Bohr effect is unchanged after treatment of hemoglobin with iodoacetamide (Brunori *et al.*, 1967). It should be pointed out that in human hemoglobin modified by treatment with iodoacetamide the value of n in the redox equilibrium curve is equal to 2 and independent of pH between 6 and 9. In this case, therefore, we must invoke the presence of a compensation between two opposite effects, namely, the pH-dependent functional heterogeneity and the allosteric cooperativity, which of course are also present in normal hemoglobin, though to a different extent.

A comparative analysis of the sequences (Dayhoff and Eck, 1968) shows that there are only two regions which would seem to meet the requirements. One is the C-terminal region (Lys_{146} in Mb, His_{146} in β, and Arg_{141} in α); however, this can hardly be involved because digestion of hemoglobin with carboxypeptidases (A or B) produces no modification in the residual oxidation Bohr effect. Another is that near the proximal histidine (α_{87}, β_{92}, and Mb_{93}). This has the following sequence

Mb	...Lys_{96}-His_{97}-Lys_{98}...
β	...Lys_{95}-Leu_{96}-His_{97}...
α	...Lys_{90}-Leu_{91}-Arg_{92}...

Examination of the X-ray model of sperm whale ferric myoglobin (Kendrew *et al.*, 1961) shows that His_{97}, which belongs to the FG corner (FG 3), has its imidazole ring near and parallel to the heme plane. This would make this residue a good candidate for the group responsible for the residual oxidation Bohr effect in myoglobin. In the β chains, the same role could be played by His_{97}, although the exact position of this residue in the hemoglobin model is not known to us. The corresponding position in the α chain is occupied by an arginyl residue, which remains presumably protonated over the whole pH range involved. It may be of interest to point out that Arg_{92} α and His_{97} β are invariant residues, which have been found to occupy the same position in all normal hemoglobins from mammals studied up to now (Dayhoff and Eck, 1968). Notable exceptions are Hb Chesapeake and Hb J. Capetown, in which Arg_{92} in the α chain is replaced, respectively, by leucine and glutamine. In any case it is tempting to ascribe the residual oxidation Bohr effect to some groups in the FG corner.

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