The Influence of Oxygenation on the Reactivity of the —SH Groups of Hemoglobin*

RUTH E. BENESCH AND REINHOLD BENESCH†

From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York

Received May 28, 1962

It was found that oxygenation and deoxygenation affect the reactivity of some hemoglobin —SH groups towards certain reagents, such as *N*-ethyl maleimide and, particularly, iodoacetamide, but not *p*-mercuribenzoate or iodoacetate. These results are interpreted in terms of configurational changes *in the protein*, which are associated with reversible oxygen binding. The absence of such changes in $4\beta^{A}$ hemoglobin (hemoglobin H) as well as in myoglobin supports the conclusion that interchain interactions are responsible for the differences in conformation between oxygenated and deoxygenated hemoglobin.

There is now a substantial body of evidence to indicate that there are differences in the conformation of the protein in oxygenated and deoxygenated hemoglobin.[1] Edsall (1958) has discussed some of the properties of hemoglobin which vary with oxygenation. The well-known spectral and magnetic changes can probably be ascribed mainly to changes in the electronic structure of the iron-porphyrin-globin complex as originally proposed by Pauling and Coryell (1936). A number of other differences, on the other hand, must reflect alterations in the conformation of the hemoglobin molecule as a whole.

Perhaps the most striking of these is the difference in solubility and crystal structure between the oxygenated and deoxygenated protein, although considerable variations occur from species to species. Lumry and his collaborators (Lumry and Maysumiya, 1959; Takashima and Lumry, 1958) have shown that the dielectric increment and the viscosity also vary as a function of oxygenation, but in this instance the changes are evident only at intermediate stages of oxygenation, while the initial and final values are almost identical. The best-known chemical difference between oxy- and deoxyhemoglobin is the reversible change in acid dissociation of certain groups which is responsible for the Bohr effect. Very recently Ottesen and Schroeder (1961) have observed a remarkable differential susceptibility of "native" oxy- and deoxyhemoglobin to proteolysis by subtilisin.

Very little seems to have been reported about more specific reactions between other molecules and hemoglobin as they are affected by oxygenation. One striking observation of this kind was made many years ago by Chargaff *et al.* (1939), who found that cephalin, but not lecithin, split the heme-globin linkage in carbon monoxy- or oxyhemoglobin *but not in deoxyhemoglobin*. More recently Riggs (1961) observed differences in the rate of reaction of *N*-ethyl maleimide with the —SH groups of oxy- and deoxyhemoglobin.

The chemical reactivity of hemoglobin side-chains as a function of oxygenation is thus of great interest. The "reactive" —SH groups present an ideal site for such studies, since a variety of reagents can be used to modify these groups under very mild conditions.

EXPERIMENTAL

Preparation of Hemoglobin Solutions

These were prepared essentially by the method of Drabkin (1946) except that the treatment of the erythrocytes with $AlCl_3$ solution was omitted.

Blood was obtained from normal adult males by venipuncture. Four different methods were used to prevent coagulation.

(1) Addition of neutral citrate. In this case 7.5 ml of a 3.2% solution of sodium citrate dihydrate was used per 40 ml of blood (Drabkin, 1946). Unless otherwise specified this method was employed.

(2) Addition of acid citrate dextrose (ACD) anticoagulant, which has the composition: 2.2% trisodium citrate·$2H_2O$, 0.8% citric acid H_2O, 2.45% dextrose; 6 ml of this solution was used per 40 ml of blood.

(3) Addition of acid citrate (AC), which is identical with the above except for the omission of dextrose. It was used in the same ratio as above.

(4) Defibrination by agitation with glass beads.

* A preliminary report of this work was presented at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1962. These investigations were supported by grants from the National Science Foundation and the National Heart Institute, United States Public Health Service.

† Established Investigator of the American Heart Association.

[1] 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.

TABLE I
REACTIONS OF HEMOGLOBIN —SH GROUPS

Reagent	Oxyhemoglobin	Deoxyhemoglobin
p-Mercuribenzoate, *p*H 7.0, instantaneous reaction	2–2.5 —SH/mole	2–2.5 —SH/mole
N-Ethyl maleimide, *p*H 7.3	2.5 —SH/mole $t_{1/2}$ 2–3 min.	2 —SH/mole $t_{1/2}$ 4–7 min.
Iodoacetamide, *p*H 7.3, 2 hrs.	2–2.5 —SH/mole	no reaction
Iodoacetate, *p*H 7.3	no reaction	no reaction

The erythrocytes were separated from the plasma within 30 minutes of the addition of the anticoagulant. They were washed four times with 3–4 volumes of 0.9% sodium chloride, and the buffy coat was removed during this procedure. The packed erythrocytes were mixed with one volume of water and 0.4 volumes of toluene and the mixture shaken vigorously for 3 minutes. After centrifugation, the lower layer containing the hemoglobin was siphoned off and dialyzed[2] overnight at 4° with gentle agitation against two changes of 200 volumes of deionized water.

After dialysis the hemoglobin solution was centrifuged at 18,000 × *g* for 20 minutes. Hemoglobin concentration was determined by conversion to methemoglobin cyanide with a molar extinction coefficient of 4.6×10^4 at 540 mμ.

Hemoglobin H was isolated and concentrated as described previously (Benesch *et al.*, 1961).

Reaction of Hemoglobin—SH Groups with Various Reagents

(a) *p-Mercuribenzoate.*—These measurements were based on the spectrophotometric titration of Boyer (1954) (*cf.* also Benesch and Benesch, 1962). In the case of deoxyhemoglobin they were performed in a tonometer cuvet kindly loaned by Dr. J. Wittenberg.

(b) *Iodoacetamide.*—The reaction with iodoacetamide was followed by the liberation of hydrogen ions at a constant *p*H of 7.3 in a Linderstrøm-Lang Jacobsen *p*H stat. The closed reaction vessel, which contained about 4 ml of solution, was immersed in a constant-temperature water bath at 25°. The solution was stirred by setting the reaction vessel on a submersible magnetic stirrer (Rank Bros., Bottisham, Cambs., England). Oxygenation and deoxygenation of the hemoglobin were carried out by passing oxygen and prepurified nitrogen, respectively, over the stirred solutions. The reaction was started by adding a ten-fold excess of iodoacetamide over the hemoglobin in the form of a 0.1 M solution. In a few experiments the iodide formed was also determined as described previously (Benesch and Benesch, 1961). For the alkylation in 2 M sodium chloride the salt solution and the hemoglobin solution were deoxygenated separately and mixed anaerobically.

(c) *Reaction with N-Ethyl Maleimide.*—The spectrophotometric procedure described previously (Benesch and Benesch, 1961) was used in this case. The initial molar ratio of *N*-ethyl maleimide to hemoglobin was 5 to 1. The reactions were carried out at room temperature in 0.1 M phosphate buffer, *p*H 7.34. Prepurified nitrogen was again used for deoxygenation, and separately deoxygenated samples were used for each time interval. Blank determinations on solutions containing no *N*-ethyl maleimide were done in all cases.

Measurement of the Bohr Effect

The Bohr effect was measured by the differential titration method described previously (Benesch and Benesch, 1961).

RESULTS

All the reactions described in this paper involve only the "reactive" —SH groups, of which one is located on each β^A chain in position 93 (Goldstein *et al.*, 1961). A wide and rather well-defined spectrum is observed in the reactivity of these —SH groups towards different reagents. A summary appears in Table I.

(1) *Reaction with p-Mercuribenzoate at pH 7.*—Figure 1 shows that both oxy- and deoxyhemoglobin react with 2.3 equivalents of *p*-mercuribenzoate at *p*H 7.0. The reaction was essentially instantaneous in both cases.

(2) *Reaction with N-Ethyl Maleimide.*—Although this compound also reacts completely with two —SH groups in both oxy- and deoxyhemoglobin, the reaction differs from the previous one in three respects. It is irreversible, it is slower, and the rates with oxy- and deoxyhemoglobin are not identical. The treatment of the blood from which the hemoglobin was obtained as well as the age of the hemoglobin solutions influence the rate of the reaction with deoxy- but not with oxyhemoglobin (Fig. 2 and 3). Attention should be drawn to the fact that in 5-day-old hemoglobin solutions, prepared from red cells treated with acid citrate dextrose, two —SH groups react completely with *N*-ethyl maleimide in deoxyhemoglobin in 30 minutes (Fig. 3). It was again confirmed in the course of this work that deoxyhemo-

[2] All viscose tubing was boiled in water for 5 minutes before use to remove reactive sulfur compounds and other contaminants.

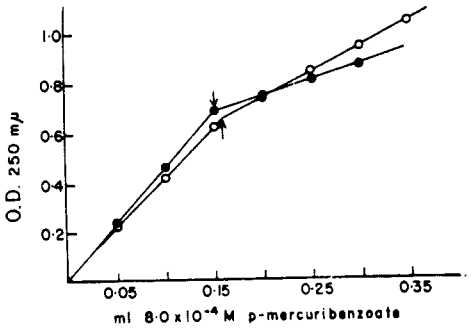

FIG. 1.—Titration of hemoglobin with *p*-mercuribenzoate. 2.5 ml of 2.1 × 10^{-5} M hemoglobin in 0.05 M phosphate buffer, *p*H 7.0, were used for each titration. Open circles: oxyhemoglobin; filled circles: deoxyhemoglobin. The optical densities are corrected for dilution. The end points, marked by arrows, correspond to 2.34 and 2.28 -SH groups per mole for oxy-and deoxyhemoglobin respectively.

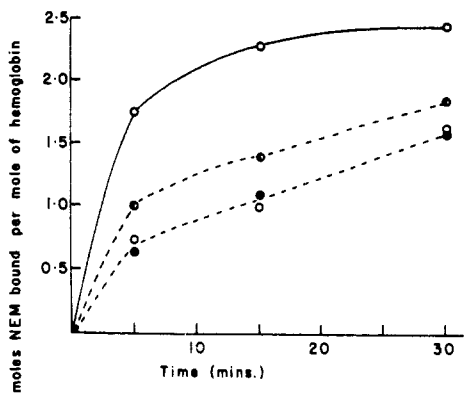

FIG. 2.—Reaction of hemoglobin with *N*-ethylmaleimide: the influence of anticoagulant treatment of the blood from which the hemoglobin was prepared. ○———○, oxyhemoglobin; identical results were obtained regardless of the method of preparation. ◐- - - -◐, deoxyhemoglobin prepared from blood treated either with ACD or AC anticoagulant. ○- - - -○, deoxyhemoglobin prepared from defibrinated blood or with neutral citrate as anticoagulant. ●- - - -●, deoxyhemoglobin prepared exactly as described by Riggs (1960).

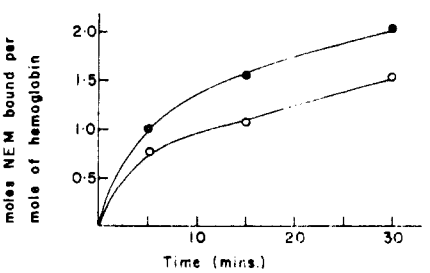

FIG. 3.—Effect of the age of the hemoglobin solution on the reaction of deoxyhemoglobin with *N*-ethyl maleimide. The hemoglobin was prepared from erythrocytes treated with ACD anticoagulant. Open circles: immediately after the preparation. Filled circles: hemoglobin stored for 5 days at +4°.

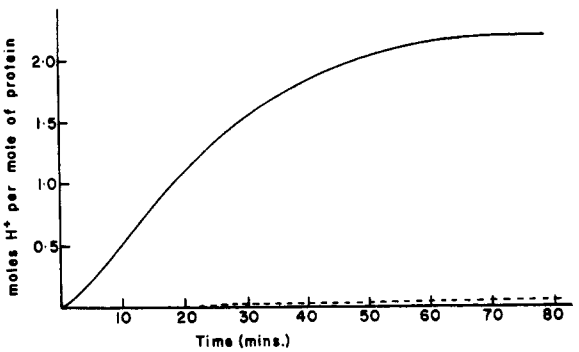

FIG. 4.—Reaction of hemoglobin with iodoacetamide. 4 ml of 4.5 × 10^{-4} M hemoglobin was used. 0.18 ml of 0.1 M iodoacetamide was added at zero time. The *p*H was kept constant at 7.34. Solid line: oxyhemoglobin; dashed line: deoxyhemoglobin.

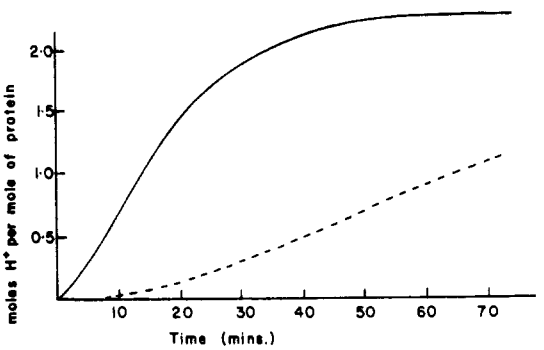

FIG. 5.—Reaction of hemoglobin with iodoacetamide in sodium chloride. 4 ml of 4.5 × 10^{-4} M hemoglobin in 2.0 M NaCl was used. 0.18 ml of 0.1 M iodoacetamide was added at zero time. The *p*H was kept constant at 7.34. Solid line: oxyhemoglobin; dashed line: deoxyhemoglobin.

globin treated in this way showed an unimpaired Bohr effect of 2.6 H^+ per mole (*cf.* Benesch and Benesch, 1961).

(3) *Reaction with Iodoacetamide.*—Iodoacetamide shows the greatest selectivity in its reaction with the —SH groups of oxy- and deoxyhemoglobin, since it reacts fully with the former and not at all with the latter under the conditions used (Fig. 4). This difference is still maintained to a considerable degree even in 2 M sodium chloride, as can be seen from Figure 5. It has been shown previously (Benesch *et al.*, 1961) that hemoglobin H has eight "reactive" —SH groups in the oxygenated state. In contrast to hemoglobin A these —SH groups react as readily with iodoacetamide in the deoxygenated as in the oxygenated protein (Table II).

(4) *Reaction with Iodoacetate.*—The —SH groups of native hemoglobin do not react at all with iodoacetate in neutral solution. This remarkable lack of reactivity is not influenced by oxygenation and is even maintained in 2 M sodium chloride.

TABLE II
REACTION OF HEMOGLOBIN H WITH IODOACETAMIDE

	moles H^+ Liberated per mole Protein
Oxyhemoglobin H	8.0
Deoxyhemoglobin H	7.4

Hemoglobin H from patient L.G. (Benesch *et al.*, 1961) was used.
Hemoglobin 1.06×10^{-4} M
Iodoacetamide 2×10^{-3} M
NaCl 0.1 M
*p*H 7.40
Reaction time: 2 hours at room temperature

DISCUSSION

The exact nature of the reaction between *N*-ethyl maleimide and the reactive —SH groups on the β chains of hemoglobin was of particular interest, since we had previously shown that the binding of 2 moles of *N*-ethyl maleimide per mole of protein leads to a 50% decrease in the Bohr effect, when this compound is reacted with oxy- but not with deoxyhemoglobin (Benesch and Benesch, 1961).

In the course of the present work we have confirmed Riggs' observation (1961) that *N*-ethyl maleimide reacts more slowly with deoxy- than with oxyhemoglobin. A quantitative comparison is, however, not possible for at least three reasons. (1) The data given by Riggs are insufficient for the calculation of reaction rates. (2) We used a larger excess of *N*-ethyl maleimide (5-fold instead of 3-fold). (3) The method for following the reaction was different (*N*-ethyl maleimide binding on the one hand and disappearance of mercury binding sites on the other).

The finding that deoxyhemoglobin can be reacted with 2 moles of *N*-ethyl maleimide/mole without influencing the Bohr effect is, in any case, independent of these rather minor differences in reaction rate.

The reaction with iodoacetamide provides the most clear-cut evidence for the profound difference in the environment of the —SH groups which must exist in the two forms of the protein. This chemical evidence for a configurational change recalls the suggestion of Wyman and Allen (1951) that the special functional properties of hemoglobin such as the heme-heme interaction and the Bohr effect could be more readily understood if it were assumed that oxygenation is associated with configurational changes in the molecule as a whole. If this line of reasoning is correct, then a hemoglobin which does not show these functional properties would also be expected not to undergo such configurational changes. We have shown previously that hemoglobin H has no heme-heme interaction or Bohr effect (Benesch *et al.*, 1961). The prediction that the reactivity of its —SH groups should therefore not change with oxygenation is borne out by the results shown in Table II. Since hemoglobin H consists of $4\beta^A$ chains (Jones *et al.*, 1959) instead of the $2\alpha^A 2\beta^A$ found in hemoglobin A, it seems likely that *interchain* interactions between unlike chains are an important factor in maintaining the special configuration of deoxyhemoglobin. This supposition derives further support from the recent studies of Briehl (1962), who concluded on the basis of U.V. rotatory dispersion measurements that deoxygenation of hemoglobin produced no major changes in the secondary structure of the globin chains.

It is also highly pertinent that in the case of myoglobin Kendrew found that the combination of this protein with oxygen involves no structural changes detectable by x-ray analysis (Perutz, 1960). The absence of oxygenation-linked conformational changes in this single-chain heme protein again bears out the conclusion that forces between the chains are responsible for the difference in structure between oxy- and deoxyhemoglobin.

REFERENCES

Benesch, R., and Benesch, R. E. (1961), *J. Biol. Chem. 236*, 405.
Benesch, R., and Benesch, R. E. (1962), *in* Methods of Biochemical Analysis, Vol. X, Glick, D., editor, New York, John Wiley and Sons, p. 43.
Benesch, R. E., Ranney, H. M., Benesch, R., and Smith, G. M. (1961), *J. Biol. Chem. 236*, 2926.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Briehl, R. W. (1962), *Fed. Proc. 21*, 72.
Chargaff, E., Ziff, M., and Hogg, B. M. (1939), *J. Biol. Chem. 131*, 35.
Drabkin, D. L. (1946), *J. Biol. Chem. 164*, 703.
Edsall, J. T. (1958), *in* Conference on Hemoglobin, Nat. Acad. Sci., Nat. Res. Council Pub. No. 557.
Goldstein, J., Guidotti, G., Konigsberg, W., and Hill, R. J. (1961), *J. Biol. Chem., 236*, PC77.
Jones, R. T., Schroeder, W. A., Balog, J. E., and Vinograd, J. R. (1959), *J. Am. Chem. Soc. 81*, 3161.
Lumry, R., and Matsumiya, H. (1958), Abstracts of Papers, 134th Meeting of the American Chemical Society, Chicago, p. 24C.
Ottesen, M., and Schroeder, W. A. (1961), *Acta Chem. Scand. 15*, 926.
Pauling, L., and Coryell, C. D. (1936), *Proc. Nat. Acad. Sci. U. S. 22*, 159, 210.
Perutz, M. F. (1960), *in* Protein Structure and Function, Brookhaven Symposia in Biology, no. 13, p. 178.
Riggs, A. (1960), *J. Gen. Physiol. 43*, 737.
Riggs, A. (1961), *J. Biol. Chem. 236*, 1948.
Takashima, S., and Lumry, R. (1958), *J. Am. Chem. Soc. 80*, 4238.
Wyman, J., Jr., and Allen, D. W. (1951), *J. Polymer Sci. 7*, 499.