Kinetics of the Reaction of the Sulphydryl Groups of Human Hemoglobin and Globin with *p*-Mercuribenzoate in Alkaline Solution

The dissociation of hemoglobin in alkaline solution has been the subject of relatively few investigations[1,2]. HASSERODT and VINOGRAD reported the reversible dissociation of human carbonmonoxyhemoglobin into dimers in glycine-glycinate-NaCl buffer which began at pH 10 and was complete at pH 11 unaccompanied by denaturation[2]. Denaturation occurs above pH 11 and has been studied by other investigators[3]. An interesting two-step process in the dissociation of horse hemoglobin at alkaline pH has been reported[4].

To provide insight into this dissociation process in alkaline solution and to explore further the comparison between hemoglobin and globin under various conditions we have investigated the kinetics of the reaction of the masked sulphydryl groups of hemoglobin and globin with *p*-mercuribenzoate (PMB) in glycine-glycinate buffer, pH 10.5. In addition starch gel electrophoresis studies were carried out to determine whether the reaction with PMB being followed spectrophotometrically was paralleled by dissociation into α- and β-chains.

Materials and methods. Human hemoglobin was prepared by the ammonium sulphate method[5] and, after dialysis against distilled water, was deionized by passage through ion-exchange resin AG 501-X8 (D) (Bio-Rad Laboratories, Richmond, California). The human globin was prepared from hemoglobin according to the method of ROSSI-FANELLI, ANTONINI and CAPUTO[6]. The protein concentrations of the final products were calculated on the basis of the extinction coefficients $E^{1\%}_{1\,cm} = 8.0$ at 280 nm for globin (tetramer) and $E^{1\%}_{1\,cm} = 8.5$ at 541 nm for oxyhemoglobin (tetramer).

Ferrihemoglobin was prepared from oxyhemoglobin by addition of stoichiometric amounts of $K_3\,Fe(CN)_6$[7] and dialyzed in the cold against distilled water for 44 h. The concentration was calculated from $E = 9.5 \times 10^3$ (heme) at 500 nm. PMB was obtained from L. Light and Company, Colnbrook, England, and used without further purification. The solutions were prepared by dissolving a weighed amount of PMB in 0.04 *N* NaOH. The final concentration was determined at 232 nm by using the extinction coefficient $E = 1.69 \times 10^4\ M^{-1}cm^{-1}$. The buffer used was glycine-sodium glycinate, $\mu = 0.1$, pH = 10.5.

Titrations of the 'free' sulphydryls were carried out by the method of BOYER[8]. In those systems in which a reaction of the 'masked' sulphydryls begins before the addition of sodium chloride the result can only be an approximation. In some experiments EDTA was added to the reaction solution to prevent possible oxidation of the sulphydryl groups through catalysis by heavy metal cations. Addition of EDTA produced no significant changes in the results.

The reaction rate of the masked-SH groups with PMB was measured spectroscopically at 10 °C by following the change of optical density at 250 nm by a method described previously[9]. The total optical density change between time zero and time infinity was calculated from titrations of the protein with PMB in phosphate buffer, pH 7 and extrapolation to 4 sulphydryls. This method has proved to be accurate in those cases in which the reaction was extremely rapid, thus allowing a check to be made. An exception was occasionally noted in the reaction of globin in glycine-glycinate buffer in which the total optical density change observed was somewhat higher than that calculated. This latter effect could be due to the onset of a denaturation reaction which was sometimes noted also in the first order rate plots as a drift upward which began after 30 min. All spectrophotometric measurements were made with a Beckman Model DB-G spectrophotometer. The pH was measured with a Radiometer Copenhagen 25 or 4 pH meter. Starch gel electrophoresis was carried out with the discontinuous buffer system of POULIK[10]. After electrophoresis the gels were sliced into halves, one of which was stained with benzidine, the other with nigrosine.

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Table I. Titration of human globin[a] and hemoglobin[b] with PMB in various buffers

Protein	Buffer	Final pH	Number of reactive sulphydryls per hemoglobin tetramer or globin dimer
Oxyhemoglobin	Glycine-glycinate	9.6–10.5	2.05
Oxyhemoglobin[c]	Glycine-glycinate + EDTA[d]	9.6	2.08
Globin	Glycine-glycinate	10.5	1.9

[a] Globin concentration (dimer) = $1.4–1.8 \times 10^{-5}\ M$. [b] Hb concentration (tetramer) = $0.70–1.2 \times 10^{-5}\ M$. [c] Concentration = $0.6 \times 10^{-4}\ M$. [d] Concentration = $1 \times 10^{-3}\ M$.

The reconstituted ferrihemoglobins were obtained by coupling native human globin in glycine-glycinate buffer with measured amounts of ferriprotoheme in stoichiometric amount and in the ratio of heme globin dimer 0.5/1; 1/1; 1.5/1; 2/1 based on a titration by the method of ANTONINI et al.[11]. The ferriprotoheme was prepared by the method of LABBE and NISHIDA[12].

Control experiments to determine whether denaturation had taken place during the reaction of PMB with globin were performed by addition of a stoichiometric amount of heme to the reaction mixture followed by reaction with dithionite and then CO. Comparison of the spectra with those of the corresponding ferri, deoxy- and carbonmonoxyhemoglobin derivatives revealed no significant differences between 700 nm and 500 nm in agreement with the stability of globin at pH 11 reported by HAUROWITZ, HARDIN and DICKS[3].

Table II. Rates of reaction of masked sulphydryl groups of human globin and hemoglobin with PMB in glycine-glycinate buffer, pH 10.5, t = 10 °C

Protein	NaCl Concentration (M)	$K \times 10^3/min^{-1}$
Globin[a]	None	39.6
Globin[a]	0.1	38.0
Globin[a]	0.5	45.6
Oxyhemoglobin[b]	None	5.1
Oxyhemoglobin[b]	0.5	4.2
Ferrihemoglobin[c]	None	8.5

[a] Final concentration: Globin, 1.0×10^{-5} — 1.8×10^{-5} M (dimer; PMB, 0.542 — 0.686×10^{-4} M. [b] Final concentration: Hemoglobin, 0.86×10^{-5} (tetramer); PMB, 0.60×10^{-4} M. [c] Final concentration: Hemoglobin, 0.934×10^{-5} M (tetramer); PMB, 0.734×10^{-4} M.

Table III. Comparison of the rate constants for the slow phase in the reaction of globin[a] with PMB after recombination with heme in glycine-glycinate buffer, pH = 10.5, t = 10 °C

Hemes/dimer	$K \times 10^3/min^{-1}$
0	32.5
0.5	19.6
1	14.8
1.5	9.4
2	7.4

[a] Final concentration (dimer) = 1.82×10^{-5} M; Final PMB concentration = 0.670×10^{-4} M.

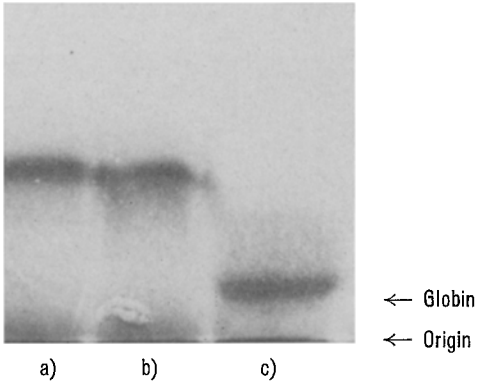

Fig. 1. Starch gel electrophoresis of globin A, 4 mg/ml, in glycine-glycinate buffer pH 10.5, μ = 0.1, with and without NaCl, 0.5 M, treated with PMB 10 moles/protein tetramer after 4 h of incubation at 4 °C. Nigrosine stain: a) globin + PMB, no NaCl; b) globin + NaCl + PMB; c) untreated globin.

Results and discussion. Titration of the reactive sulphydryls of human oxyhemoglobin with PMB in glycine-glycinate buffer, pH 10.5, indicated the presence of 2 such groups per tetramer (Table I). These results taken together with the report of HASSERODT and VINOGRAD[2] that carbonmonoxyhemoglobin exists largely as a dimer under similar conditions suggests that the species present is the $\alpha_1\beta_1$-dimer[13] in which only 1 sulphydryl is 'free'. By contrast titration of the sulphydryl groups in globin under the same conditions indicated ca. 4 reactive groups per tetramer suggesting the existence of either globin monomers or the $\alpha_1\beta_2$-dimer.

The rate of the reaction of the masked sulphydryl groups of globin and oxyhemoglobin with PMB in glycine-glycinate buffer, pH 10.5 followed first order kinetics (first order in protein but independent of the concentration of PMB). This behaviour is in contrast with the behaviour of globin or hemoglobin in phosphate buffer, pH 7[9,14] in which the rate is first-order in PMB, suggesting that a different mechanism is operative in glycine-glycinate buffer, perhaps one involving a slow, rate-determining change in protein followed by a fast reaction with PMB. The rate of the reaction of globin in glycine-glycinate buffer (Table II) was faster than in phosphate buffer, pH 7[9]. These kinetic results parallel the relative degree of dissociation of globin in the various buffers predicted on the basis of the number of 'free' sulphydryls titrated. Further, globin reacts with PMB in glycine-glycinate buffer, pH 10.5, faster than does oxyhemoglobin (Table II), suggesting that globin is dissociated to a much greater extent than is oxyhemoglobin under these conditions. The effect of sodium chloride on the reaction rate of the masked sulphydryls of globin or oxyhemoglobin with PMB appears to be very slight (Table II). That the reaction begins without sodium chloride can be taken as further evidence of dissociation of the protein into subunits. Additional evidence is provided by the experiments in which PMB was added to solutions of reconstituted ferrihemoglobin (Table III). It can be seen that the reaction rate decreased as the ratio heme/globin increased and the composition approached that of ferrihemoglobin. Comparison of the rate of reaction of the system containing 2 hemes/dimer with the rate of ferrihemoglobin (Table II) reveals that the two rates are approximately equal. Rate plots of the reaction in which PMB was added to reconstituted ferrihemoglobin were biphasic apparently due to the presence of denatured globin in the preparation used since the rapid phase amounted to ca. 10% of the total reaction. The electrophoresis studies of the reaction of globin (Figure 1),

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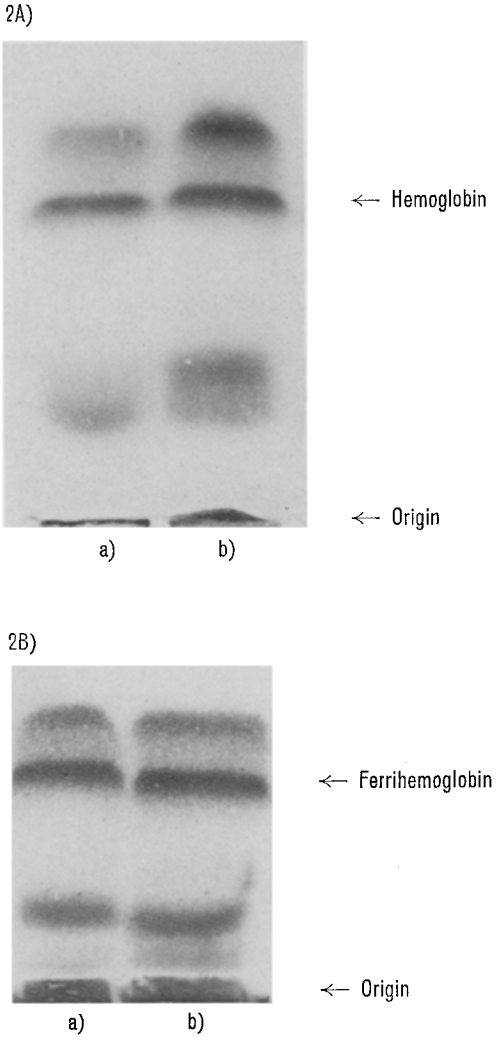

Fig. 2. Starch gel electrophoresis of HbO_2, and ferrihemoglobin, 4 mg/ml, in glycine-glycinate buffer with and without NaCl, 0.5 *M*, treated with PMB 10 moles/Hb tetramer after 4 h incubation at room temperature. Nigrosine stain: A) HbO_2 in glycine-glycinate buffer, pH 10.5, $\mu = 0.1$. a) HbO_2 + PMB + NaCl; b) HbO_2 + PMB, no NaCl. B) Ferrihemoglobin in glycine-glycinate buffer, pH 10.5, $\mu = 0.1$. a) ferriHb + PMB + NaCl; b) ferriHb + PMB, no NaCl.

oxyhemoglobin (Figure 2A) and ferrihemoglobin (Figure 2B) in glycine-glycinate with PMB indicate complete separation into 2 new bands after 4 h for globin and partial separation into 2 new bands for oxy- and ferrihemoglobin after 4 h. The new bands presumably correspond to the α^{PMB}- and β^{PMB}-chains.

Conclusions. Change in conformation and/or increases in dissociation that occur in hemoglobin at pH 10.5 and that appear in globin upon removal of the heme result in greater reactivity of the sulphydryl groups toward PMB. The results reported herein are consistent with the greater dissociation of hemoglobin at alkaline pH[1,2] and with the known differences in conformation and dissociation between globin and hemoglobin[15–18].

Résumé. La mesure des taux de réaction des sulfhydryles masqués de l'hémoglobine et de la globine humaines avec le *p*-mercurichlorobenzoate montre que ce taux de réaction est plus grand en tampon glycine-glycinate au pH 10,5 qu'en tampon phosphate au pH 7. Il est plus grand pour la globine que pour l'hémoglobine, dans les mêmes conditions. Le titrage des groupes de sulfhydryles «libres» dans le dimère d'hémoglobine présent en tampon glycine-glycinate au pH 10,5 indique la présence d'un tel groupe ce qui fait supposer que l'espèce en question est le dimère $\alpha_1 \beta_1$.

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The Effect of Oxygen on the Hemoglobin-Binding Capacity of Haptoglobins

Earlier it was demonstrated that, depending on the phenotype, the peroxydase activity of the haptoglobin-hemoglobin complex formed in serum heat-treated at 56 °C decreases[1]. Parallel with the decrease of peroxydase activity, and likewise depending on the phenotype, the hemoglobin-binding capacity (HbBC) of haptoglobin decreases[2]. In the same study it was also demonstrated that the decrease of HbBC due to of heat treatment, might be restored completely in the case of phenotype Hp 1-1 and partially in the case of phenotype Hp 2-1 and Hp 2-2, if into the heat-treated serum an oxygen stream was let in for 10 min[2]. The present study discusses the effect of oxygen on HbBC of native serum.

Method. Sera of all 3 phenotypes were divided into 2 parts. One part was left untreated, the second oxygenated for 10 min in a tube measuring 15 × 105 mm. The amount of oxygen was 0.6 to 0.8 l in total. At intervals, the scum produced was destroyed with a Pasteur's pipette, and after oxygenation completely disappeared by centrifugation. Then the HbBC of both the native and oxygenated samples was determined on the agar-plates of the Hyland's 'haptoglobin electrophoresis test'.

Results. It was found in all sera of the 3 phenotypes that the HbBC of oxygenated samples increased as compared to the original HbBC of serum. The increase was most distinct in sera of the phenotype Hp 1-1: 20–40 mg/100 ml HbBC. The Figure shows the value of HbBC in the native and oxygenated sample of serum of the phenotype Hp 1-1.

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