# EFFECT OF HAPTOGLOBIN ON IMMUNOCHEMICAL PROPERTIES OF HEMOGLOBIN

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The immunochemical properties of human oxyhemoglobin and the haptoglobin–hemoglobin complex obtained from human $HbO_2$ and dog Hp were compared relative to antihemoglobin serum. It was shown by the complement fixation test that the complex reacts more strongly than oxyhemoglobin with the serum. It is suggested that the conformation of oxyhemoglobin is modified when it is bound with haptoglobin.

Changes in certain properties of hemoglobin (Hb) on binding with haptoglobin (Hp), i.e., in the Hp–Hb complex, have recently been extensively investigated. The results serve to identify the binding centers. The investigations have included determination of the accessibility of titratable carboxyl groups and imidazole rings of histidine residues [6] and the decrease in reactivity of SH-groups in the position of the $\beta$-(93) chain in Hp–Hb complexes by comparison with Hb [8]. Changes in the oxygen dissociation curve of Hb on binding with Hp [11] have been demonstrated. However, the immunochemical properties of the Hp–Hb complex relative to antihemoglobin serum have not hitherto been studied. The object of the investigation described below was to study changes in the immunochemical properties of human oxyhemoglobin ($HbO_2$) and to compare them with those of dog Hp by means of a quantitative immunochemical method – the complement fixation test (CFT).

## EXPERIMENTAL METHOD

$HbO_2$ was isolated by the method of Rossi-Fanelli et al. [14], and deoxy-Hb was obtained with the aid of crystalline $Na_2S_2O_4$. Rabbits were immunized with $HbO_2$ in accordance with the following scheme: first injection (30 mg $HbO_2$) subcutaneously together with Freund's complete adjuvant, 10 days later 30 mg $HbO_2$ twice a week; in the next 2 weeks $HbO_2$ in doses of 40 mg and 50 mg twice a week. Seven days after the last injection the starving animals were exsanguinated. If the antibody titer was too low, the animals were left for the second cycle of immunization. The specificity of the resulting antisera was verified immunochemically: from the absence of precipitation arcs with plasma proteins and by immunoelectrophoresis by Grabar's method in Scheidegger's modification [5]. The complement fixation test was carried out by the method described by Uspenskaya et al. [1], and partially modified by the authors (the Hp was purified on a column with Sephadex G-200 in 0.1 M phosphate buffer, pH 7.2). The Hp concentration was determined spectrophotometrically using the coefficient $E^{1\%}_{1cm} = 12$ at 280 nm and by Lowry's method [4]. Hp–Hb complexes were obtained by mixing equimolar amounts of Hp and $HbO_2$ followed by purification on a column with Sephadex G-75 in 0.1 M phosphate buffer, pH 7.25. The concentration of $HbO_2$ and of Hp–Hb complex was determined spectrophotometrically using the coefficient of extinction $E^{1\%}_{1cm} = 8.5$ at 540 nm.

The complement fixation curves illustrated in this paper were checked with two antihemoglobin sera and identical results were obtained.

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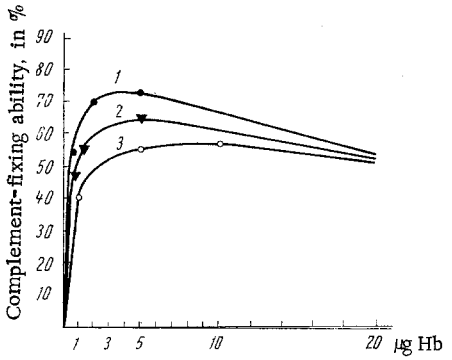


Fig. 1. Complement-fixing ability of $HbO_2$, of Hp–$HbO_2$ complex, and of deoxy-Hb with antihemoglobin serum: 1) Hp–$HbO_2$ complex; 2) $HbO_2$; 3) deoxy-Hb. Abscissa, content of Hb (in μg); ordinate, complement-fixing ability (in %).

## EXPERIMENTAL RESULTS

Dog Hp is a comparatively well studied protein. In its ability to bind with Hb, it is identical with human Hp 1-1 [4]. The specimen of Hp isolated in these experiments was electrophoretically homogeneous on free boundary electrophoresis in 0.1 M veronal buffer (pH 8.6) and in starch gel by Smithies' method [15]. When investigated in the Spinco E ultracentrifuge, it moved as a single symmetrical peak with sedimentation coefficient of 3.8 S (0.5% solution in 0.66 M phosphate buffer, pH 7.5, temperature 20°C). The molecular weight of Hp, determined by the unstable equilibrium method and calculated from its distribution at the meniscus (7200 rpm) was 80,000, in agreement with data in the literature [4]. Investigation of the hemoglobin-binding ability of the Hp by the method of Lawrell and Nyman [7] showed that 1 mole Hp binds 1 mole $HbO_2$. These findings were all evidence of the purity of the Hp used.

The CFT control showed that Hp does not bind antiserum against Hb. For this reason, during isolation of the Hp–Hb complex, attention was concentrated on the absence of free Hb. For this purpose the complex was purified by chromatography on Sephadex G-75. The product obtained after chromatography was electrophoretically homogeneous on a starch block and corresponded completely to the saturated Hp–Hb complex.

Complement fixation curves for $HbO_2$ and for the Hp–$HbO_2$ complex are shown in Fig. 1. It is clear that the complex binds antihemoglobin serum better than $HbO_2$. The change in the complement-fixing power of the complex compared with $HbO_2$ suggests that a change takes place in the conformation of $HbO_2$ when it binds with Hp. This conclusion is confirmed by recently published work on the immunochemistry of Hb and its derivatives. For instance, conformation differences between deoxy-Hb and $HbO_2$ found by Perutz by the method of x-ray structural analysis [10] have recently been demonstrated also by Reichlin et al. [13] by an immunochemical method. The identity of the changes in the conformational and immunochemical characteristics of a series of modified hemoglobins has been observed by Atassi [3]. In additon, the fact that the β-(93) SH groups in the Hp–Hb complexes are less accessible for reaction than those in $HbO_2$ may also indicate conformational changes in $HbO_2$ during binding with Hp. Since a similar decrease in reactivity of these groups has been established for the deoxy-form of Hb, Malchy and Dixon [9] have postulated that $HbO_2$ in the Hp–Hb complex has a conformation analogous to that of deoxy-Hb.

These immunochemical results show directly that conformational changes take place in the $HbO_2$ molecule when it binds with Hp, and they are also evidence of differences in the conformations of deoxy-Hb and $HbO_2$ in the complex, for they have different complement fixation curves. For instance, it follows from Fig. 1 that, by contrast with the Hp–Hb complex, deoxy-Hb is more weakly bound with antihemoglobin serum than $HbO_2$. This weaker binding of deoxy-Hb is in agreement with the earlier observations of Reichlin [13]. The conformational changes in Hb on binding with Hp demonstrated in the present investigation can evidently be compared with the results of Japanese workers who studied the breakdown of heme into Hb by the enzyme heme-α-methyloxygenase. For example, good substrates for this enzyme have been shown to be HbA in the form of the Hp–HbA complex of myoglobin (Mb), and HbH; free HbA is split by the enzyme only to a very slight degree [12]. The more rapid enzymic degradation of Hb when bound with Hp in a complex can be attributed to conformational changes in the Hb. These conformational changes also correlate with the disappearance of heme-heme interaction in Hb in the Hp–Hb complex, and it is also absent in Mb and in HbH.

The reasons why the Hp–Hb complex binds antibodies to a greater degree than $HbO_2$ are not yet known. Asconas et al. [2] found that there is a latent center of antigenicity in the β-chains of Hb, and it can accordingly be postulated that the conformational changes in Hb during its binding with Hp affect the β-chains in $HbO_2$, so that the latent centers of antigenicity become more accessible for reaction with antibodies.

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