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THE INTERACTION OF BROMTHYMOL BLUE WITH HEMOGLOBIN AND ITS EFFECT ON THE OXYGEN EQUILIBRIUM

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SUMMARY

The equilibria and kinetics of the reaction of the dye bromthymol blue with various derivatives of human hemoglobin, as well as with several other proteins, have been studied. In the case of normal hemoglobin the affinity of the dye is much greater for the deoxy than for the oxy form and this difference is reflected in the kinetics of the reaction. Conversely the dye has a marked effect on the oxygen equilibrium. The characteristic difference of behaviour shown by the oxy and deoxy forms of normal hemoglobin is lacking in myoglobin and those modified forms of hemoglobin which have simple hyperbolic oxygen-equilibrium curves.

INTRODUCTION

The present study originated in an attempt to measure the kinetics of the liberation of the Bohr protons by human hemoglobin during oxygenation. We had hoped to follow the pH change colorimetrically of an unbuffered solution containing a suitable indicator in a stopped flow apparatus and relate it to the uptake of oxygen. This proved impossible owing to the indicator, bromthymol blue, itself taking part in a reaction with hemoglobin proceeding roughly at the same rate as that with oxygen. The resulting optical changes masked the expected effects but the reaction itself proved to be of some interest.

Bromthymol blue combines rapidly and reversibly with hemoglobin, thereby changing its pK and becoming a weaker acid. The pK of the free dye is 7.1 at 20°; that of the dye bound to the protein appears to be above 8. Hemoglobin is capable of combining with a considerable amount of dye and there is no evidence of saturation at a dye/heme ratio of 10:1. The affinity of the dye for the protein changes with pH, being greatest at acid pH, where the dye is largely unionized. It is markedly greater for hemoglobin than for oxyhemoglobin. As a reciprocal effect the dye greatly reduces the oxygen affinity at pH above the isoelectric point. The velocity of the combination of dye and protein in most of our experiments had a half time in the range 5–100 msec.

Abbreviations: BTB, bromthymol blue; HbCPA, HbCPB, HbCPA + B, hemoglobin digested with, respectively, carboxypeptidase, A, B and A + B.

Under a given set of conditions the velocity of the reaction with deoxyhemoglobin is between two and four times greater than with oxyhemoglobin, depending on the pH. Other derivatives of hemoglobin which were studied, CO-hemoglobin, ferrihemoglobin, ferrihemoglobin cyanide, and ferrihemoglobin fluoride, are all closely similar to oxyhemoglobin in the speed with which they combine with dye. The three modified hemoglobins HbCPA, HbCPB, HbCPA + B which result from digestion of hemoglobin with carboxypeptidase A or B or A + B (see ref. 1) show somewhat different velocities; but it is suggestive that only in the case of HbCPB, in which alone the strong heme–heme interactions are preserved, is there a significant difference between the rates for the oxy and deoxy forms. In horse myoglobin also, which contains only a single heme and for which the exponent n of the Hill equation is unity, just as for HbCPA and HbCPA + B, there is no significant difference between the oxy and deoxy forms although the absolute rate of the reaction is much less than for the hemoglobins and falls off markedly as the reaction proceeds. In human globin the rate also changes as the reaction proceeds; here, however, the initial rapid phase of the reaction may be estimated as at least 10 times faster than that of deoxyhemoglobin. Thus it would seem that the rate of the dye reaction is a highly characteristic feature of the protein molecule and reflects in a sensitive way any changes in its structure or configuration. In the case of a very different protein, bovine serum albumin, the reaction is complex and under certain conditions there is an "overshooting" phenomenon.

EXPERIMENTAL

Materials

Human hemoglobin was prepared by the same method employed in earlier studies[2] from blood obtained from the blood bank of the hospital.

The three hemoglobins HbA, HbB, and HbA + B were prepared by digestion with carboxypeptidase A, B, or a mixture of the two as described by ANTONINI *et al.*[1].

The horse ferromyoglobin was prepared from a stock of ferrimyoglobin by enzymic reduction[3]. The stock ferrimyoglobin was prepared according to ROSSI-FANELLI[4].

Human globin was prepared by the method of ROSSI-FANELLI *et al.*[5].

Bovine serum albumin was a crystalline preparation obtained from Armour. It was not purified.

Bromthymol blue (MW 624) was obtained from Merck.

Spectrophotometry

Most of the static spectrophotometric measurements were made with a Beckman DK 1 instrument, a few in a Beckman DU.

Concentrations

The concentrations of hemoglobin were determined spectrophotometrically at $\lambda = 540$ mμ after converting the material into the oxygen derivative. The extinction coefficient was taken as $\varepsilon_{540\ m\mu} = 1.45 \cdot 10^7$ cm²/mole heme.

The concentrations of the dye, whenever they were not known stoichiometrically, were likewise determined spectrophotometrically at $\lambda = 620$ mμ, after adjusting the pH to 10, on the basis of $\varepsilon_{620\ m\mu} = 2.25 \cdot 10^7$ cm²/mole.

The concentrations of myoglobin were determined at $\lambda = 500$ mμ on the basis $\varepsilon_{500\ m\mu} = 0.98 \cdot 10^7$ for the ferri form at pH 7.

The concentrations of globin and bovine serum albumin were calculated from stoichiometric data.

Oxygen equilibrium

The oxygen equilibrium measurements were made by the spectrophotometric method of ROSSI-FANELLI AND ANTONINI[3].

Kinetic measurements

These were made with a GIBSON stopped flow apparatus[6]. The dead time of the instrument, as determined by calibration with the well characterized myoglobin-carbon monoxide reaction, was 3 ± 0.3 msec. The band width employed did not exceed 4 mμ. The light path of the observation tube was 2 cm.

RESULTS

Spectrophotometric titration of BTB in the presence of oxyhemoglobin

A proof of the interaction of dye and protein is provided by titrations of the dye in the presence of varying amounts of protein.

In these titrations pH was measured directly and α, the average fractional ionization of the dye, partly free, partly bound, was determined from spectrophotometric measurements at $\lambda = 620$ mμ, a wavelength at which the extinction coefficient of the ionized form of the free dye is a maximum ($2.25 \cdot 10^{-7}$) and the extinction coefficient of the unionized form is negligible. A correction was applied for the absorption due to the hemoglobin as determined from a blank.

Values of $\log \alpha/(1-\alpha)$ *versus* pH were plotted for various fixed hemoglobin concentrations. When there was no added hemoglobin the result was a straight line of unit slope with a pK of 7.10, as was to be expected. In other cases the plot also gave a straight line, but the slope of the line, designated by n, decreased progressively with increasing hemoglobin concentration and the pH corresponding to $\alpha = 1/2$, which gives the average apparent pK, increased. For technical reasons experiments of this kind were limited to oxyhemoglobin. The results are summarized in Fig. 1.

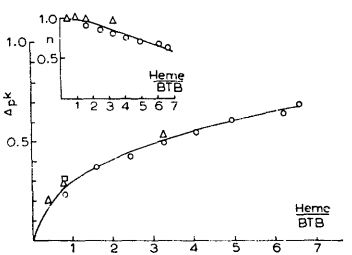

Fig. 1. Apparent pK shift of BTB at a concentration of 10^{-4} M in presence of varying amounts of hemoglobin in 0.2 M phosphate buffer at 20°. Abscissa gives molar ratio of total heme to total dye in the solution. Insert shows values of n (see text) under same conditions. Different symbols represent different experiments.

They indicate qualitatively that the dye is bound by the protein and that the pK of the bound dye is higher than that of the free dye. This implies a difference of affinity of the two forms of the dye for oxyhemoglobin.

Absorption spectrum of the dye in the presence of protein

It will be seen that although the procedure used in the above analysis does not involve any knowledge of the absolute value of the extinction coefficient at $\lambda = 620\,m\mu$, since it depends only on the ratio of two optical densities, the tacit assumption is that there is no change, or at least no considerable change, in the absorption spectrum of the dye when it is bound by hemoglobin. The effect of the combination of dye and protein on the dye spectrum is indeed fundamental not only to the interpretation of the titration data but also to the study as a whole and it was therefore investigated with bovine serum albumin. Hemoglobin could not be employed over the whole range of wavelengths because of its own absorption.

Fig. 2 shows two families of spectra, one for the dye alone and the other for the dye in the presence of bovine serum albumin. Each curve of each family corresponds to a different pH value, but in the figure pH may be regarded as having been eliminated as a parameter. The close similarity of the two families is apparent; the positions of the maxima and the isosbestic points are much the same for both, although under conditions where the dye is partially dissociated the curves seem to be slightly less sharp when protein is present than when the dye is alone.

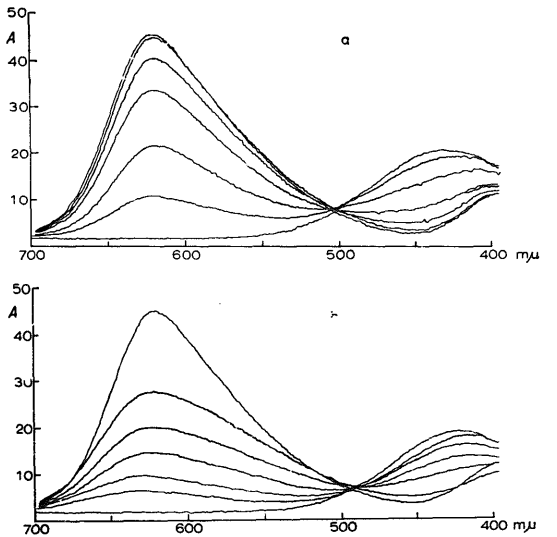

Fig. 2. Absorption spectra of BTB: a, alone; b, in the presence of 1 g/l bovine serum albumin. Each curve corresponds to a different pH, in 0.2 M phosphate buffer.

Ignoring the significance of such details, however, it is clear that there are no major spectral differences between free and bound dye. Certainly at $\lambda = 620$ mμ the absorption of the undissociated form of the dye, whether free or bound, is almost zero and that of the dissociated form is essentially constant. It was also verified independently that the extinction coefficient of a solution of BTB at $\lambda = 620$ mμ is independent of the presence of large amounts of oxyhemoglobin between pH 9.5 and 10.5, where the dye is completely dissociated.

Equilibrium dialysis

In order to explore the equilibrium between dye and protein we carried out a series of dialysis experiments in 0.2 M phosphate buffers of various pH values. After equilibration for 48h in the cold room (at about 4°) with continuous shaking the absorbancies of dialysate and residue were measured both at the original pH and again after making the solutions strongly alkaline with carbonate. From the results obtained the concentrations of bound and free dye in the residual solution were calculated, as well as the pK of the bound dye, assuming n to be unity.

Fig. 3 shows the amount of dye bound per heme as a function of the concentration of free dye at two different pH values. It will be seen that both sets of results, those at pH 6.5 and those at 7.5, give an essentially linear relationship up to 10 molecules of dye per heme, as if the dye binding sites were more or less alike and the hemoglobin remained always far from saturated. At higher dye concentrations the protein was precipitated. The pK values of the bound dye (as distinct from the average pK values for the bound and free dye discussed above) are dependent on small differences and showed a correspondingly large amount of scatter. They ranged from 8 to 8.5.

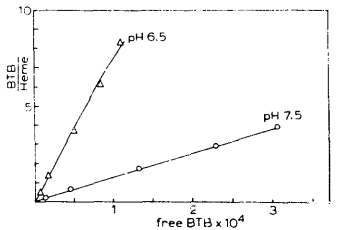

Fig. 3. Results of an equilibrium dialysis experiment in 0.2 M phosphate buffer. Ordinates give moles BTB bound per heme. Abscissae give concentration of free BTB in moles per litre. Hemoglobin concentration $1.3 \cdot 10^{-4}$ M (in heme).

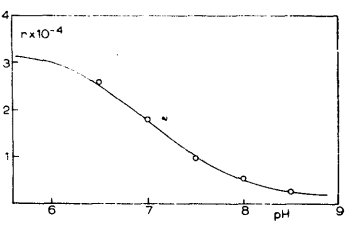

Fig. 4. Affinity of BTB for hemoglobin in relation to pH. Points shown represent averages from 8 different experiments. Ordinates give BTB bound per heme divided by concentration of free BTB. Equilibration temperature 4°. Solvent 0.2 M phosphate buffer. Hemoglobin concentration varied from $0.6 \cdot 10^{-4}$ to $0.20 \cdot 10^{-4}$ M in heme between different experiments.

Fig. 4 shows the pH dependence of the affinity of BTB for hemoglobin. In eight experiments the protein concentration varied from 0.6 to $2.0 \cdot 10^{-4}$ M heme. The ordinates give moles of dye bound per heme divided by the concentration of free dye in moles per litre, *i.e.* the slopes of curves such as those shown in Fig. 3. In these

experiments the dye/heme ratio varied up to about 3.75. The original data showed a large amount of scatter and the plotted points represent an average.

The above results all refer to oxyhemoglobin. A few similar experiments were attempted on deoxyhemoglobin. The procedure used was the same except that argon was bubbled through the dialysate solution during the equilibration. The results were much less consistent than those involving oxyhemoglobin, possibly due to removal of dye from the solution on the surface of the bubbles. They leave little doubt, however, that the affinity of the dye for hemoglobin is greater than that for oxyhemoglobin, the ratio of the two increasing from about 1.5 at pH 6.5 to roughly twice that value at pH 8.5.

Kinetic observations of combination of BTB with protein

In these experiments a solution of dye was placed in one syringe of the GIBSON stopped flow apparatus and a solution of protein in the other. The apparatus is so constructed that on operation of a lever equal volumes (about 0.3 ml) of the two solutions are suddenly mixed and forced into an observation chamber where they are viewed under monochromatic light by a photomultiplier tube whose response is recorded by an oscilloscope. From the record of the oscilloscope changes with time of the absorbancy can be readily obtained; but it should be emphasized that since no observation is made in the absence of solution no information as to absolute values of the absorbancy is provided.

Since the dead time of the instrument was 3 msec it was necessary to choose the conditions of the experiments such that no large part of the reaction would occur within this interval. In most cases this was not a serious problem, though globin and bovine serum albumin were exceptions. In all but these cases it was possible to estimate absorbancy changes referred to zero time with reasonable assurance by extrapolation, treating the reaction as first order (*i.e.* taking $d\log A/dt$ as constant) over the small time interval involved (3 msec). This was done whenever the resulting corrections were significant. In kinetic measurements of this kind a comparison of the observed values of the absorbancy changes with the results of static spectrophotometry is valuable as a check. Such a check was often made in analyzing our results.

Reaction of dye with hemoglobin and oxyhemoglobin

In these experiments a buffered solution of the dye was completely deoxygenated in a tonometer and transferred at once to one of the storage syringes of the apparatus. The hemoglobin solution, in the same buffer, was also deoxygenated in a tonometer and transferred to the other syringe. After the reaction was measured the syringe containing the hemoglobin was removed from the apparatus and the solution equilibrated with air. The syringe was then replaced and the reaction with oxyhemoglobin measured. This procedure ensured that the measurements on the oxygenated and deoxygenated forms were made under identical conditions.

Figs. 5 and 6 show the striking difference in the rates at which hemoglobin and oxyhemoglobin react with dye at pH 7.38. The exact conditions of this experiment are given in the legend of Fig. 5, but it may be noted here that the heme/dye ratio was 1.7. Since as many as 10 dye molecules can combine with one heme equivalent of protein without producing saturation, the number of dye binding sites was in

great excess. In Fig. 5 the dotted lines give the asymptotes obtained from the final limiting deflection of the oscilloscope corresponding to equilibrium. They show that in the case of oxyhemoglobin the last plotted point corresponds to about 70% attainment of equilibrium; in the case of deoxyhemoglobin, to over 90%.

It will be seen from Fig. 6 that the rate of the reaction falls off progressively with time, as might be expected. The initial slopes of the two curves, however, provide a good basis of comparison. That of curve a (for hemoglobin) corresponds to a rate of 21/sec, or nearly 4 times that given by curve b (for oxyhemoglobin) which is 5.5/sec (in assigning these dimensions we treat the reactions as pseudo first order).

Fig. 5 shows that the total absorbancy change, 0.197, for hemoglobin is about twice as great as that for oxyhemoglobin, 0.097. This is in conformity with the results of the equilibrium dialysis experiments, in which the absorbancy data, at least for deoxyhemoglobin, are, however, a good deal less reliable.

Although the general picture provided by Figs. 5 and 6 is typical, the absolute values of the absorbancy changes and the rate constants are dependent on the conditions. Of these the pH is of particular interest. Fig. 7 shows the initial values of

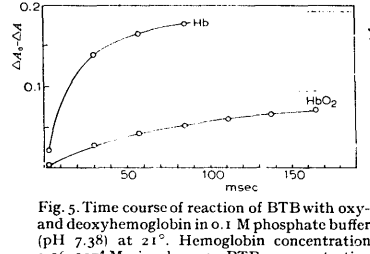

Fig. 5. Time course of reaction of BTB with oxy- and deoxyhemoglobin in 0.1 M phosphate buffer (pH 7.38) at 21°. Hemoglobin concentration $0.36 \cdot 10^{-4}$ M in heme; BTB concentration $0.21 \cdot 10^{-4}$. Here and elsewhere concentrations refer to the reaction mixture in the chamber, not to solutions before mixing. Ordinates give absorbancy change at time t, measured from $t = 0$. Dashed lines give asymptotes, *i.e.* total absorbancy changes. Absorbancies were measured at $\lambda = 620$ mμ.

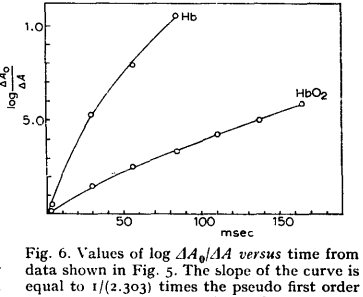

Fig. 6. Values of $\log \Delta A_0/\Delta A$ *versus* time from data shown in Fig. 5. The slope of the curve is equal to $1/(2.303)$ times the pseudo first order rate constant in sec^{-1}.

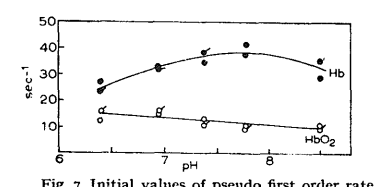

Fig. 7. Initial values of pseudo first order rate constant for combination of BTB and oxy- and deoxyhemoglobin as a function of pH, from two experiments in 0.1 M phosphate buffer at 21°. Protein concentration $0.36 \cdot 10^{-4}$ M (in heme). Dye concentration $0.21 \cdot 10^{-4}$ M (in heme). $\lambda = 620$ mμ.

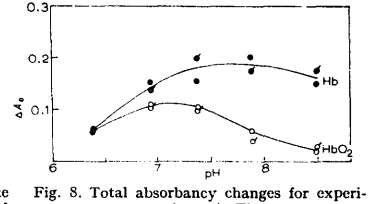

Fig. 8. Total absorbancy changes for experiments shown in Fig. 7.

the pseudo first order rate constants for hemoglobin and oxyhemoglobin and Fig. 8 the corresponding total absorbancy changes, in both cases as a function of pH.

It will be seen that the difference in the behaviour of ΔA as between hemoglobin and oxyhemoglobin shown in Fig. 8 is consistent with the results of the equilibrium dialysis experiments.

Figs. 9 and 10 relate to the effect of protein concentration on the reaction. Fig. 9 shows that the initial values of the pseudo first order rate constant are roughly linear in the concentration of hemoglobin or oxyhemoglobin but that the curves do not pass through the origin, a phenomenon which will be discussed later. The lowest points plotted correspond to a ratio of heme/dye of about 1/6. Fig. 10 shows that the difference $\Delta A_0(\mathrm{Hb}) - \Delta A_0(\mathrm{HbO_2})$ has a maximum at a dye/heme ratio close to 1 under the conditions defined in the legend of Fig. 9.

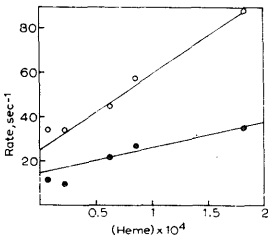

Fig. 9. Initial values of pseudo first order rate constant for combination of oxy- and deoxyhemoglobin with BTB as a function of protein concentration. From experiments in 0.2 M phosphate buffer (pH 7.0) at 2[illegible]. Dye concentration $0.32 \cdot 10^{-4}$ M; $\lambda = 620$ mμ.

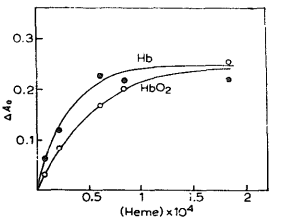

Fig. 10. Total absorbancy changes for experiments shown in Fig. 9.

TABLE I

INITIAL VALUES OF THE PSEUDO FIRST ORDER RATE CONSTANT FOR REACTION OF HEMOGLOBIN DERIVATIVES WITH BTB

Reaction in 0.2 M phosphate buffer (pH 7.5) at 20°. Dye concentration $0.32 \cdot 10^{-4}$ M. Hemoglobin concentration $0.67 \cdot 10^{-4}$ M (in heme).

Derivative	*Hb*	*HbO_2*	*Hb⁺*	*HbCN*	*HbCO*
Rate constant, sec^{-1}	30	9	9	7	11

TABLE II

INITIAL VALUES OF PSEUDO FIRST ORDER RATE CONSTANTS OF HEMOGLOBIN DIGESTS

Reaction in 0.1 M phosphate buffer at (pH 7.0) at 18°. Protein concentrations as listed.

Compound	*Hb concentration (in heme)*	*Rate (sec^{-1}) Hb*	*Rate (sec^{-1}) HbO_2*
HbCPB	$0.9 \cdot 10^{-4}$ M	37	9.5
HbCPA	$1.07 \cdot 10^{-4}$ M	20	17
HbCPA + B	$0.85 \cdot 10^{-4}$ M	27	25

Reaction of dye with various hemoglobin derivatives

Two experiments were carried out in order to compare the rates of reaction of the dye with various hemoglobin derivatives. The conditions of both experiments were the same, except for pH, and are described in Table I, which gives the results at pH 7.5. The results at pH 7.0 are shown graphically in Fig. 11.

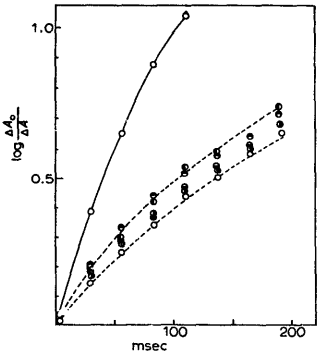

Fig. 11. Values of log $\Delta A_0/\Delta A$ *versus* time for combination of various hemoglobin derivatives with BTB in 0.2 M phosphate (pH 6.95) at 19°. The upper curve is for deoxyHb. The dashed lines are the higher and lower limits for the curves obtained with HbO_2 (○ – ○), HbCO (◑ – ◑), Hb^+ (◐ – ◒), HiHbCN (◓ – ◓) Hb^+F (◓ – ◓).

Reaction of dye with digested hemoglobins

Similar experiments were carried out with the three carboxypeptidase products HbCPA, HbCPB, and HbCPA + B. The results, in terms of the initial values of the pseudo first order rate constants, are shown in Table II.

Reaction of dye with myoglobin

Myoglobin was also studied in 0.2 M phosphate buffer (pH 7). The dye concentration was $0.32 \cdot 10^{-4}$; the myoglobin concentration was $0.5 \cdot 10^{-4}$ in one experiment and $1.0 \cdot 10^{-4}$ in another. The temperature was 19°. The initial rate was very slow (about 5/sec even at the higher protein concentration) and became much slower still as the reaction proceeded. There appeared to be no significant difference between myoglobin and oxymyoglobin.

Reaction of dye with globin

The experiments on globin are summarized in Table III. As in the case of myoglobin the reaction rate diminished with time. The initial values, even at the lower protein concentrations, were so high that it was evident that a large part of the reaction had taken place in the dead time of the apparatus. This is shown also by the values of the absorbancy change given in the table. On the basis of the total absorbancy changes obtained in static spectrophotometric measurements at a protein concentration of $2 \cdot 10^{-4}$ M it was estimated that the initial rate was of the order of 500/sec.

Reaction of dye with bovine serum albumin

Bovine serum albumin was studied in 0.2 M phosphate buffer (pH 7.15). The reaction was fast and complex and it was clear that at the higher concentrations a significant part took place in the dead time of the apparatus, just as with globin.

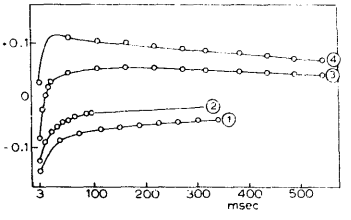

Fig. 12. Combination of BTB with bovine serum albumin. Ordinates give ΔA referred to infinite time at 19°. BTB concentration $0.32 \cdot 10^{-4}$ M. Protein concentration (g/l): curve 1, 0.225; curve 2, 0.45; curve 3, 0.90; curve 4, 1.80.

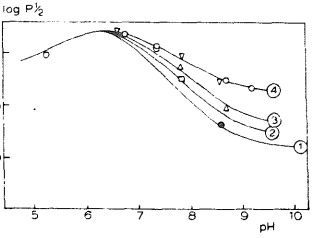

Fig. 13. Effect of BTB on the oxygen equilibrium of normal hemoglobin in 0.15 M phosphate buffer at 20°. Hemoglobin concentration 3–5%. Curve 1 is from previous studies without BTB (●—●). The single point represents a control experiment on the same material used in this work. Ratios of total dye to heme for other curves are as follows: curve 2 (□—□), 0.3; curve 3 (△—△), 0.6; curve 4 (○—○), 1.2; curve 4 (▽—▽), 2.4.

TABLE III

VALUES OF ΔA_0 AND INITIAL VALUES OF PSEUDO FIRST ORDER RATE CONSTANTS FOR REACTION OF DYE WITH GLOBIN

Reaction in 0.2 M phosphate (pH 7.0) at 18°. Dye concentration $0.32 \cdot 10^{-4}$ M. Globin concentrations are given in terms of a unit molecular weight of 17000.

Globin concentration × 10^4 *(M)*	*Rate constant* *(sec⁻¹)*	ΔA_0
2	> 140, 500*	0.038
1	> 120	0.051
0.5	120	0.094
0.2	120	0.134
0.1	70	0.160

* Based on static spectrophotometry.

The observed absorbancy changes at each concentration studied are shown as a function of time in Fig. 12. Due to uncertainty as to the unobserved absorbancy changes which occurred in the first 3 msec we have plotted ΔA against time, rather than $\Delta A_0 - \Delta A$ as in the other experiments. The overshooting phenomenon in curves 3 and 4 is not observed in any of the other cases and is characteristic of this protein.

The dissociation of dye

A few measurements were made of the dissociation of the dye–protein complex. A solution containing dye and hemoglobin in 0.04 M phosphate buffer (pH 6.5) was

mixed in the stopped flow apparatus with a 0.2 M K_2HPO_4 solution. This brought the pH to approx. 8 and caused a partial dissociation of the dye, which, as shown by the dialysis experiments, is less strongly bound at high than at low pH. The results revealed no obvious difference between the rates of dissociation for oxy- and deoxyhemoglobin. When the concentrations of dye and heme were both $0.3 \cdot 10^{-4}$ M the half time of the reaction was of the order of 100 msec at 20°. The total absorbancy change, however, was greater for oxyhemoglobin than for hemoglobin.

The effect of the dye on the oxygen equilibrium

Both the static and the kinetic observations show that the dye interacts differently with normal oxy- and deoxyhemoglobin, particularly at the more alkaline pH values. It might be expected therefore that the dye would have an effect on the oxygen equilibrium of hemoglobin. In order to explore this point a study was made of the oxygen equilibrium in the presence of various amounts of dye under conditions described in Fig. 13.

The value of n, the interaction parameter of the Hill equation for the oxygen equilibrium, was found to be unaffected by the presence of the dye at any of the dye concentrations used. The sole effect of the dye was on the values of the oxygen affinity. The nature of this effect, which is a function of pH, is shown in Fig. 13, which includes as curve 1 the Bohr effect for normal hemoglobin.

As a check one measurement on normal hemoglobin was made: the point lies almost exactly on the curve.

It will be seen that the dye leads to a marked reduction in the Bohr effect and that this is due to an increase of log $p_{1/2}$ at the more alkaline pH values. This increase seems to reach a limiting value when the dye/heme ratio is 1.2, although, as is shown by the dialysis results, the dye continues to combine with protein roughly in proportion to the free dye concentration up to a ratio of 10.

Another experiment was performed to test the reversibility of the effect of the dye. An approximately 0.5 % solution of hemoglobin in 0.2 M K_2HPO_4 (pH 8.75) in which the dye/heme ratio was 2.4, was placed in a dialysis sack and dialyzed 3 days at 4°, with shaking, against pure 0.2 M K_2HPO_4. At the end of this period no dye could be seen in the dialysate. As a control a similar hemoglobin solution without dye was treated in the same way. The oxygen equilibrium curve was determined (a) for the dye–protein solution before dialysis, (b) for the same solution after dialysis, (c) for the same dialyzed solution after readdition of dye, this time at a dye/heme ratio of 1.2, (d) for the control solution after dialysis. The results, in terms of log $p_{1/2}$ and n (at 20°) are shown in Table IV. They establish the complete reversibility of the dye–hemoglobin reaction.

TABLE IV

VALUES OF LOG $p_{1/2}$ AND n FOR THE VARIOUS SOLUTIONS DESCRIBED IN THE TEXT

Solution	*log* $p_{1/2}$	n
Undialyzed	0.75	2.7
Dialyzed	0.24	2.6
Dialyzed plus dye	0.64	2.6
Control	0.27	2.6

In contrast to normal hemoglobin, the oxy and deoxy form of HbCPA + B show no difference in reactivity with dye (see Table II). For this reason we performed a similar set of experiments on the effect of BTB on the oxygen equilibrium of HbCPA + B at pH 8.8. In this case addition of the dye produced no change either in n, which remained at unity or in the oxygen affinity although there is a large effect on both quantities in normal Hb.

DISCUSSION

The central feature of this study is the results on the combination of the dye with the various hemoglobin derivatives. Obviously, when considered in detail this reaction must be extremely complex, involving as it does many sites which are almost certainly different and may interact. Nevertheless, as we shall now show, the general features of the process can be accounted for on the basis of a very simple model and are in accord with the other results of this study.

We assume that the sites are all alike and independent and that there are so many of them that even at the maximum dye adsorption attained the number of free sites is not noticeably diminished. The differential equation which governs the combination of the dye with the protein is then

$$\frac{dB}{dt} = -lB + \bar{l}(B_T - B) \tag{1}$$

Here B denotes the concentration of free dye; B_T the total concentration of the dye, free and bound; and l and $\bar{l}$ are respectively the total "on" and "off" constants of the reaction. These two l's may be decomposed as follows.

$$l = \alpha' l_1 + (1 - \alpha') l_2 \tag{2}$$

where α' is the fraction of the bound dye in the ionized form and l_1 and l_2 are the "off" constants for the ionized and unionized forms of bound dye respectively. Similarly

$$\bar{l} = \alpha \bar{l}_1 + (1 - \alpha)\bar{l}_2 \tag{3}$$

where α is the fraction of free dye in the ionized form and $\bar{l}_1$ and $\bar{l}_2$ are the "on" constants of the ionized and unionized forms of free dye respectively; both $\bar{l}_1$ and $\bar{l}_2$ are proportional to the concentration of free sites, assumed constant. l_2 and $\bar{l}_2$ may be supposed to remain essentially constant, but on electrostatic grounds we might expect $\bar{l}_1$ to decrease and l_1 to increase somewhat with pH.

Integration of Eqn. 1 yields, for the most general set of boundary conditions,

$$\frac{B - B_e}{B_o - B_e} = e^{-(\bar{l} + l)t} \tag{4}$$

where B_0 is the concentration of free dye at $t = 0$ and B_e is the concentration of free dye when $t \to \infty$, *i.e.* the equilibrium concentration. B_e and B_T are related by the equation

$$\frac{B_e}{B_T} = \frac{\bar{l}}{l + \bar{l}} \tag{5}$$

Eqn. 4 is quite general within the scope of the model. Provided only the absorbancy of the solutions is linear in B and B_T, it can be rewritten in terms of absorbancy changes, which are observable in the stopped flow experiments, as follows

$$\frac{\Delta A}{\Delta A_0} = e^{-(l + \bar{l})t} \tag{6}$$

or, if preferred,

$$\Delta A_0 - \Delta A = \Delta A_0 (1 - e^{-(l + \bar{l})t}) \tag{6a}$$

a form which corresponds to the method of presenting the data employed in Fig. 5. Here ΔA is defined as the difference between the absorbancy of the solution at time t and that at time ∞, when $B \longrightarrow B_e$; ΔA_0 is the difference between the absorbancy of the solution at time 0 and that at time ∞. For convenience we add the expression for the half time of the reaction, $t_{1/2}$.

$$t_{1/2} = \frac{0.69}{(l + \bar{l})} \tag{7}$$

As the most general expression for A we have

$$A - A^* \propto B[\alpha\beta_1 + (1 - \alpha)\beta_2] + (B_T - B)[\alpha'\beta'_1 + (1 - \alpha')\beta_2'] \tag{8}$$

where β_1 and β_2 denote the extinction coefficients of the free dye in the ionized and unionized forms, β_1' and β_2' those of the bound dye, likewise in the ionized and unionized forms, and A^* is the residual absorbancy arising from the solvent. At constant pH where α and α' are constant the conditions required by Eqn. 6 are therefore satisfied regardless of whether absorbancy changes arise from pK effects or spectral effects, or both.

If our over-simplified model were a true description of the facts, then the velocity constant for the combination of dye with protein, as determined from absorbancy changes, should be a true constant. Actually, as we have seen, it diminishes substantially as the reaction proceeds. This might be due either to the fact that the sites are not all alike and that the more reactive ones are exhausted first, or to a decrease in the number of free sites, or both. The former effect is probably the more important.

According to this analysis the pseudo first order rate constant $(l + \bar{l})$ should, at any pH, be linear in the protein concentration, which is proportional to the concentration of free sites; and the intersection of the straight line given by plotting $(l + \bar{l})$ against protein concentration with the ordinate should give l. It will be seen from Fig. 9 that $(l + \bar{l})$ is in fact linear in protein concentration within the errors of observation for both hemoglobin and oxyhemoglobin. In order to test the results a little further we can make use of the values of $\bar{l}$ and l obtained from Fig. 9 to calculate B_e/B_T, the ratio of combined dye to total dye at equilibrium, by means of Eqn. 5. For a protein concentration of $1.25 \cdot 10^{-4}$ M we obtain, B_e/B_T for Hb, 64%; B_e/B_T for HbO, 48%. These figures are for pH 7.0. That for HbO_2 may be compared with that calculated for the same pH from the equilibrium dialysis data shown in Fig. 3, which shows combined results obtained at various protein concentrations whose average is close to $1.25 \cdot 10^{-4}$ M. In this case the calculated value of B_e/B_T is 73%. The value estimated for deoxyhemoglobin would be somewhat higher. The figures given by the kinetic results are thus of the right order of magnitude and those for deoxy- and oxyhemoglobin differ in the right direction.

Next consider the change in the observed rate constant with pH. The expression for the rate constant is, on the basis of Eqns. 1, 2, and 3,

$$(l + \bar{l}) = \alpha(\bar{l}_1 - \bar{l}_2) + \alpha'(l_1 - l_2) + \bar{l}_2 + l_2 \tag{9}$$

This varies between $(\bar{l}_2 + l_2)$ at strongly acid pH, where $\alpha = \alpha' = 0$, to $(\bar{l}_1 + l_1)$ at

strongly alkaline pH, where $\alpha = \alpha' = 1$. If both $(l_1 - l_2)$ and $(\bar{l}_1 - \bar{l}_2)$ were constant and of the same sign the increase or decrease would be monotonic. Otherwise the situation would be more complicated, as indeed it is seen to be from Fig. 7. Since we know from equilibrium dialysis that the free dye is a stronger acid than the bound dye and therefore that α rises from 0 with increasing pH more rapidly than α', Fig. 7 might be interpreted to mean that

$$(\bar{l}_1 - \bar{l}_2) > 0 \text{ and } (l_1 - l_2) < 0 \tag{10}$$

The total absorbancy changes observed in the kinetic experiments are never more than about 0.2 or 0.3 and are sometimeless one tenth of this. It is a tribute to the GIBSON apparatus that it can provide such good results when the total absorbancy changes are so small, the transmitted light changing on occasion by less than 10%. In interpreting the data we shall, on the basis of Fig. 2, assume that the absorbancy changes result entirely from changes in the pK of the bound dye, and that at the wavelengths employed (620 mμ) only the ionized form of the dye has an appreciable extinction coefficient, which is the same for the free and the bound dye. Then Eqn. 8 becomes

$$A - A^* \propto (\alpha - \alpha') B + \alpha' B_F \tag{11}$$

Consequently

$$\Delta A_0 \propto (\alpha - \alpha') (B_0 - B_e) \tag{12}$$

Since in our experiments B_0 may be identified with B_T this gives

$$\frac{\Delta A_0}{B_T} = (\alpha - \alpha') \left(1 - \frac{B_e}{B_T}\right) \tag{13}$$

We know that B_e is a function of pH, being greatest at low pH and least at high pH. Its exact variation with pH need not concern us in this qualitative discussion. Since $(\alpha - \alpha')$ varies from 0 to 0 when pH increases from low to high values, passing through a maximum at pH $= (pK + pK')/2$, where pK and pK' are the pK values of free and bound dye respectively, it follows that ΔA_0 must likewise pass through a maximum somewhere not too far from pH $= (pK + pK')/2$, just as it is found to do (see Fig. 7).

This brings us to the question of the difference of pK between free and bound dye and its relation to the kinetic data. This difference has been estimated from dialysis data as at least 1 unit in the case of oxyhemoglobin. Now the equilibrium constant for the combination of ionized dye with protein is

$$L_1 = \frac{\bar{l}_1}{l_1} \tag{14}$$

Similarly that for the unionized dye is

$$L_2 = \frac{\bar{l}_2}{l_2} \tag{15}$$

But

$$\frac{L_2}{L_1} = \frac{K}{K'} \cong 10 \tag{15}$$

Therefore

$$\frac{\bar{l}_2}{\bar{l}_1} \cdot \frac{l_1}{l_2} \cong 10 \tag{15}$$

Without a knowledge of one of these ratios it is, of course, impossible to estimate the other. However, as we have seen, the kinetic data suggest that perhaps $\bar{l}_1 > \bar{l}_2$. If so, l_1 must be more than 10 times greater than l_2 under the conditions of these experiments.

The foregoing analysis is, admittedly, based on a gross over-simplification, but

it has been useful in showing the essential plausibility of the results and the basic consistency which prevails among all of them.

Probably of greater interest than these semi-quantitative considerations are the implications of the observations in terms of structure. Certainly the kinetics of the dye reaction reflect in a most sensitive way differences or changes of conformation of the protein molecules involved. The contrast between globin and hemoglobin is striking; so is the totally different behaviour encountered in the case of bovine serum albumin. Here the overshooting phenomenon observed under conditions where the reaction is fast would seem to demand, as part of the reaction sequence, a step or transition in which there is no uptake of dye, something not found in the other cases. The difference between hemoglobin and oxyhemoglobin and other ligand complexes supports the idea of a configurational change accompanying reaction with ligand which has been invoked to explain the stabilizing interactions between the sites[9,10]. The absence of a difference in cases where the ligand reaction gives no evidence of such interactions, as in HbA and HbA + B lends further support to the same idea.

The results on the effect of dye on the oxygen equilibrium form a part of this picture. It follows unequivocally from the decrease of oxygen affinity caused by combination with dye at constant pH in the alkaline range, that in this range hemoglobin binds more dye than oxyhemoglobin[8]. The effect is in fact formally identical with the familiar Bohr effect, dye replacing proton as second ligand; and in principle it could be used to calculate the difference of dye-binding by the two forms. The fact that the effect reaches a limiting value when the dye/heme ratio in the solution is only 1.2, although hemoglobin, either oxy or deoxy, can combine with 10 molecules per heme without becoming nearly saturated, suggests that there are certain dye binding sites which play a special role and that these sites are preferentially occupied during the early stages of the reaction. The fact that the effect disappears at more acid pH implies that only the ionized form of the dye is involved. Evidently the unionized form has the same affinity for hemoglobin as for oxyhemoglobin. The situation presents an interesting case of linkage involving three ligands, oxygen, proton, and dye.

In conclusion we emphasize once more the high degree of specificity in the kinetics of the dye/protein reaction. The response is so sensitive as to suggest that the reaction might be useful in exploring conformational changes associated with other processes involving proteins.

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