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Observations on Rapidly Reacting Hemoglobin*

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SUMMARY

The time course of appearance of quickly reacting hemoglobin at pH 7 in dilute solutions (<10 μM in heme) was studied by means of a stopped flow-flash apparatus. When the flash is fired as soon as possible after the reaction of human deoxyhemoglobin with carbon monoxide or immediately after dilution of a concentrated human carbon monoxide hemoglobin solution, little of the quickly reacting form is present; the amount of quickly reacting material increases with time and reaches its final value in a few seconds. The results may be due to dissociation of ligand bound hemoglobin.

Some years ago it was reported that sheep deoxyhemoglobin produced by photodissociation of carboxyhemoglobin recombined more rapidly with carbon monoxide than does normal hemoglobin (1). The phenomenon was influenced by several factors, and especially by pH. At pH 9.1, with about 30 μM hemoglobin in terms of heme, it appeared that the quickly reacting hemoglobin was the principal photoproduct: it reverted to ordinary hemoglobin with a half-time of a few milliseconds at 3°. At pH 7 only a small fraction of the photoproduct reacted quickly, and no evidence of reversion of this small fraction was obtained. At the lower pH, however, the behavior of the system was strongly dependent on concentration, and in solutions more dilute than about 10 μM sheep hemoglobin an increasing fraction of rapidly reacting photoproduct was obtained. This paper reports some experiments on the time dependence of changes in reactivity of human hemoglobin at pH 7.0, which may also be related to observations of Roughton (2) on quick and slow equilibria between oxygen and hemoglobin, and on changes in the reactivity of deoxyhemoglobin immediately after its formation from oxyhemoglobin by the action of dithionite.

The results are of importance in interpreting kinetic work with hemoglobin, which has necessarily been carried out chiefly with dilute solutions, and they point to a need for further investigation of the degree of aggregation of hemoglobin molecules in dilute solutions of the various hemoglobin derivatives.

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EXPERIMENTAL PROCEDURE

Human hemoglobin was prepared by precipitation with ammonium sulphate from hemolysates of washed red cells, and was stored at −12° in the form of a thick paste. Before the experiments, the oxyhemoglobin paste was dissolved in dilute buffer solutions and the solution was deoxygenated by the addition of small amounts of solid dithionite. This procedure ensured that no methemoglobin was present during the kinetic experiments. Hemoglobin concentrations were determined spectrophotometrically with a Zeiss PMQ II spectrophotometer, and are given in heme equivalents.

Stopped Flow-Flash Photolysis Apparatus—The apparatus was similar in principle to that previously described (3). The flash photolysis cell, in most experiments, was a cylindrical tube with a light path of 8 cm and a diameter of 5 mm, connected to two syringes at one end and to an outflow tube at the other. An alternative cell had a path of 1 cm, while, for flash experiments without flow, a cell with a 1.5-mm path was also available. By application of gas pressure to a pneumatic piston (Airco, Angola, Indiana, model 11 GSR) the contents of the two syringes were rapidly forced through a 6-jet mixer which delivered directly into the observation cell, the flow being suddenly stopped at the end of the displacement of the pistons. The photolysis flash was fired manually at different time intervals after the stop; these times were measured by means of an oscilloscope which was triggered on stopping flow and which also recorded the occurrence of photodissociation. When the concentration of hemoglobin permitted, the reaction with carbon monoxide was followed at 430 mμ: if the amount of light transmitted by the solution at this wave length was not sufficient, longer wave lengths were used. The bandwidth of the observing beam from a Bausch and Lomb 500-mm focal length grating monochromator was restricted to 1.6 mμ nominal. The dead time of the flash apparatus was limited by the duration of the flash itself, and was about 20 μsec. The shortest interval between flow stopping and firing the flash was determined primarily by the rate of the reaction between hemoglobin and carbon monoxide, and was about 30 msec when the carbon monoxide-containing buffer was equilibrated with 1 atmosphere of the gas.

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RESULTS

Time-dependent Changes in Reactivity of Hemoglobin—The results of experiments in which the course of recombination of carbon monoxide with hemoglobin was influenced by a previous flow procedure fall into a single pattern which is shown in Figs. 1 and 2. Fig. 1 shows the time course of the absorbance change at 430 mμ after flash photolysis of a solution of carboxyhemoglobin: *1*, when the flash was fired 70 msec after dilution with buffer from 30 μM to 1.5 μM in heme, *2* when the flash was fired 3.5 sec after dilution. In both cases the reaction is obviously biphasic, but the distribution between the phases is different in the two experiments; the slower phase preponderates in (*a*) while the faster phase is the more evident in (*b*). Fig. 2 shows the time course of the transition between result *1* and result *2* of Fig. 1 (although for different experimental conditions). It was obtained by firing the flash at different time intervals after the flow procedure and analyzing the resulting curves into slow and fast phases. This can be done with good accuracy (±5%) because the steps are widely different in rate. In a few cases the values obtained graphically were compared with those yielded by fitting the data points with two exponentials, minimizing the squared residuals by a steepest descents procedure with the use of a digital computer. In each case the final fit was close to that obtained by the graphical method.

Experiments with Deoxyhemoglobin—Dilute solutions (about 1 μM or 0.0016%) of deoxyhemoglobin in 0.1 M phosphate buffer at pH 7 were mixed with equal volumes of buffer equilibrated with 1 atmosphere of carbon monoxide and the behavior of the resulting carboxyhemoglobin was followed as a function of time by flash photolysis. When the flash was fired as soon as possible after the reaction of carbon monoxide with deoxyhemoglobin, very little of the quickly reacting form was observed: the amount increased with time and reached its final value after a few seconds. The results of two sets of experiments at different initial concentrations of hemoglobin are shown in Fig. 2, in which the concentration of quickly reacting material is plotted as a function of the interval between mixing the reagents and firing of the flash. In these experiments, the hemoglobin was diluted 1:1 on mixing with the carbon monoxide solution. As a control, similar experiments were performed in which the deoxyhemoglobin solution was mixed with 0.05 volume of buffer equilibrated with 1 atmosphere of carbon monoxide. In this case, although the concentration of hemoglobin decreased only 5% on mixing, the proportion of rapidly reacting material showed changes in time after mixing similar to those in the experiments in which the dilution was 1:1, which suggests that the effects were consequent on ligand binding rather than on the dilution of deoxyhemoglobin.

Experiments with Carboxyhemoglobin—In these experiments the flow procedure consisted of mixing carbon monoxide hemoglobin with a buffer equilibrated with carbon monoxide and flashing the solution at various time intervals after flow. The only variable was the concentration of the hemoglobin. A part of one such experiment is shown in Fig. 1, which shows that there is a marked increase in the proportion of quickly reacting hemoglobin between 70 msec and 3.5 sec. A fuller series of points covering a range like that shown in Fig. 2 showed that the rate of change was the same (within a factor of 2) whether the flow procedure was to mix deoxyhemoglobin with carbon monoxide-containing buffer or to dilute a solution of carboxyhemoglobin with a similar buffer. The range of starting conditions which could be used was different, however, since if the carboxyhemoglobin was initially too dilute, a rapid reaction was observed on photolysis immediately after dilution. Control experiments in which a

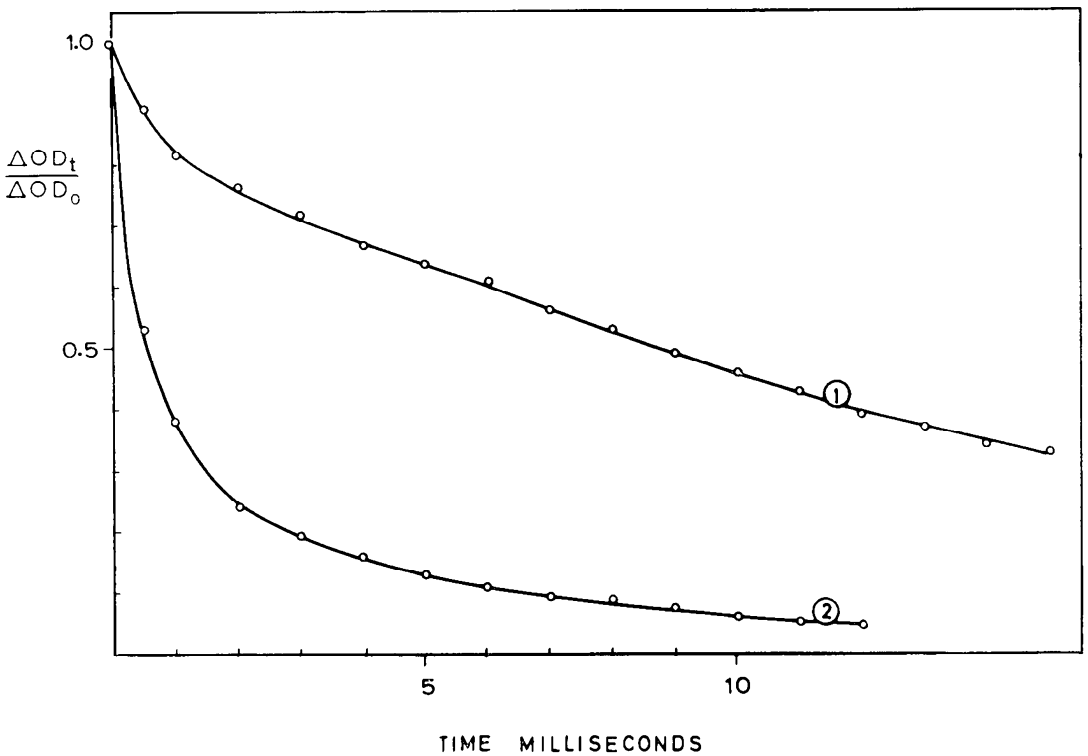

FIG. 1. Time course of combination of carbon monoxide on flash photolysis after mixing 30 μM carboxyhemoglobin in 0.1 M phosphate buffer, pH 7.0, with 20 volumes of buffer equilibrated with 1 atom of carbon monoxide. *Curve 1*, 70 msec after dilution. *Curve 2*, 3.5 sec after dilution. Temperature, 28°; light path 8 cm; wave length, 430 mμ; total absorbance change measured, 0.88.

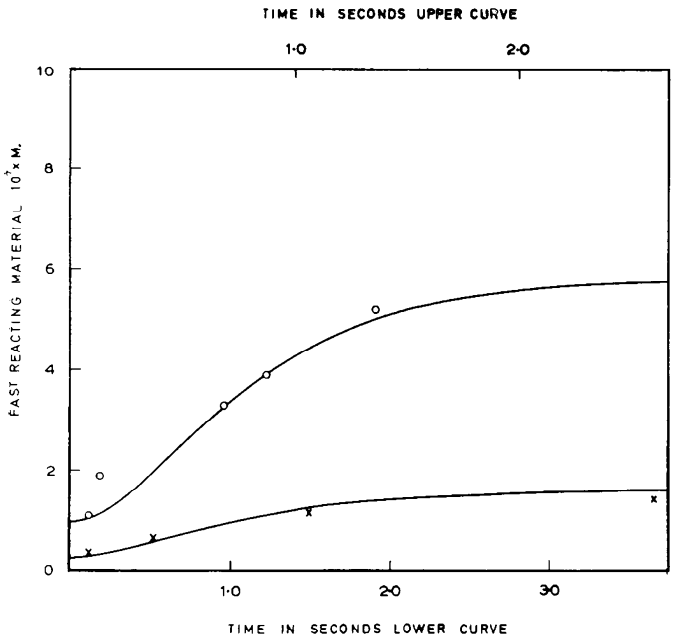

FIG. 2. Amount of fast reacting material on flash photolysis as a function of time between mixing deoxyhemoglobin with carbon monoxide and firing of the flash. Total hemoglobin concentration, 1 μM, *upper curve*, 0.2 μM, *lower curve*. Phosphate buffer, 0.1 M, pH 7.0, 28°, 430 mμ, 8-cm light path. Note that combination of hemoglobin with carbon monoxide is essentially complete in 30 msec; hence, the light flash is always acting on completely liganded hemoglobin, just as it does under the conditions of the experiments shown in Fig. 1.

large volume of carboxyhemoglobin solution was mixed with a small volume of buffer showed that the effects observed were not due to manipulations of the solutions, since no time-dependent changes were observed after flow.

Experiments Under Other Conditions—In confirmation of earlier experiments with sheep hemoglobin (1) it was shown that photolysis of human hemoglobin at pH 9.1 yields a quickly reacting material which reverts rapidly (half-time, 4 msec at 5°) to normal hemoglobin. This behavior was observed at concentrations of hemoglobin of up to 0.1 mM (1.5-mm path cell), and was not influenced by the time interval between mixing deoxyhemoglobin with carbon monoxide and flash photolysis. These results are qualitatively different from the ones described at pH 7: at the lower pH there is no evidence of reversion from rapid to normal rates of reaction, and a rapid reaction is seen only at very low protein concentrations.

Again in confirmation of earlier experiments with sheep hemoglobin (4), it was shown that partial photodissociation of human carboxyhemoglobin at pH 7 leads to the production of a rapidly reacting form even at high concentrations of hemoglobin (up to 0.1 mM). Under these conditions complete photodissociation gave almost exclusively slowly reacting material. No time dependence of this rapidly reacting form was evident.

DISCUSSION

The experiments reported here show that a change in reactivity follows the combination of dilute solutions of deoxyhemoglobin with carbon monoxide. A similar change follows sufficient dilution of carboxyhemoglobin. These results suggest that dissociation of liganded hemoglobin into subunits is occurring under conditions in which deoxyhemoglobin is not dissociated in a way which influences its kinetic behavior. These experiments do not permit identification of the type of subunit involved, but the observations can be explained in terms of the known properties of isolated chains of hemoglobin which react much faster with ligands than the intact molecule does (5). The concentrations of hemoglobin involved are very low and fall at the bottom end of the range of application of ultracentrifuge methods, so that relevant comparisons are difficult. Moreover, ultracentrifugal measurements yield the mean molecular size, and need not reveal the separate existence of species in rapid equilibrium with one another. Nevertheless, measurements by Schachman and Edelstein (6) with the use of a new and more sensitive method of observation have suggested that significant dissociation of liganded human hemoglobin into monomer may indeed occur in the range of concentrations used in our experiments.

It should perhaps be pointed out that dissociation of hemoglobin into subunits probably does not explain all the types of rapid reaction of hemoglobin with ligands which have been observed. For example, the rapid reaction seen at pH 9 after photodissociation of carboxyhemoglobin disappears in a few milliseconds, apparently in a first order reaction (1). There is, moreover, no evidence of extensive dissociation of liganded hemoglobin at this pH on the one hand, while the phenomenon has recently been observed with hemoglobin concentrations of 33%.[1] There are difficulties also with the rapid reaction seen on partial photodissociation of carboxyhemoglobin, since a normal slow reaction may be obtained from the same solution by increasing the flash energy. An explanation in terms of monomeric subunits would imply dissociation into chains followed by reassociation on continued illumination.

The experiments described here can be correlated with several observations in the literature. Thus Roughton (2), with the use of very dilute solutions of hemoglobin, observed that equilibrium with oxygen was not approached directly, but in two stages, the first rapid, the second slow. Roughton (7) also observed that when two mixers were set up in series, the first introducing dithionite into oxyhemoglobin and the second introducing carbon monoxide into the mix after a known time interval, the rate of reaction of carbon monoxide with freshly formed hemoglobin was several times greater than that observed with hemoglobin which had been allowed to stand. Both of these observations seem immediately related to the results reported here, and may be explained if differential aggregation of subunits in liganded hemoglobin and deoxyhemoglobin is assumed.

The findings described here help to explain the marked differences between the kinetics of combination of oxygen and of carbon monoxide with deoxyhemoglobin. The carbon monoxide reaction is relatively straightforward (8), while the oxygen combination reaction is simple only if the oxygen concentration is great enough to establish an equilibrium with 90% or more oxyhemoglobin. With lower concentrations of oxygen, equilibrium is approached in two stages,[2] the second of which occupies several seconds and cannot be reconciled with simple mass action schemes. These difficulties can be explained in terms of the observations described here because the kinetic experiments were

[1] L. J. Parkhurst and Q. H. Gibson, unpublished observations.
[2] F. J. W. Roughton and Q. H. Gibson, unpublished observations.

necessarily carried out with dilute solutions in which differences in aggregation would play a prominent part in the oxygen reaction, the high affinity of the dissociated chains leading to a drift in percentage saturation. With carbon monoxide, however, no such effect can be observed because the affinity for ligand is already so high that no change in the properties of the protein can affect the saturation to a measurable extent since there is scarcely any free ligand which remains in solution. Changes in the protein would, however, affect the equilibrium with carbon monoxide in much the same way as for oxygen, which thus resolves the paradox of the similarity of equilibrium and dissimilarity of kinetic behavior for the two ligands.

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