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Communication

Observations on the Kinetics of the Reaction of Hemoglobin with Oxygen*

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SUMMARY

The kinetics of the reaction of human deoxyhemoglobin with oxygen at high concentrations of ligand ($\sim 10^{-4}$ M) has been studied with the use of a fast stopped flow apparatus with a dead time of approximately 300 μsec. The shape of the progress curve reveals that the apparent second order rate constant for the reaction with oxygen (k') increases with the extent of the reaction, similar to what was reported for other ligands (*i.e.* carbon monoxide). Within the oxygen concentration range studied, the rate of the reaction at any stage is proportional to the ligand concentration, notwithstanding the change in rate as the reaction proceeds.

These results are interpreted to indicate that the ligand-linked intramolecular change in hemoglobin is a fast process which occurs in a fraction of a millisecond.

Although the combination of deoxyhemoglobin with oxygen was among the first reactions studied in the classical rapid mixing experiments of Hartridge and Roughton (1), little work has been done on this reaction since that time. Most of the recent work on the kinetics of the combination of hemoglobin with ligands has concentrated on the reaction with carbon monoxide, which is much slower and which presents a technically simpler problem (2–5). The difficulties encountered in the study of the kinetics of the reaction of hemoglobin with oxygen have been discussed by Gibson (2) and appear to be primarily because with this ligand the "on" and "off" constants are both very high. Thus, in rapid mixing experiments, with the use of apparatus with a dead time of no less than 1 msec, the reaction of hemoglobin with oxygen can be studied only at reagent concentrations so low that the rate of the off reaction is not negligible in comparison with that of the on reaction. So far this has prevented any accurate comparison of the kinetics of the reaction with oxygen and that with other ligands under the conditions in which the kinetics of the process is dominated by the "forward" process, the "back" reaction making a negligible contribution. The recent availability of a new type of stopped flow apparatus with a much shorter dead time (6) alleviates some of the difficulties mentioned above and provides an opportunity for the study of the reaction of hemoglobin with oxygen at high oxygen concentrations. Such studies are reported below.

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EXPERIMENTAL PROCEDURE

The stopped flow apparatus has been described before (6). The flow velocity through the observation tube was 22 m per sec, which was obtained with a driving pressure of 16 atmosphere of N_2 on the syringe pistons. The measured dead time was approximately 300 μsec. The diameter of the observation tube, which is the same as the length of the light path, was 3 mm. The band width of the monitoring light varied from 0.5 to 2 mμ in the different experiments. In most of the experiments, an instrument artifact was observed to occur approximately 50 to 100 msec after flow stopped. This resulted in a progressive drift of the base-line representing a change in the output of the photomultiplier; a situation which was later found to be due to a defective part in the stop valve which permitted a slow leak, thus causing poorly mixed reagents to drift up the observation tube. Nevertheless, since the reaction being studied was completed prior to this, the optical density change during the reaction could be calculated from the energy change recorded with the oscilloscope and from the total optical density change obtained from static spectrophotometric measurements on the same solutions as those used in the kinetic experiments.

To test the over-all performance of the apparatus, control runs were made by using two well known reactions, (*a*) the deoxygenation of hemoglobin by dithionite at pH 7 (half-time, ~15 msec), and (*b*) the combination of hemoglobin with CO (initial second order rate constant, $\sim 10^5$ M^{-1} sec^{-1}). In both cases the results with the present apparatus agreed with previous data and with measurements done using the same solutions with a Gibson or a Durrum-Gibson apparatus (7).

Human hemoglobin was prepared by the toluene procedure (8). The stock oxyhemoglobin solution (~5%) was deoxygenated by vacuum and stored in a tonometer under argon. Just before the experiments it was diluted with deoxygenated buffer to obtain the final working solution. Oxygen and other gases of greater than 99% purity were from Società Italiana Ossigeno (Rome). Oxygen concentrations in the solutions were calculated from partial pressures of the gases and from solubility coefficients.

RESULTS

A set of oscilloscope traces, which represent duplicate experiments with the same solutions, is reproduced in Fig. 1. The traces from the different runs have all been superimposed on the storage screen of the oscilloscope to give an indication of the re-

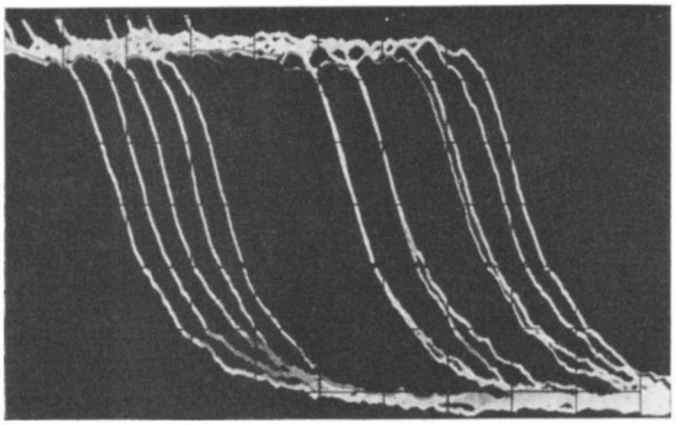

FIG. 1. Oscilloscope traces of the reaction of hemoglobin with oxygen at pH 7, 0.05 M phosphate buffer. Observations were made at $\lambda = 430$ mμ; band width = 1.5 mμ; ΔO.D. total = 0.240. Sweep time = 2 msec per cm. Hemoglobin concentration = 9×10^{-6} M; oxygen concentration = 1.36×10^{-4}; M temperature = 23°.

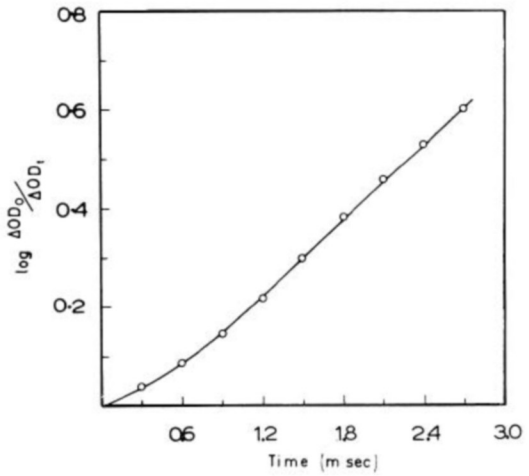

FIG. 2. Time course of the combination of hemoglobin with oxygen. Experimental conditions are reported in Fig. 1. The experimental points are the average of 14 separate traces of the type reported in Fig. 1. Zero time corresponds to 0.3 msec after mixing.

producibility and the signal-to-noise ratio in a typical experiment. The point where the flow stops was taken to be where the traces deflect from the upper horizontal line. It must be noted that, as reported in detail elsewhere (6), the stopping time of the instrument is negligible (approximately 20 μsec) in comparison to the time scale of the experiments.

Fig. 2 shows the time course of the reaction of deoxyhemoglobin (9×10^{-6} M) with a great excess of oxygen (1.36×10^{-4} M). The points shown in the figure are the average of 14 kinetic runs on a single hemoglobin sample. Consistent results were obtained in several other sets of experiments. The shape of the progress curve, when the process is treated as a pseudo first order reaction, shows that there is an increase in rate as the reaction progresses. If the apparent second order rate constant (k') is calculated at different time intervals, and these values are plotted against the extent of reaction (*i.e.* fractional oxygenation, $\bar{Y}$), the graph shown in Fig. 3 is obtained. It is evident that k' increases from an initial value of approximately 2×10^6 M^{-1} sec^{-1} to nearly twice this value during the first half of the reaction, and then remains roughly constant. Progress curves of this same shape were obtained in other experiments by using different oxygen concentrations, provided the concentration was higher than approximately 7×10^{-5} M. At lower oxygen concentrations there was evidence of a change in shape of the progress curve, the reaction tending to slow down as it progressed. This is in agreement with the observations of Gibson and Roughton in their experiments at low oxygen concentrations (2). No attempt was made to explore in any detail the kinetics at low oxygen concentrations, because of the unsuitableness of the apparatus to follow relatively slow reactions and because of the extensive, although primarily unpublished studies of Gibson and Roughton, in which difficulties were encountered in the interpretation of the data.

Within the range of oxygen concentrations studied (0.7 to 1.7×10^{-4} M), these concentrations always being in great excess with respect to the protein, the rate of the reaction at any stage is proportional to the oxygen concentration, notwithstanding the change in rate as the reaction progresses. This is shown in Fig. 4, where the apparent pseudo first order rate at $\bar{Y} \geq 0.50$ is plotted against the oxygen concentration. The apparent second order velocity constant calculated from this plot is 4.7×10^6 M^{-1} sec^{-1}. It may be noted that the highest pseudo first order rate measured was about 10^3 sec^{-1} corresponding to a half-time of about 700 μsec. Under these conditions, a substantial part of the reaction (~20%) is lost in the dead time of the apparatus, so that no information on the initial stages of the reaction could be obtained.

A few experiments were also done at still higher oxygen concentrations; however, since the fraction of the reaction lost in the

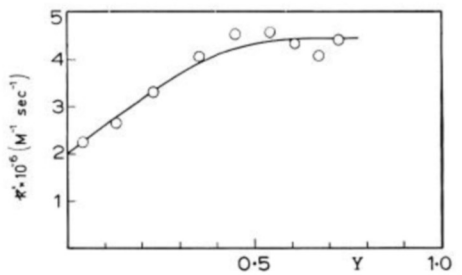

FIG. 3. Apparent second order velocity constant (k') for the reaction of hemoglobin with oxygen as a function of $\bar{Y}$, the fractional saturation with the ligand. Experimental conditions are reported in Fig. 1.

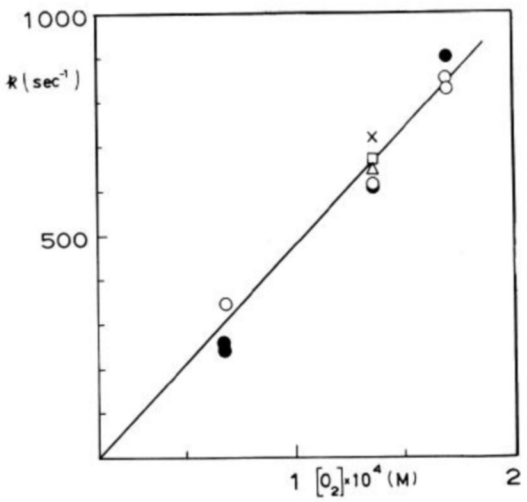

FIG. 4. Dependence of the pseudo first order rate constant for the reaction of hemoglobin with oxygen on ligand concentration. The values of k have been computed for values of $\bar{Y} \geq 0.5$. Solvent = 0.05 M phosphate buffer, pH 7; temperature = 22–23°.

dead time of the apparatus was greater than 50%, no analysis was attempted.

CONCLUSIONS

Although limited in range, the present results cover a hitherto inaccessible region, and they lead to significant conclusions regarding hemoglobin kinetics. First, when the rate of the off reaction is negligible in comparison with that of the on reaction, the kinetics of combination of hemoglobin with oxygen and CO are very similar, even though the absolute rates for the reaction with the two ligands differ about 20-fold. This is brought out by comparison of the shape of the curve shown in Fig. 3 with the corresponding curve obtained for the reaction with CO (2, 5). In both cases the initial value of the apparent second order rate constant increases by a factor of approximately 2 during the first 50% of the reaction and then remains constant.

Also, in both cases the rate at any stage of the reaction is proportional to the ligand concentration, provided the ligand is present in excess. This is only true at high oxygen concentrations; at low oxygen concentrations (when the conditions are such that both the on and off rates play a significant part), the kinetics change in character and show a more complex pattern. The same has been observed in unpublished stopped flow experiments on the reaction of hemoglobin with ethyl isocyanide[1] and in temperature jump experiments with both oxygen and ethyl isocyanide (9).

The second important point which emerges from these experiments concerns the kinetic aspects of the heme-heme interaction. The autocatalytic shape of the combination reaction curve demands that the velocity constant for the reaction with the ligand increases with the amount of ligand bound. The situation has been discussed in terms of protein subunits in another connection (5) where it was shown that for the reaction of hemoglobin with CO, the combination velocity might be fitted by a scheme involving only $\alpha\beta$ dimers, such as

$$\alpha\beta + X \xrightarrow{k'_1} \alpha\beta X$$

$$\alpha\beta X + X \xrightarrow{k'_2} \alpha\beta X_2$$

with k'_2 somewhat higher than k'_1. Such a scheme implies the occurrence of some intramolecular change (presumably conformational) following the combination of the first ligand molecule, as a result of which k'_2 becomes greater than k'_1. The invariance of the shape of the kinetic curves with changes in the absolute speed of the process demands that the rate of the intramolecular change be rapid in comparison with the rate of uptake of ligand. For the reaction with oxygen, the shape of the reaction curve was found to be autocatalytic up to rate constants of the order of 1000 sec^{-1}, the conclusion being that the intramolecular change has a rate constant greater than this. Thus, in agreement with the results of previous work (10), the present experiments give strong indications that the ligand-linked intramolecular change in hemoglobin is a very rapid process occurring in a time scale of fractions of milliseconds.

[1] E. Antonini and M. Brunori, unpublished experiments.

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