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Magnetokinetic Studies of the Reaction of Hydrogen Peroxide with Haemoglobin in Dithionite Solutions

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The changes of magnetic susceptibility which accompany the reaction have been recorded. The results have been interpreted in relation to the simultaneous changes of spectral absorption, and show that a transient compound of haemoglobin with distinctive spectral absorption and relatively low magnetic susceptibility is formed. A tentative value of 4 500 $\times$ 10^{-6} cgs emu has been obtained for the molar paramagnetic susceptibility of the compound. It cannot be decided at the moment whether the transient compound is ferric or ferrous.

The addition of hydrogen peroxide to a neutral or slightly alkaline solution of haemoglobin containing an excess of sodium dithionite ($Na_2S_2O_4$) results in the destruction of some of the haemoglobin and the formation of small amounts of choleglobin. In spectrokinetic studies of this reaction, Dalziel and O'Brien [1-3] detected the formation of a transient intermediate compound of haemoglobin. They showed that within a few seconds of the addition of hydrogen peroxide the spectrum of haemoglobin, λ_{max} 430 mμ and 555 mμ, was completely displaced by an entirely different absorption curve, with distinct and symmetrical maxima at 417, 545, and 582 mμ. This new spectrum persisted, unchanged except for a slow decrease of intensity, for periods of seconds or minutes, according to the reactant concentrations and the pH, and then gradually gave way to a haemoglobin spectrum of reduced intensity, with an additional small peak at 630 mμ due to choleglobin. The life of the transient spectrum increased with the initial peroxide concentration and was reduced by the presence of catalase. The spectrum differed significantly from that of known haemoglobin derivatives, including methaemoglobin-hydrogen peroxide, but was quantitatively reproduced over quite a wide range of reactant concentrations. The experimental facts suggested that the spectrum might characterise a hitherto unrecognised peroxide com-

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pound of haemoglobin; the formation of "haemoglobin-hydrogen peroxide" as an intermediate in such reactions had been postulated by Lemberg, Legge and Lockwood [4,5]. Conclusive evidence of the nature of the compound, or even of the state of oxidation of the haem iron, could not be obtained, however, in this peculiar reaction mixture, and the possibility that the transient spectrum was due to a mixture of methaemoglobin-hydrogen peroxide and some unknown degradation product could not be excluded.

Theorell and Ehrenberg [6] determined the molar magnetic susceptibilities of the unstable peroxide compounds of peroxidase, catalase and metmyoglobin by means of simultaneous measurements of light absorption and susceptibility. Their apparatus [7] was specially designed for rapid measurements on dilute solutions. It seemed worth while to attempt to study the haemoglobin-dithionite-peroxide reaction by this method, in spite of the evident complexity of the reaction, and the likelihood that the degradation of a considerable proportion of the haemoglobin to choleglobin and other unidentified products would preclude an unequivocal interpretation of the results. It was realised at the outset that only if the life of the transient compound was associated with diamagnetism of the reaction mixture would the results be of definite value in identifying the compound. This has not proved to be the case. Nevertheless, the results obtained are additional proof of the formation of a transient compound which slowly reverts to haemoglobin, and yield a tentative value for the molar susceptibility of the compound.

MATERIALS

Oxyhaemoglobin. Solutions of human oxyhaemoglobin were prepared from haemolysates of washed red cells, from which catalase was removed by adsorption on alumina. The solutions were dialysed against water, and finally against borate buffer solutions of pH 8.5 or 9.5. The haem content was estimated spectrophotometrically as the cyanmethaemoglobin derivative (Drabkin and Austin [8]). Details of such preparations have been given by Dalziel and O'Brien [2]. The iron content, estimated by a slight modification of the method of Lorber [9], was always about 5 % greater than the haem content.

Sodium Dithionite was a fresh, dry commercial sample stored in a sealed bottle.

Hydrogen Peroxide. A stock solution, *ca.* 1.5 *M*, was prepared by dilution of perhydrol with glass distilled water, and was analysed periodically by titration with potassium permanganate.

Buffer Solutions. To dilute the stock reagent solutions and prepare reaction mixtures of pH 10.0, Clarke and Lubs borate buffer solutions of four times the usual concentration were used, *viz.* 0.2 *M* H_3BO_3—KCl with the addition of the appropriate volume of 1 *N* NaOH. Even with this high buffer capacity, the acidic products of the reaction of H_2O_2 and $Na_2S_2O_4$ caused the pH to fall by 0.6 units during the reaction.

METHODS

Preparation and Sampling of the reaction mixture. In preparing the reaction mixture and filling the magnet tube and the optical cell, it was necessary to work as quickly as possible and to minimise exposure of the solutions to air. 5 ml portions of the reaction mixture were prepared in an Erlenmeyer flask fitted with a cork with a small cut for gas outlet. A glass tube passing through a hole in the cork was connected to a nitrogen cylinder; the reactants were introduced, and samples of the mixture withdrawn, through a second hole in the cork.

3 ml 0.715 mM (haem) HbO_2 was pipetted into the flask, and the air was displaced by a stream of nitrogen, which was maintained during the subsequent manipulations. 1 ml $Na_2S_2O_4$ solution, containing 2 g/100 ml and prepared and stored under nitrogen in a Thunberg tube, was added, followed after mixing by 0.1 ml 0.1 M H_2O_2. The contents of the flask were immediately mixed and a stopwatch started. A sample was quickly withdrawn into a Pasteur pipette, from which portions were delivered into the optical cell and the magnet tube, all previously flushed with nitrogen.

The magnet tube and optical cell were kept at the working temperature in their respective compartments until just before filling. Strict temperature control of the solutions during the preparation and sampling was not possible, but for the experiments below room temperature the reagents and pipettes *etc.* were first brought to the experimental temperature in the magnet thermostat.

Spectrophotometric Measurements. A Beckman quartz spectrophotometer model DU was used. The cell compartment was kept at constant temperature by thermostat water circulation in double-layer thermospacers. Optical depths of 0.1—0.02 cm were obtained by means of 1 cm cells into which solid quartz prisms 0.90—0.98 cm thick were inserted. The prisms carry lids which overlap the edges of the cell, and a layer of vaseline round these lids ensured an air tight seal.

The extinction of the reacting mixture at 430 mμ, the wavelength of the haemoglobin Soret maximum, was recorded at intervals until a constant value was reached. Time was measured with a stopwatch. The earliest measurement which could be made by this technique was about 2 minutes after the start of the reaction. The results are recorded as millimolar extinction coefficients, $\varepsilon_{mM\ (haem)} = \log I_0/I \times 1/cd$, where c = initial haem content of the reaction mixture in mM, d = optical depth in cm. The optical depths of the cell-prism combinations were determined by calibration with oxyhaemoglobin solutions against a 1.000 cm cell.

Magnetic Measurements. The apparatus described by Theorell and Ehrenberg [7] was used in its present condition with improved air shielding, temperature control and filling technique. Details will be published elsewhere. The instrument was calibrated with a dilute solution of nickel chloride for which the molar susceptibility was assumed to be $4\,434 \times 10^{-6}$ cgs emu at 20° C[7]. The calibration constant was 2.015×10^{-11} volume susceptibility units per scale division (μ). As the errors in the deflections were about $\pm$ 4 μ it was desirable to work with solutions containing not less than 0.4 mM haem iron so that molar susceptibilities could be determined within $\pm 200 \times 10^{-6}$ cgs emu. Preliminary spectrokinetic experiments showed that the life of the transient at this concentration would be long enough for magnetokinetic measurements if the initial reaction mixture contained 0.4 g/100 ml $Na_2S_2O_4$ and 20 mmole/l H_2O_2.

The diamagnetic correction due to the protein was determined by measurements on two samples of carboxyhaemoglobin, prepared by omitting hydrogen peroxide from the reaction mixture and saturating the Hb-$Na_2S_2O_4$ solution with carbonmonoxide. The mean value was 10 μ for a sample with 0.479 mM haem and 0.504 mM iron. This is slightly less than would be expected in comparison with previous data on myoglobin [10]. The difference can be explained by a moderate persisting paramagnetism of the non-haem iron. As the behaviour of this iron in the reaction mixture is not known, we have preferred to correct with the lower figure and base all susceptibility values as well as extinction coefficients on the haem-iron content. In that way the possible error due to the foreign iron will be minimized.

The procedure and evaluation of a magnetokinetic experiment was as follows. First the counter-balance part of the magnet tube was filled with water. A blank mixture was prepared, containing all the reactants and buffers except the oxyhaemoglobin; a buffer solution, which had been equilibrated with the oxyhaemoglobin solution by dialysis, was added in place of the latter. The peroxide was added last and a stopwatch was started. The sample part of the magnet tube was quickly filled and the tube brought in measuring position. Readings were taken alternately with 0 A and 20 A through the magnet-coil until stable readings were obtained. The time was taken at every reading. The procedure was repeated with a sample of the reaction mixture, and finally once again with the buffer-blank. The readings at 0 and 20 A for the buffer blanks, and at 0 A for the reaction mixture were plotted against time and smoothed-out curves drawn. Values at the instants when the reaction mixture was read at 20 A were interpolated on these five curves. The difference reading of the reaction mixture was $\Delta S_{\text{reaction mixture}}$

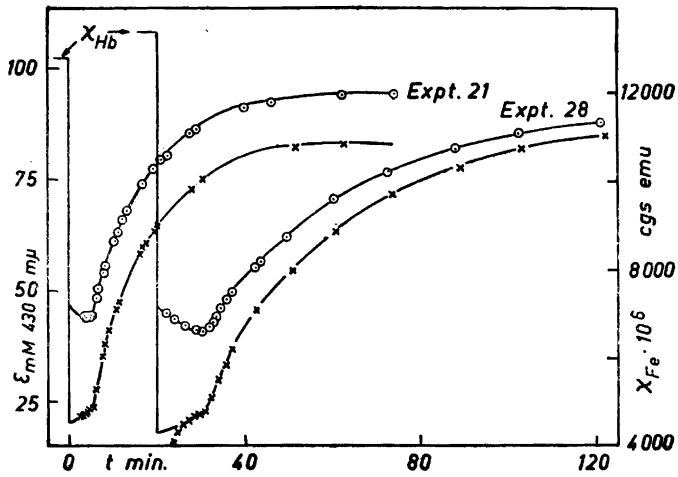

Fig. 1. The magnetic susceptibility, χ_{Fe} (×), and the extinction at 430 mμ, ε_{mM} (o), of two experiments as functions of time. Expt. 21 is made at 21.6° C and starts at 0 time, while expt. 28 is made at 13.0° C and starts at 20 min. The initial susceptibility values, χ_{Hb}, are indicated.

$= S_{20} - S_0$. This value minus the mean difference reading of the two buffer experiments gives the deflection S'. To that value we have further to add the diamagnetic correction $S_{diam} = 10 \cdot \frac{c_{haem,\ actual}}{c_{haem,\ diam}}$, where $c_{haem,\ diam}$ is the haem content in the experiment that gave the diamagnetic correction 10 μ. Thus the corrected deflection S is obtained and can be inserted in the formula

$$\chi_{Fe,\ haem} = \frac{K \cdot S}{c_{haem}}$$

where K is the calibration constant and c_{haem} is in moles per ml so that χ is obtained in cgs emu. The susceptibility values were then plotted *versus* the time.

Because of drifts in buffer readings and readings at 0 A during the first minutes of the measurements, and because of the foreign iron present, we estimate the probable errors in the χ values during that period of the reaction to be as high as $\pm$ 500 × 10^{-6} cgs emu.

RESULTS

The results of two typical experiments, one at 21.6° C (Expt. 21) and the other at 13.0° C (Expt. 28), are shown in Fig. 1. The composition of the reaction mixture in both experiments was 0.43 m*M* haem, 0.4 % $Na_2S_2O_4$ and 20 m*M* H_2O_2. The pH decreased from 9.9 to 9.3 during the overall reaction. The magnetic susceptibility, χ_{Fe}, and the extinction at 430 mμ, ε_{mM}, are plotted against the time, t. The peroxide was added to the haemoglobin-dithionite solution at $t = 0$ in Expt. 21 and at $t = 20$ minutes in Expt. 28. The initial susceptibility values $\chi_{Fe} = 12\,700$ and $13\,300 \times 10^{-6}$ cgs emu, respectively, are shown in Fig. 1, but the initial extinction level, $\varepsilon_{mM} = 145$, is omitted; the data for room temperature were obtained in control experiments, in which the peroxide was replaced by buffer solution, and the susceptibility value of Hb at 13° C was calculated by means of Curie's law.

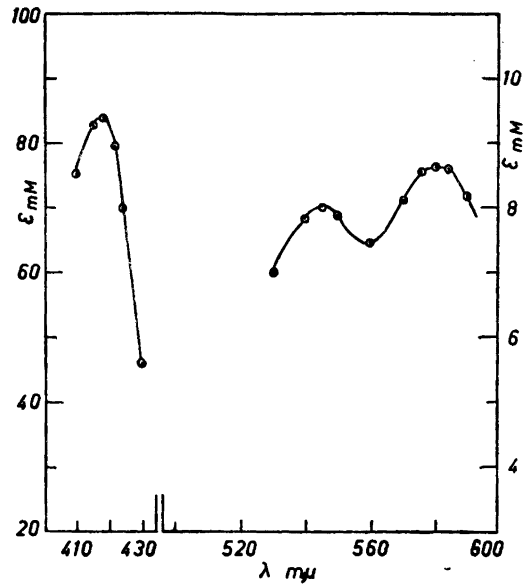

Fig. 2. The absorption spectrum of the reacting mixture at 13.0° C, in the Soret-region 4 min. and in the visible 5 min. after the start of the reaction.

It is obvious that ε_{mM} and χ_{Fe} change in a similar manner during the reaction. In both experiments there are marked decreases from the initial haemoglobin levels to the first measurements 2—3 minutes after the addition of peroxide. There is then a period during which both parameters change quite slowly, ε_{mM} decreasing and χ_{Fe} increasing, which lasts for 5 minutes at 21.6° and 11 minutes at 13.0°. Finally, both parameters increase in a roughly exponential manner at a similar rate, with a half-time of approximately nine minutes at 21.6° and twenty-one minutes at 13.0°. The stable end-values are ε_{mM} = 90 —95 and χ_{Fe} = 10 800. In Expt. 28 there is an inflexion in the χ_{Fe} curve at 5 minutes for which there is no parallel in the ε_{mM} curve. This is almost certainly an artefact, however, due to errors in the first two susceptibility measurements associated with the sample and the magnet tube regaining the experimental temperature of 13° after handling at room temperature. Some control experiments were made at 13° C with samples of known and constant susceptibility. A few of them showed steep starting branches similar to that of Expt. 28. The slow increase of χ_{Fe} from 5 to 11 minutes, however, is real and reproducible.

Both the magnetokinetic and the spectrokinetic curves indicate the occurrence of at least three successive reactions. The first stage was complete by the time the earliest measurements were made, and was accompanied by profound changes of spectral absorption and magnetic susceptibility; the haemoglobin must therefore have undergone reaction involving the iron atoms. The final reaction was the regeneration of haemoglobin, and results in partial reversal of the initial changes of both parameters. The overall reaction results in a decrease of about 35 % in ε_{mM} at 430 mμ, and at least this proportion of the

haemoglobin must therefore have been destroyed. The choleglobin formed does not account for the whole of this loss, but apart from a choleglobin maximum at 630 mμ there was no evidence for the formation of other products with significant spectral absorption (Dalziel and O'Brien [3]). The net decrease of magnetic susceptibility is about 15 %, so that the mean susceptibility of the non-haemoglobin iron in the products must be about $8\,000 \times 10^{-6}$ cgs emu. The liberation of free ionic iron by oxidative fission of haem is obviously likely to occur.

During the middle period, which lasts from the earliest measurements up to 5 minutes in Expt. 21 and 11 minutes in Expt. 28, ε_{mM} decreases in an approximately linear manner. It has been shown by Dalziel and O'Brien that during the same period the extinction at many other wavelengths decreases at a similar rate, and that consequently a distinctive spectrum persists without appreciable distortion. The presence of this spectrum under the conditions of Expt. 28 was established by measurements of the spectral absorption of fresh reaction mixtures made up from the same reagents under identical conditions. The important spectral regions were scanned back and forth in the period 2—8 minutes after the addition of the peroxide, so that the means of pairs of extinction measurement at each wavelength corresponded to the same time from the start of the reaction. The absorption spectrum of the reacting mixture 5 minutes after the start of the reaction is shown in Fig. 2 and agrees closely with the transient spectrum recorded by Dalziel and O'Brien[3].

The magnetic susceptibility measurements during the last half of the life of this transient spectrum in both the experiments (Fig. 1) show clearly that it is associated with a slowly increasing susceptibility value of about 4 500. The coincidence, in both experiments, of an inflexion in the χ_{Fe} curve with the minimum in the ε_{mM} curve which marks the end of the life of the transient spectrum, and the onset of haemoglobin regeneration, is striking.

DISCUSSION

The changes of magnetic susceptibility which follow the addition of hydrogen peroxide to a haemoglobin solution containing excess dithionite confirm the conclusions drawn by Dalziel and O'Brien [3] from spectrokinetic studies regarding the general course of the reaction. The haemoglobin is temporarily converted, in a rapid reaction involving the iron atoms, into at least one unstable derivative which possesses distinctive spectral absorption and rather low magnetic susceptibility, and from which a high proportion of the haemoglobin is subsequently regenerated. As regards the detailed course of the reaction, the magnetic data taken alone do not give any new information.

The formation of a distinctive transient spectrum was demonstrated again under the conditions of the present experiments a few minutes after the start of the reaction. When the measured extinction coefficients (Fig. 2) are corrected for the decrease of intensity which occurs during the life of the spectrum by extrapolation to zero time values of $\varepsilon_{mM} = 88$, 8.6 and 9.2 at 418, 545, and 580 mμ are obtained. The transient spectrum is therefore in excellent quantitative agreement with that recorded by Dalziel and O'Brien [3] a few seconds after the start of the reaction.

It is possible that this transient spectrum characterises a single derivative of haemoglobin [3], and the principle objective of the present experiments was to see whether it was associated with a reasonably constant and reproducible magnetic susceptibility value. The measurements have shown that at least during the last half of the life of the transient spectrum the magnetic susceptibility of the reaction mixture increases slowly. This observation can be explained by the slow degradation of the transient compound to colourless products of high susceptibility which is suggested by a concomitant slow decrease of intensity of the spectrum, and the fact, already mentioned, that some of the degradation products formed in the overall reaction have relatively high susceptibility. If this slow increase of susceptibility persists throughout the life of the transient compound, the best estimate of the susceptibility of the compound will be obtained by extrapolation to zero time. In the two experiments described here, values of 4 500 and 4 300 $\times 10^{-6}$ cgs emu are so obtained; other experiments gave values of from 4 300 to 5 000 $\times 10^{-6}$ cgs emu. Since the errors of the measurements are rather large, and nothing is known about the validity of Curie's law in this instance, a mean value was taken, regardless of temperature differences between the experiments. Thus, a value of 4 500 $\times 10^{-6}$ cgs emu was obtained for the molar susceptibility of the transient compound, on the assumption that all the haemoglobin is converted into a single derivative by the initial rapid reaction with hydrogen peroxide. Susceptibility values of this order are common to hydrogen peroxide and methyl hydrogen peroxide compounds of type II and III of peroxidase, catalase and metmyoglobin [6]: HRP—MeOOH—II, $\chi_M = 5\,040 \times 10^{-6}$; Cat—MeOOH—II, 3 400; metMb—MeOOH—III, 3 000; metMb—H_2O_2—III, 3 500. These data have been interpreted as due to two unpaired electrons and the proposed structure is a resonance between a ferric-radical form and a ferryl form [11]. Judged from the susceptibility value the same structure might be proposed in the case of the transient.

The position of the absorption bands of the transient in the visible, 545 and 582 mμ, would also suggest a compound of type III. The relative height of the two absorption bands, $\varepsilon_{445} < \varepsilon_{582}$, is, however, contrary to what would be expected for such a compound. On the other hand, the transient spectrum is also consistent with a ferrohaem derivative. Theoretically the iron in such a compound have two unpaired electrons and square dsp^2 bonds, which would be compatible with the susceptibility reported here. Among the ferrohaem derivatives, however, no such compound has yet been demonstrated, and although there is no experimental evidence against this interpretation, it is not a likely one.

Thus the present investigation supports the view that the transient spectrum is predominantly due to a peroxide derivative of haemoglobin similar to those already recognised for peroxidase, catalase, and myoglobin, but the question whether it is due to metHb—H_2O_2—III mixed with some other derivative with an absorption maximum at about 580 mμ, or to a new compound, remains unanswered. Direct comparison with the susceptibility values of metHb-peroxide-III compounds can not be made at the moment, as these data are still lacking. We hope to determine them in the near future.

With the present technique, magnetic measurements could be made only during the last half of the life of the transient spectrum, partly because of the time taken to prepare the sample and fill the magnet tube *etc.*, and partly because inadequate temperature control caused inaccuracies in the first few measurements at low temperature, when the life of the spectrum is longest. It should be possible to improve the technique in both these respects. The first measurement could probably be made 1 ½ minutes after the start of the reaction. At a temperature of 2° C we found the life of the transient to be 21 minutes, but an attempt to make magnetic measurements at this low temperature failed because of condensation on the optical system of the magnet, which must also be cooled down. If magnetic measurements could be made under these conditions it would be possible to test the association of the transient spectrum with a definite susceptibility value much more conclusively.

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