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Biochim. Biophys. Acta, 40 (1960) 309–313

A MAGNETO-KINETIC STUDY OF THE REACTION BETWEEN FERRIMYOGLOBIN AND METHYL HYDROGEN PEROXIDE

ARTHUR S. BRILL*, ANDERS EHRENBERG** AND HENDRIK DEN HARTOG***

E. R. Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

(Received August 26th, 1959)

SUMMARY

1. The reaction between ferrimyoglobin and methyl hydrogen peroxide has been studied with a new instrument for measuring rapid changes in the magnetic susceptibility of dilute aqueous solutions of proteins.

2. A comparison of the magnetically and spectrophotometrically obtained rate data shows differences, increasing toward lower temperatures, which can be explained in part by the production of free radicals.

3. The molar susceptibility at 20° C of the myoglobin compound formed is $3300 \cdot 10^{-6}$ emu with a standard error of $500 \cdot 10^{-6}$ emu.

INTRODUCTION

The reactions of the hemoproteins with peroxides are of interest in themselves and pertinent to the elucidation of the mechanisms of peroxidase and catalase action. They have been studied spectrophotometrically and magnetometrically for many years. The recent development of rapid spectrophotometric techniques[1] has permitted

* Present address: Department of Engineering Physics, Rockefeller Hall, Cornell University, Ithaca, New York, U.S.A.
** Present address: Biochemical Department, Medical Nobel Institute, Stockholm, Sweden.
*** Present address: Natuurkundig Laboratorium, University of Amsterdam, Amsterdam, The Netherlands.

observation of the kinetics of formation and decay of labile intermediates as well as the formation of stable compounds, and hence permitted the determination of the absorption spectra of the short-lived compounds. CHANCE suggested that a Rankine-balance type magnetic susceptometer be combined with a flow system to achieve great time resolution in magnetic susceptibility measurements, so that the magnetic moment of such labile intermediates could be determined. A most sensitive magnetic susceptometer of this nature has been developed and is described eleswhere[2]. Fig. 1 shows the performance of this instrument. Fig. 2 depicts a flow experiment where the magnetic susceptibility of the reaction mixture in half-cell 2 (which is constant in time for the constant flow rates Q_M and Q_S) is compared with the magnetic susceptibility of the unreacted solution in half-cell 1. While flow is in progress, steady-state reaction mixtures are observed with a time resolution of 0.05 sec or less, corresponding to the time spent by the mixture in the neighborhood of the magnet. When flow is stopped, the magnetic changes in the reaction mixture, now stationary in the cell, are followed with a time resolution corresponding to the electronically adjustable response of the magnet. In the experiments to be described, the response was somewhat faster than in Fig. 1A; for the fastest reaction the undamped angular frequency was 2 rad sec^{-1} and the ratio of damping to critical damping was 0.55.

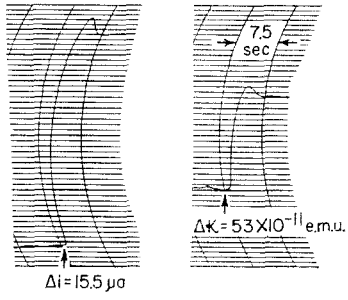


Fig. 1. Response of instrument to a current step in the directive force circuit (left), and to a nickel-chloride concentration step (right)*.

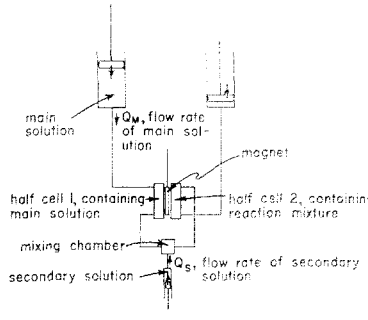


Fig. 2. Schematic of flow experiment.

The red compound formed in the reaction between horse ferrimyoglobin and methyl hydrogen peroxide was studied by THEORELL AND EHRENBERG[3] spectrophotometrically, using a Beckman apparatus, and magnetometrically, using their special Gouy-type balance[4]. Because of the instability of the compound at the ferrimyoglobin concentration of 650 μM required for the Gouy susceptometer, some of the compound had reverted to ferrimyoglobin during the relatively long time required to make a measurement with this instrument. Correcting for the amount of ferrimyoglobin present on the basis of spectrophotometric measurements, they obtained for the molar paramagnetic susceptibility of the compound a tentative value of $3000 \cdot 10^{-6}$ emu. They also measured the molar paramagnetic susceptibility of the compound formed in the reaction between ferrimyoglobin and H_2O_2, obtaining $3500 \cdot 10^{-6}$ emu. Recent calculations of GRIFFITH[5] show that a paramagnetic correction to the diamagnetism of the comparison hemoproteins should be made. Accordingly, these values are revised to 3300 and $3800 \cdot 10^{-6}$ emu.

* From this Figure it can be seen that the poor time resolution of the original flow system (see Fig. 14 of ref.[2]) has been remedied by the introduction of a cell of improved design.

In an extensive series of experiments, GEORGE AND IRVINE[6-10] have studied the reactions of ferrimyoglobin with hydrogen peroxide, methyl and ethyl hydrogen peroxides, and other strong oxidizing agents. They have shown that all the myoglobin compounds formed have the same absorption spectrum and state of oxidation, one equivalent higher than ferrimyoglobin. They have suggested that there is only one compound and that it contains iron in the quadrivalent state. Additionally they have shown that when the peroxides, which have two oxidizing equivalents, react with ferrimyoglobin, a transient oxidizing entity is produced which they suggest is a free radical.

More recently GIBSON, INGRAM AND NICHOLLS[11], utilizing electron spin resonance, have demonstrated the appearance of a free radical in the frozen reaction mixture of ferrimyoglobin and hydrogen peroxide. Their evidence indicates that the free radical is a product of a reaction of the transient oxidant rather than the peroxide, and is most likely situated in the globin where it is stabilized at the low temperature (90°K) required for the experiments. Additionally, in experiments with the magnetic field set for a g-value of 6, they find a quenching of the five unpaired electron signal of ferrimyoglobin when the peroxide compound is formed, which implies a small magnetic moment for the compound.

EXPERIMENTAL METHODS AND ANALYSIS OF DATA

The ferrimyoglobin, methyl hydrogen peroxide reaction has now been studied with the new rapid susceptometer. For reasons of temperature control, the flow scheme of Fig. 2 was used in these experiments so that the unreacted ferrimyoglobin was in one half-cell, the reaction mixture in the second half-cell, and the difference in the magnetic susceptibilities was measured. Table I is a compilation of the experimental conditions and the data obtained. The ferrimyoglobin concentrations were measured

TABLE I

EXPERIMENTAL CONDITIONS AND DATA OBTAINED

Expt.	*1*	*2*	*3*	*4*	*5*
[Ferrimyoglobin] $\times 10^6$, M	45.3	18.5	25.2	26.9	23.6
[Me OOH] $\times 10^6$, M	2300	210	240	200	190
pH*	8.36	8.23	8.25	8.29	8.28
Temperature °C	25	26	21	13.9	25.2
$\Delta\kappa \times 10^{11}$, emu (volume magnetic suscept. change)	—46.1 (mean of 4)	—21.1	—20.5 (mean of 2)	—	—27.9
k (rate constant), $sec^{-1}\ M^{-1}$	—	—	370	210	1030

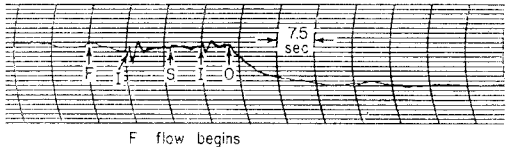
* In all experiments 0.10 M borate buffer was used.

in terms of hemin by the pyridine hemochromogen method[12]. Some hemin is destroyed in this reaction (see DISCUSSION). The concentrations given are for unreacted ferrimyoglobin solutions. The methyl hydrogen peroxide was kindly supplied by A. C. MAEHLY*. The hydrogen peroxide content, determined by the pertitanic acid complex

* The authors are pleased to thank DR. MAEHLY for his generous help.

method, was 6%. A correction was made for the O.D. of this impurity, and the concentration of the methyl hydrogen peroxide was measured spectrophotometrically at 230 mμ using an extinction coefficient of 32 M^{-1} cm^{-1}. The change in magnetic susceptibility obtained in experiment 4 is considered less reliable than other data and has not been included. The changes shown for expts. 1 and 3 are the mean values from identical reactions and carry weights of 4 and 2 respectively. The method of obtaining the rate constants is described in the paragraph on spectrophotometric experiments. Expts. 1 and 2 were done in 1955 and 1957 using an early cell with a time resolution of several seconds. Therefore, the kinetic data from these experiments are not included.

The record of expt. 5 is shown in Fig. 3. Methyl hydrogen peroxide was injected twice. The biphasic transients accompanying the injections (I) are due to jarring of the instrument when the stopcock was turned. The stopcock was turned off (S) with less disturbance. The steady-state reaction mixture in the cell during these injections corresponds to 0.10 sec after the beginning of the reaction. Essentially no deflection has occurred at this early stage. During the second injection period, the flow is stopped and the magnetic changes accompanying the remainder of the reaction are followed.



F flow begins
I injection of MeOOH
S injection stopped
O flow off

Fig. 3. Record of expt. 5.

The molar paramagnetic susceptibility of the compound formed in the reaction with methyl hydrogen peroxide is assumed to be independent of the pH because the absorption spectrum is pH independent[7]. The susceptibility is calculated from

$$\chi_{\text{compound}} = \chi_{\text{unreacted}} + \Delta\chi$$

where $\Delta\chi = 10^3 \Delta_{K\text{final}}/[\text{ferrimyoglobin}]$ is the measured change in molar susceptibility.

The unreacted ferrimyoglobin exists in two forms, a brown neutral form with a molar paramagnetic susceptibility at 20° of $\chi_{Fe} = 13{,}980 \cdot 10^{-6}$ emu and a red alkaline form with a susceptibility of $\chi_{FeOH} = 11{,}330 \cdot 10^{-6}$ emu[13]. (These values have been revised according to the calculation of GRIFFITH[5]).

The molar susceptibility of the unreacted ferrimyoglobin solution is

$$\chi_{\text{unreacted}} = x\chi_{Fe} + (1 - x)\chi_{FeOH}$$

where the mole fraction x of the neutral form is given by

$$\log \frac{x}{1 - x} = pK - pH$$

The temperature dependence of the pK has been determined[14]. After correcting the values for χ_{compound} to 20° by the Curie law, the mean value is calculated to be $3300 \cdot 10^{-6}$ emu with a standard error of $500 \cdot 10^{-6}$ emu.

At the high methyl hydrogen peroxide concentration used in the experiments, there was the possibility of a second reaction between the compound and unreacted MeOOH to form another compound, resulting in a mixture of compounds. This possibility was examined by recording the change in the absorption spectrum of ferrimyoglobin upon addition of MeOOH to make molar ratios of 1.5, 3.0, 4.5, and 7.5. The reactions were permitted to go to completion, and then the differential absorption spectrum was recorded from 500 mμ to 650 mμ. Confirming the observation of GEORGE AND IRVINE[7], the data show that a molar ratio somewhat greater than 3 : 1 is required for full formation of the compound at this pH. Within the accuracy of the spectrophotometer, the change in the absorption spectrum is the same for molar ratios of 4.5 and 7.5. At the molar ratios of 1.5 and 3.0 the change in the absorption spectrum is simply less intense. Therefore, only one spectrophotometrically identifiable compound is formed in this range of relative concentrations.

Spectrophotometric data were obtained on the kinetics of formation of the compound under the conditions of the magnetic expts. 3, 4 and 5. For the spectrophotometric studies, a split-beam recording spectrophotometer operated with a 0–67 % rise time of 1 sec was used[15]. The time course of the reaction, which was followed at 550 mμ, was found not to obey second-order kinetics even when the effective excess oxidant was taken smaller than the nominal excess ratio, as might be suggested by the high molar ratio required for complete reaction. In the absence of full knowledge of the actual reaction mechanism, one can still define a rate constant as the rate of formation of compound per unit concentration of ferrimyoglobin and per unit concentration of oxidant. Since the concentrations are known only at the beginning of the reaction, and the initial 1.5 or 2 sec are obscured by a mixing artefact, it was necessary to extrapolate the time course of the observed compound concentration back to the initial point. For this purpose of extrapolation, some empirical descriptions of our data were tried. With a power-law dependence of the form

$$y = m_0 [1 - (1 + t/T)^{-1/q}]$$

where y is the concentration of compound at the time t and m_0 is the initial ferrimyoglobin concentration, it was possible to adjust q and T to obtain an excellent fit. The second-order rate constant,

$$k = [\text{Me OOH}]^{-1} (m_0 - y)^{-1} \, dy/dt,$$

is then given by

$$k = 1/qTa_0$$

where a_0 is the initial concentration of MeOOH. q determines the shape of the reaction curve, and will therefore depend upon the conditions of the reaction. For the spectrophotometrically observed reactions, the shape factor q is found to be the following function of the excess ratio a_0/m_0,

$$q = 8.3 \, (a_0/m_0)^{-3/2}$$

The time courses of the magnetic susceptibilities fit the same empirical power function when q was calculated from the above formula and only T was adjusted. Thus there is no indication of different shape between the magnetic and spectrophotometric kinetic curves. However, the magnetic data do not allow of an accurate shape analysis. The rate constants for the magnetic curves are calculated in the same way as the spectrophotometric constants and are significantly smaller at lower temperatures.

This is shown in Fig. 4 which is an Arrhenius plot of the rate constants. Straight lines through the magnetic and spectrophotometric points were fitted by the method of least squares.

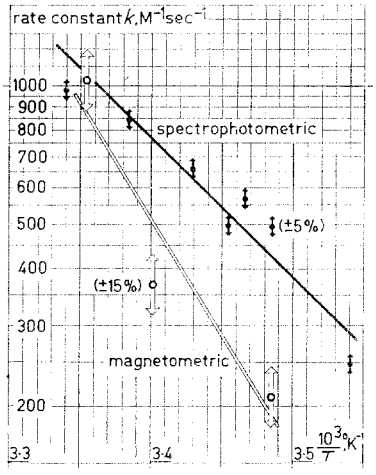


Fig. 4. Arrhenius plot of magnetic and Spectrophotometric rate data.

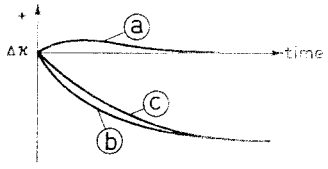


Fig. 5. *a*: the production and disappearance of free radicals; *b*: conversion of ferrimyoglobin to compound; *c*: observed change in magnetic susceptibility = *a* + *b*.

DISCUSSION

The existence of differences between the rates of change of optical density and magnetic susceptibility with time is consistent with the liberation of a free radical when ferrimyoglobin and methyl hydrogen peroxide react. This mechanism is one of two discussed by GEORGE AND IRVINE for the reaction between ferrimyoglobin and hydrogen peroxide[10]. It is interesting to note that the latter reaction proceeds about 5 times more slowly but has the same activation energy of 14,000 cal as calculated from the spectrophotometric data. The magnetically determined rate constants would not be expected to obey an Arrhenius relation if two or more magnetic processes contribute to the observed kinetic curve. The effect of the production and disappearance of free radical upon the time course of the magnetic susceptibility is shown schematically in Fig. 5. The observed change proceeds at a slower rate than the rate of conversion of ferrimyoglobin to compound alone. Spectrophotometrically only this conversion is followed (except possibly for a small effect due to the degradation of myoglobin;—see below). Because of the factor of eight between the molar magnetic susceptibility change in the myoglobin and the much smaller molar susceptibility of free radicals, it is difficult to account for the differences in the spectrophotometrically and magnetometrically determined rates on this basis alone. However, there is another observation which bears upon the problem.

Pyridine hemochromogen determinations of hemin concentration before and after the experiments show that as much as 11 % of the hemin of the myoglobin is destroyed during the reaction. The nature of the breakdown products has not yet been investigated so that their contributions to the magnetic susceptibility and to the O.D. are not quantitatively known but are almost certainly observable. It is very likely that the rate at which the degradation products are formed has an influence upon the spectrophotometric and magnetometric kinetic curves.

The value obtained for the molar susceptibility of the compound does not contain contributions from free radicals since these will have reacted to form stable diamagnetic products by the end of a magnetic run. However, the breakdown products will contribute to the extent that their molar susceptibilities differ from that of the compound. The amount, and possibly the types of breakdown product as well, depend upon the experimental conditions, and this variability may account for part of the spread in the values obtained for the molar susceptibility of the compound. The mean value of $3300 \cdot 10^{-6}$ emu at 20° is that for a complex of unit spin (two unpaired electrons). a spin which is theoretically possible for the d-shell configuration of GEORGE's postulated quadrivalent iron. It must be realized that for the small temperature range over which the magnetic experiments were made, the data are not sufficiently precise to test whether or not there is the Curie law dependence appropriate to a complex of unit spin. The molar susceptibility does not differ from the values obtained earlier by THEORELL AND EHRENBERG for both the hydrogen peroxide and methyl hydrogen peroxide reactions and supports the contention of GEORGE AND IRVINE that the same compound is formed in both reactions.

It is advisable now to extend the range of temperature and to investigate the breakdown products so that their effects upon the data can be evaluated. It would also be most useful to obtain data on the time course of the oxidant concentration. Until single large crystals of the compound are available, it is not likely that paramagnetic resonance will be of further help in elaborating the configuration of the d-shell.

ACKNOWLEDGEMENTS

The authors wish to thank F. A. MULLER for his assistance in analysing the data, and B. CHANCE, P. GEORGE Aand D. H. IRVINE for stimulating and helpful discussions.

This investigation was supported in its early stages by a Predoctoral Research Fellowship to A. S. BRILL from the Division of Research Grants of the United States Public Health Service.

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