Biochem. J. (1968) **110**, 617
Printed in Great Britain

The Catalase–Hydrogen Peroxide System

KINETICS OF CATALATIC ACTION AT HIGH SUBSTRATE CONCENTRATIONS

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(*Received* 4 *July* 1968)

1. A re-examination of the catalase–hydrogen peroxide reaction at high substrate concentrations, by using the quenched-flow technique, reveals a more complex kinetic behaviour than that previously reported. At constant reaction time the catalatic process obeys Michaelis–Menten kinetics, but the apparent Michaelis constant is markedly time-dependent, whereas the conventional catalase activity is independent of time. 2. The kinetics of the 'time effect' were analysed and it is suggested that the effect derives from the formation of an inactive species (thought to be catalase Compound II). The process shows Michaelis–Menten kinetics, with a Michaelis constant equal to that for the catalatic reaction in the limit of zero reaction time. 3. It has been confirmed that certain buffer components have marked inhibitory effects on the catalatic reaction and that, in unbuffered systems, catalatic activity is substantially independent of pH in the range 4·7–10·5.

Two different techniques have been used by previous workers to study the kinetics of catalatic action at high substrate concentrations (up to about 5M-hydrogen peroxide). Ogura (1955) used the 'two-mixer' quenched-flow technique, with reaction times in the range 0·1–0·4sec. and a very high enzyme concentration (0·12μM). He reported that the reaction obeyed Michaelis–Menten kinetics. George (1947, 1949) used a manometric method and studied the variation with time of the rates of oxygen evolution from reaction solutions over several minutes, with a very low enzyme concentration. He reported that the reaction rate fell from an initially high value (α-activity) to a 'steady' lower value (β-activity) in about 5min. In the latter phase the reaction showed a reversible inhibition at substrate concentrations greater than 0·07M. Jones & Wynne-Jones (1962) proposed a mechanism that was formally successful in reconciling these apparently discordant results, although quantitative differences remained. Bonnichsen, Chance & Theorell (1947) pointed out that the enzyme preparations used by George (1947) appeared to have very low activities. From our recent analysis (Jones & Suggett, 1968*a*) the valid assay of catalase activity at very low catalase concentrations presents severe difficulties, although this work also suggests that thermal deactivation of catalase may have been substantial in George's (1947) solutions.

In the present paper we examine the application of the quenched-flow technique to the study of catalase action. One aspect of this technique, which is of importance in the present context, is that, in a 'two-mixer' apparatus, the reaction takes place under essentially adiabatic conditions. The enthalpy of decomposition of hydrogen peroxide into water and oxygen is $-23{\cdot}5$kcal.mole^{-1}, so that, when the hydrogen peroxide concentration of a solution changes by 1 mole l.$^{-1}$ as a result of decomposition occurring under adiabatic conditions, the temperature of the solution (assuming a specific heat of unity) increases by 23·5°. Examination of the experiments of Ogura (1955) suggests that this factor was not appreciated; thus in this work a catalase concentration of 0·12μM was used with an initial hydrogen peroxide concentration of 5M and a reaction time of 0·4sec., which under adiabatic conditions would lead to a temperature rise of about 40°. We have confirmed experimentally that temperature changes corresponding to near-adiabatic conditions occur in experiments using the quenched-flow technique.

A systematic kinetic study of catalatic action at high substrate concentrations requires both maximal precision in the estimation of extent of reaction and isothermal conditions. Although these two factors are in opposition in the quenched-flow technique, it is possible, because of the low activation energy of catalase action, to devise an acceptable compromise procedure. In the present paper we report an investigation of the kinetics of bacterial catalase action at high substrate concentrations by the quenched-flow method. In our experiments a change of 0·1mole l.$^{-1}$ was taken as the maximum allowable extent of hydrogen peroxide decomposition. This procedure controls the maximum increase in the average reaction temperature from the thermostat value to about 1°.

MATERIALS AND METHODS

The preparation of bacterial catalase and other materials for this work and the analytical procedures employed have been described previously (Jones & Suggett, 1968*a*). In the 'two-mixer' quenched-flow apparatus the rate of flow could be varied to give reaction times between 0·2 and 0·5 sec. The syringes containing reactants and quenching agent (2·5 M-H_2SO_4) were surrounded by a thermostatically controlled water jacket and the flow system was contained in an air thermostat.

The kinetic experiments yield values of H_2O_2 concentration as a function of time. In analysing these results it is necessary, either to use some method of differentiation of the primary data, so that differential rate laws may be used, or to use an integrated rate law (e.g. for measurements at low substrate concentrations an integrated first-order rate law is employed (Bonnichsen *et al.* 1947; Jones & Suggett, 1968*a*)). We have compared our experimental data with the integrated Michaelis–Menten equation in the form:

$$-\frac{\Delta \ln [P]}{\Delta [P]} = \frac{k_3}{K_m} \cdot \frac{\Delta t[E_0]}{\Delta [P]} + \frac{1}{K_m} \qquad (1)$$

a modified form of the equation used by Kremer (1965). $[E_0]$ represents the stoicheiometric enzyme concentration, and [P] the initial H_2O_2 concentration.

In the quenched-flow technique it is necessary to make up the catalase reaction solution in buffer of the appropriate composition and pH before addition of substrate. It was therefore to be expected that the catalase 'activity' determined by the quenched-flow procedure [given by k_3/K_m from plots according to eqn. (1) if Michaelis–Menten kinetics are obeyed] would correspond to the quantity k_0 as defined by Jones & Suggett (1968*a*), rather than to the activity (k_B) as measured by the conventional assay procedure (Bonnichsen *et al.* 1947; Jones & Suggett, 1968*a*).

In our experiments it was possible to use catalase concentrations of about 10 nM. Catalase reactant solutions of these concentrations may be prepared from stock solutions and stored for several days near 0° without decrease in activity arising from thermal deactivation (Jones & Suggett, 1968*a*).

RESULTS

The results of our experiments show a considerably more complex kinetic behaviour than that reported by Ogura (1955). The type of complexity observed is illustrated in Fig. 1, where data from experiments in potassium dihydrogen phosphate–disodium hydrogen phosphate buffer, pH 7, at 20° are plotted according to eqn. (1). The initial hydrogen peroxide concentrations were 0·30, 0·41, 0·59 and 0·74 M and the corresponding enzyme concentrations were 16·9, 14·4, 11·8 and 8·5 nM respectively. Four reaction times between 0·2 and 0·5 sec. were used for each set of initial conditions. For each reaction time the points lie on a straight line. However, the points for different reaction times are not collinear, but yield four separate lines that are parallel and separated by distances proportional to the difference in reaction time. For simple

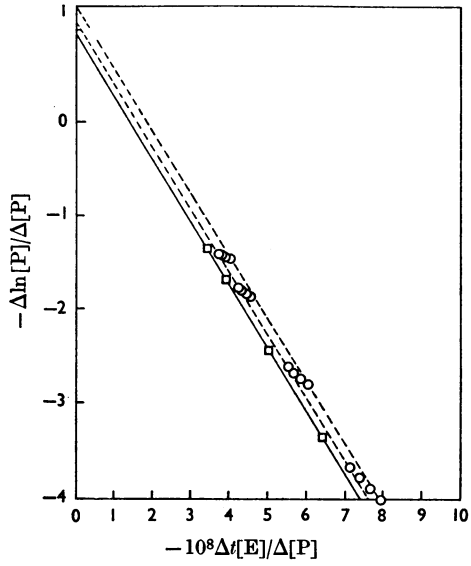


Fig. 1. Integrated Michaelis–Menten plot for pH 7·0 at 20°. ○, Experimental points; □, data extrapolated to zero time.

Michaelis–Menten kinetic behaviour all 16 experimental points should be collinear.

The direction of this 'time effect' corresponds to a deactivation of catalase with increasing reaction time. In terms of eqn. (1) the results imply that the apparent Michaelis constant for the reaction is time-dependent (0·96 M with a reaction time of 0·2 sec., 0·81 M with 0·5 sec.), although the catalase activity, k_3/K_m, is independent of time. By extrapolation of the data for each initial hydrogen peroxide concentration we obtain the Michaelis–Menten kinetic behaviour of catalase in the limit of zero reaction time (squares and solid line in Fig. 1). From the extrapolated data the Michaelis–Menten parameters obtained in phosphate buffer, pH 7, at 20° are $K_m^0 = 1{\cdot}1$ M and $k_3/K_m = 6{\cdot}61 \times 10^7 \text{M}^{-1}\text{sec.}^{-1}$. The latter value compares with $k_0 = 6{\cdot}62 \times 10^7 \text{M}^{-1}\text{sec.}^{-1}$ obtained by assay at low substrate concentration (Jones & Suggett, 1968*a*) with the same catalase preparation. From studies of the effect of temperature variation (13° to 30°), K_m^0 was found to be independent of temperature. The temperature coefficient of k_3/K_m corresponded to an activation energy of $0{\cdot}90 \pm 0{\cdot}05$ kcal.mole^{-1}, which compares with the value of $1{\cdot}01 \pm 0{\cdot}06$ kcal.mole^{-1} reported with low substrate concentrations.

From measurements in other buffers (citric acid–disodium hydrogen phosphate, pH 4·65, potassium dihydrogen phosphate–disodium hydrogen phosphate, pH 8·25, and borax–sodium carbonate, pH 9·45) K_m^0 was found to be independent of buffer

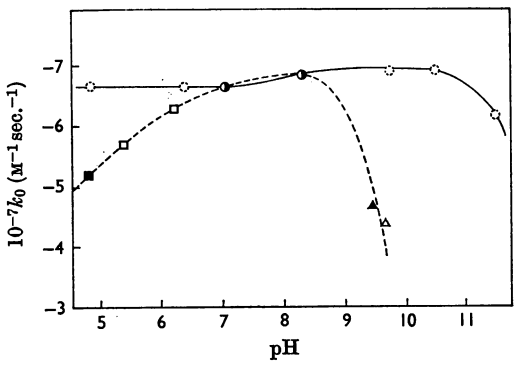


Fig. 2. Dependence of k_0 on pH at 20°. Filled symbols refer to quenched-flow data, open symbols to low-concentration data (Jones & Suggett, 1968*a*) and broken symbols to quenched-flow data in virtually unbuffered systems; ◑ indicates points common to the first two types of measurement (in KH_2PO_4–Na_2HPO_4 buffers). □, Citric acid–Na_2HPO_4 buffers; △, borax–Na_2CO_3 buffers.

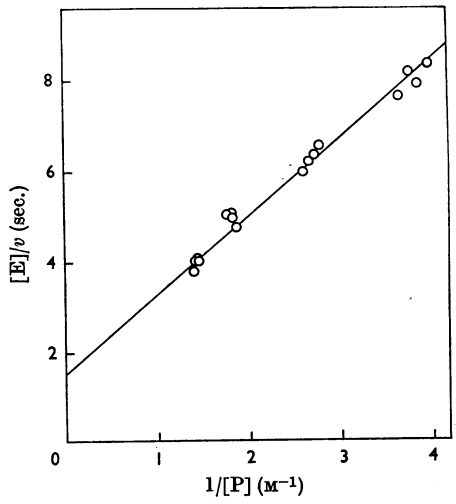


Fig. 3. Relationship between [E]/v and 1/[P] (Lineweaver–Burk plot) for the 'time-effect' data derived from Fig. 1.

composition and pH, but the values of k_3/K_m changed in the same manner as found in k_0 determinations at low substrate concentrations. The two sets of data are compared in Fig. 2. Also shown in Fig. 2 are values of k_3/K_m obtained from quenched-flow experiments with essentially unbuffered solutions. In these measurements the pH was adjusted by adding small amounts of either 0·1M-hydrochloric acid or 0·1M-sodium hydroxide to a catalase reactant solution in dilute phosphate buffer. The results confirm the idea that there are substantial specific effects of some buffer components on catalase activity if the enzyme is equilibrated with buffer before addition of substrate. In the unbuffered systems the activity is almost independent of pH in the range 4·7–10·5. The sharp fall in activity found at about pH 11 has almost universally been accepted as being the result of an irreversible, or partially reversible, denaturation. Ogura's (1955) suggestion that an apparent pH effect on catalase activity in borax–sodium carbonate and citrate buffers was due to non-competitive inhibition by H^+ and OH^- ions is not confirmed by the above results.

By using the extrapolated Michaelis–Menten catalatic kinetic data as a base line it is possible to analyse the 'time effect' as a phenomenon that arises from a decrease with time of the concentration of active catalase. The decrease in active enzyme concentration, $\Delta[E_a]$, was calculated from:

$$\Delta[E_a] = [E_0].\frac{\Delta[P]_{obs.}}{\Delta[P]_{calc.}} \qquad (2)$$

where $[E_0]$ is the stoicheiometric catalase concentration and $\Delta[P]_{calc.}$ is the change in substrate concentration predicted by the 'zero-reaction-time' Michaelis–Menten curve. The velocity of the 'time-effect' deactivation process is then $v^* = -d[E_a]/dt$, or for small reaction time $v^* = -\Delta[E_a]/\Delta t$. The kinetic data obtained in this way showed that the 'time-effect' reaction itself follows Michaelis–Menten kinetics, i.e.:

$$v^* = \frac{k^*[E][P]}{K_m + [P]} \qquad (3)$$

Fig. 3 illustrates the data obtained in phosphate buffer, pH 7, at 20° plotted according to the Lineweaver–Burk procedure. This graph yields $K_m^* = (1{\cdot}1 \pm 0{\cdot}25)$ M and $k^* = 0{\cdot}65 \pm 0{\cdot}1$ sec.$^{-1}$. From the experiments in other conditions it was found that k^* and K_m^* were independent of pH, and the temperature coefficient of k^*/K_m^* corresponds to an activation energy of 10 ± 2 kcal.mole^{-1}. An interesting and possibly important result of this analysis is that, within the experimental error, $K_m^0 = K_m^*$.

DISCUSSION

The excellent agreement between our value K_m^0 and that reported by Ogura (1955) seems at first sight remarkable, in view of the considerations presented above, although this is to some extent explained by the insensitivity of K_m^0 to temperature changes. Ogura (1955) used differential kinetic plots in analysing his results and this procedure necessarily involves a differentiation of the primary experimental data. The illustrative zero-order plot shown by Ogura (1955, Fig. 2) could not apply, except by fortuitous cancellation of effects, under all conditions, and it seems likely that the reaction

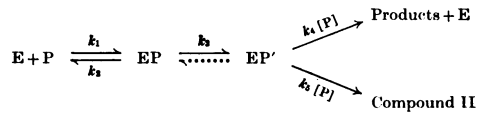
velocity was obtained by taking the initial slope of plots of extent of reaction against time. Such a procedure would extrapolate out the thermal effect and be expected to lead to a K_m value comparable with K_m^0 as determined in our experiments. It would also obscure the effect of time on the kinetic parameters of the reaction.

In the analysis of the kinetics of catalase action given by Jones & Wynne-Jones (1962) it was suggested that the difference in kinetic form reported by Ogura (1955) and George (1949) arose because the quenched-flow work of the former referred to a 'pseudo-steady state' in which reversible deactivation processes had not proceeded appreciably, whereas the latter results (in the β-activity phase) referred to a 'steady state' with respect to these deactivation processes. A difficulty with the model presented was that the Michaelis constants obtained from the two sets of data differed substantially.

The present work shows that catalase action cannot be studied directly in the absence of deactivation phenomena, because catalatic action and the 'time-effect' deactivation phenomenon are similar in kinetic form, so that the latter process is significant at all finite reaction times. Nevertheless the effects of deactivation may be sensibly extrapolated out and the kinetic data for the 'time effect' show an apparent decrease in the reaction Michaelis constant with reaction time. Projection of the 'time-effect' data from the present study to the time-scale used by George (1949) suggests that this effect can account for the discrepancy in Michaelis constant between the results of Ogura (1955) ($K_m = 1{\cdot}1$M) (and our own value for $K_m^0 = 1{\cdot}1$M) and that obtained from George's (1949) data (0·03M).

Circumstantial evidence suggests that the 'time effect' probably arises from the accumulation of catalase Compound II. After catalase and hydrogen peroxide have been mixed and the reaction has been allowed to subside the spectrum of Compound II is observed (Jones, Pain & Suggett, 1968), and Compound II has been shown (Chance, 1949) to be catalatically inactive. The kinetics of Compound II formation (above very low peroxide concentrations; Chance, 1949) are similar to the kinetics of the 'time effect'.

To describe the aspects of catalase action studied in this work we therefore suggest the model shown in Scheme 1, where EP and EP′ are the primary and secondary complexes proposed by Jones & Wynne-Jones (1962) (i.e. Compound I is a mixture of EP and EP′). At short reaction times the amount of Compound II formation is small and we make the approximation $\Delta[\text{Compound II}]/\Delta t = \mathrm{d}[\text{Compound II}]/\mathrm{d}t$ so that $[\text{Compound II}] = k_5[\text{EP}'][\text{P}]\Delta t$. Assuming a steady state for the intermediates EP and EP′ yields the rate equation:



Scheme 1.

$$-\frac{\mathrm{d}[\text{P}]}{\mathrm{d}t} = \frac{k'[\text{E}_0][\text{P}]}{K_m + [\text{P}]} \tag{4}$$

where:

$$k' = \frac{k_1k_2k_4}{k_1k_4 + k_1k_5 + k_1k_3k_5\Delta t} \tag{5}$$

and

$$K_m = \frac{k_2k_4 + k_2k_5 + k_3k_5 + k_3k_4 + k_1k_3}{k_1k_4 + k_1k_5 + k_1k_3k_5\Delta t} \tag{6}$$

An expression of the same form as eqn. (6) applies to the rate of formation of Compound II with the same K_m, but with a constant k'' that differs from k' in that k_5 replaces k_4 in the numerator of eqn. (5). Thus this scheme fulfils the three requirements of the present experimental work: (i) K_m is time-dependent; (ii) k'/K_m is time-independent; (iii) the same K_m value is obtained for the 'time effect' and the catalatic reaction. Although this scheme is formally satisfactory it is unlikely that it affords a complete description of the process, since the formation of Compound II involves a major disruption of the catalase molecule (Jones *et al.* 1968). A detailed discussion of this point is presented in the next paper (Jones & Suggett, 1968*b*).

We are indebted to Professor J. Baddiley, F.R.S., for making available facilities for growth and manipulation of bacteria in the Science Research Council Microbiological Chemistry Research Unit Laboratories, and to Dr A. L. Davison for assistance with microbiological work. We also thank the Science Research Council for a studentship and the University of Newcastle upon Tyne for a Sir James Knott Fellowship (both to A.S.).

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