APPENDIX

Analysis of the Catalase–Hydrogen Peroxide Intermedi in Coupled Oxidations

By BRITTON CHANCE and NOZOMU OSHINO
Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104, *U.S.A.*

(*Received* 13 *September* 1972)

In previous communications, we considered the kinetics of the catalase–H_2O_2 intermediate in coupled oxidations of the isolated liver catalase (Chance, 1949) and of the mixed mitochondrial–peroxisomal fraction (Chance & Oshino, 1971). In those cases, it was possible to record the kinetics of formation of the intermediate by H_2O_2 pulses, and solve for the H_2O_2 concentration. In most of the cases, however, only the analysis of steady-state effects are feasible and will be useful for the further study on the catalase reaction, especially in a system such as the perfused liver. It seems appropriate, therefore, to extend the analysis. The chemical equations for the catalase reactions are:

$$\underset{(e-p)}{\text{Cat}} + \underset{(x)}{H_2O_2} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(p)}{\text{Cat–}H_2O_2}$$

$$\underset{(p)}{\text{Cat–}H_2O_2} + \underset{(a)}{C_2H_5OH} \xrightarrow{k_4} \underset{(e-p)}{\text{Cat}} + H_2O + CH_3CHO$$

$$\underset{(p)}{\text{Cat–}H_2O_2} + \underset{(x)}{H_2O_2} \xrightarrow{k_4'} \underset{(e-p)}{\text{Cat}} + O_2 + 2H_2O$$

Symbols* in parentheses represent the concentrations of corresponding molecular species. In the following analysis the designations of rate constants and the symbols for molecular species do not conform with current practice but instead retain the nomenclature originally used, to avoid confusion and to simplify a comparison of these equations with those previously derived (Chance, 1949; Chance *et al.*, 1952).

The three differential equations for catalase action (Chance *et al.*, 1952) are modified by inserting into eqn. (2) the rate of generation of H_2O_2, dx_n/dt:

$$\frac{dp}{dt} = k_1 x(e-p) - k_4' xp - (k_2 + k_4 a)p \qquad (1)$$

$$\frac{dx}{dt} = \frac{dx_n}{dt} - k_1 x(e-p) - k_4' xp + k_2 p \qquad (2)$$

$$\frac{da}{dt} = -k_4 ap \qquad (3)$$

The equations are solved for the steady state of the catalase intermediate $\left(\frac{dp}{dt}=0\right)$, for the steady state of H_2O_2 generation $\left(\frac{dx}{dt}=0\right)$ and for the particular case where k_2, the rate of dissociation of H_2O_2 from catalase intermediate, is negligible. The initial concentration of the hydrogen donor is a_0. The equations were also studied by Clayton (1959) and a graphic solution representing the various parameters was presented. Summing eqns. (1) and (2), and noting that when $\frac{dx}{dt}=0$, $x = x_m$ and $p = p_m$, the steady-state concentration of the intermediate (as distinguished from p_M, the maximum concentration of the intermediate), gives eqn. (4a):

$$\frac{dx_n}{dt} = 2k_4' x_m p_m + k_4 a_0 p_m \qquad (4a)$$

The steady-state solution for p_m/e is derived from a solution of eqn. (1), giving eqn. (5a):

$$\frac{p_m}{e} = \frac{1}{1 + \frac{k_4'}{k_1} + \frac{k_4 a_0}{k_1 x_m}} \qquad (5a)$$

In general terms, $k_4' = nk_1$, and eqn. (4a) becomes:

$$\frac{dx_n}{dt} = 2nk_1 x_m p_m + k_4 a_0 p_m \qquad (4b)$$

* The following symbols are used: p, concentration of the catalase–H_2O_2 intermediate (Compound I) at any time, based on haem molarity; p_m, concentration of catalase–H_2O_2 at the steady state; p_M, maximal attainable value of p_m for a particular e value; e, total concentration of enzyme, catalase haem iron molarity; a, concentration of hydrogen donor; x, concentration of H_2O_2 at any time; x_m, concentration of H_2O_2 at the steady state; $\frac{dx_n}{dt}$, rate of H_2O_2 generation; p/e, proportion of haem binding with H_2O_2 in the total haem.

Similarly, eqn. (5a) becomes:

$$\frac{p_m}{e} = \frac{1}{1+n+\frac{k_4 a_0}{k_1 x_m}} \quad (5b)$$

A general equation for $\frac{dx_n}{dt}$ can be obtained by substituting eqn. (5b) into eqn. (4b) and eliminating x_m. From eqn. (5b):

$$x_m = \frac{k_4 a_0}{k_1}\left[\frac{p_m}{e-(n+1)p_m}\right] \quad (5c)$$

then, from eqn. (4b) by substituting for x_m and rearranging:

$$\frac{dx_n}{dt} = k_4 a_0 p_m\left[1+2n\left(\frac{1}{\frac{e}{p_m}-(n+1)}\right)\right] \quad (4c)$$

This equation generally relates the rate of H_2O_2 generation to the rate of the 'peroxidatic' reaction. When, from eqn. (3), $-\frac{da}{dt}$ is substituted for $k_4 a_0 p_m$:

$$\frac{dx_n}{dt} = \left(-\frac{da}{dt}\right)\left[1+2n\left(\frac{1}{\frac{e}{p_m}-(n+1)}\right)\right] \quad (4d)$$

Rearranging:

$$\frac{p_m}{e} = \frac{1}{n+1+2n\,\frac{1}{\left[\frac{\frac{dx_n}{dt}}{\left(-\frac{da}{dt}\right)}-1\right]}} \quad (4e)$$

Therefore the p_m/e value directly relates to the proportion of the 'peroxidatic' reaction in the overall H_2O_2-decomposition reaction. Experimentally a linear relationship is obtained and the deviation from theory (eqn. 4e) is largely set by the error of the experiments from $p_m/e = 0.02$ to 0.39 as shown in Fig. 1.

Simplified forms of eqn. (4c) are obtained if a_0 is adjusted to $a_{1/2}$, the value that brings p_m to $p_M/2$. From eqn. (5b) as $x_m \to \infty$, $p_m \to p_M$, thus:

$$\frac{p_M}{e} = \frac{1}{1+n} \quad (5d)$$

At $a_0 = a_{1/2}$,

$$p_m = \frac{p_M}{2} = \frac{e}{2(1+n)} \quad (5e)$$

Substituting for p_m in eqn. (4c) and simplifying:

$$\left[\frac{dx_n}{dt}\right]_{a_{1/2}} = k_4 a_{1/2} p_m\left(\frac{3n+1}{n+1}\right) = k_4 a_{1/2} e\left[\frac{3n+1}{2(n+1)^2}\right] \quad (4f)$$

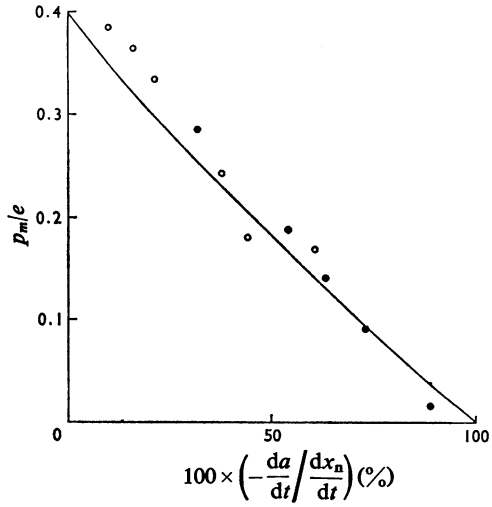

Fig 1. *Relationship between* p_m/e *and* $\frac{da}{dt}\Big/\frac{dx_n}{dt}$

The steady-state concentration of the catalase–H_2O_2 intermediate (p_m/e) and the proportion of the 'peroxidatic' reaction (da/dt) in the overall H_2O_2 decomposition (dx_n/dt) were measured as described for Fig. 7 and Fig. 1 in the main paper (Oshino *et al.*, 1973*b*) respectively. ——, Theoretical curve of eqn. (4e). o, Experimental values in the presence of 1 mM-ethanol; ●, experimental values in the presence of 10 mM-ethanol.

Eqn. (4f) is rearranged:

$$a_{1/2} = \left[\frac{1}{\frac{3n+1}{2(n+1)^2}k_4}\right]\frac{1}{e}\cdot\frac{dx_n}{dt} \quad (4g)$$

For rat liver catalase $n = 1.5$ (Chance & Oshino, 1971) and, assuming k_4 for ethanol $= 10^3\,M^{-1}\cdot s^{-1}$ (Chance, 1949), eqn. (4g) becomes:

$$a_{1/2} = 2.28\times10^{-3}\left(\frac{1}{e}\cdot\frac{dx_n}{dt}\right) \quad (4h)$$

Thus the $a_{1/2}$ value can represent the ratio of H_2O_2-generation rate to the catalase concentration in the system. An experimental support for eqn. (4f) was obtained; k_4 values for ethanol and methanol calculated from eqn. (4f) by measuring the $\frac{1}{e}\frac{dx_n}{dt}$ and $a_{1/2}$ values are in good agreement with directly determined values (Chance, 1949) (Table 1).

In a similar treatment, eqns. (4d) and (4f) become:

$$\frac{dx_n}{dt} = \left(-\frac{da}{dt}\right)\left(1+\frac{3}{\frac{e}{p_m}-2.5}\right) \quad (4i)$$

Table 1. *k_4 values for methanol and ethanol estimated from eqn. (4f)*

The alcohol concentration producing $p_m/e = 0.2$ at various $\frac{1}{e}\cdot\frac{dx_n}{dt}$ values was determined as described for Fig. 7 in the main paper (Oshino *et al.*, 1973*b*). Since $n = 1.5$ with rat liver catalase, k_4 was calculated from the equation: $\frac{1}{e}\cdot\frac{dx_n}{dt} = 0.44 k_4 a_{1/2}$. The temperatures used for methanol and ethanol experiments were 30°C and 25°C respectively. The k_4 values for methanol and ethanol, which were directly measured at room temperature, were $10^3 \text{M}^{-1}\cdot\text{s}^{-1}$ (Chance, 1949).

$\frac{1}{e}\cdot\frac{dx_n}{dt}$ (s^{-1})	Alcohol	$10^3 \times a_{1/2}$ (M)	$10^{-3} \times k_4$ ($M^{-1}\cdot s^{-1}$)
0.067	Methanol	0.10	1.5
0.096		0.20	1.1
0.125		0.30	1.0
0.200		0.40	1.1
0.322		0.60	1.2
0.200	Ethanol	0.5	0.9
0.367		1.0	0.8
1.02		3.0	0.8
2.40		6.0	0.9
4.84		10.0	1.1

$$\left[\frac{dx_n}{dt}\right]_{a_{1/2}} = -2.2\left[\frac{da}{dt}\right]_{a_{1/2}} \quad (4j)$$

Eqns. (4i) and (4j) define the relationship between the rate of H_2O_2 generation and the rate of alcohol disappearance or aldehyde formation under conditions where the hydrogen donor, a_0, has been adjusted to a value of $a_{1/2}$: where the steady-state concentration of the catalase intermediate, p_m, is one-half its maximum concentration, i.e. $p_m = p_M/2$, and assuming that a steady state can be maintained, i.e. $dx/dt = 0$. The latter represents a significant constraint upon the system, and implies that there is enough catalase present to 'control' the H_2O_2 concentration and maintain it at a constant value. When this constraint is applicable, then it is apparent that the fraction of the H_2O_2 generated that is utilized in converting alcohol into aldehyde in the presence of sufficient donor to give $p_m = p_M/2$ is 45% of the total, for a catalase for which $m = 1.5$ (eqn. 4j) [cf. Fig. 3 in the main paper (Oshino *et al.*, 1973*b*)]. The data further show that the fractional conversion is independent of the rate of H_2O_2 generation and of the molarity of the enzyme (Sies *et al.*, 1973).

The steady-state H_2O_2 concentration, x_m, can further be related to the rate of alcohol disappearance or aldehyde formation by the following series of steps. Eqns. (5c) and (5e) are arranged:

$$(n+1)k_1 x_m = k_4 a_{1/2} \quad (6)$$

From eqns. (3) and (5e),

$$k_4 a_{1/2} = -\frac{2(n+1)}{e}\cdot\frac{da}{dt} \quad (7)$$

Substituting eqn. (7) into eqn. (6):

$$(n+1)k_1 x_m = -\frac{2(n+1)}{e}\cdot\frac{da}{dt} \quad (8a)$$

Simplifying:

$$\frac{da}{dt} = \left[-\frac{k_1 x_m e}{2}\right]_{a_{1/2}} \quad (8b)$$

In this equation, it is observed that the rate of alcohol disappearance or aldehyde formation at a hydrogen donor concentration that causes $p_m = p_M/2$ is, first, independent of the value of n for the particular catalase. Secondly, it is constant for a wide range of x_m values and e values. In this respect, the system is self-adjusting; with low values of e, x_m will be high and vice versa. Thus we find an explanation for the insensitivity of alcohol disappearance or aldehyde formation to the washing of catalase out of microsomal fraction (Roach *et al.*, 1969) or to the inhibition of catalase by cyanide or azide; only the balance between x_m and e values is altered.

By substituting da/dt in eqn. (4f), with its $k_4 a_{1/2} p_m$ value in eqn. (8b), we get:

$$\left[\frac{dx_n}{dt}\right]_{a_{1/2}} = \left(\frac{3n+1}{n+1}\right)\left(\frac{k_1 x_m e}{2}\right) \quad (9a)$$

or:

$$\left[\frac{1}{e}\frac{dx_n}{dt}\right]_{a_{1/2}} = \left(\frac{3n+1}{n+1}\right)\left(\frac{k_1 x_m}{2}\right) \quad (9b)$$

Thus the term $\frac{1}{e}\frac{dx_n}{dt}$ is linearly related to x_m when $a_0 = a_{1/2}$.

The agreement of the equations developed here with the experimental results on coupled oxidations gives further support to the validity of the general equations for the 'catalatic' and 'peroxidatic' reactions of catalase (Chance *et al.*, 1952; Chance, 1969), and provides the theoretical basis for applying the steady-state analysis in the perfused liver (Sies *et al.*, 1973; Oshino *et al.*, 1973*a*).

This work was supported by Research Grant MH-20573-1 from the National Institute of Mental Health, U.S.A.

References

Chance, B. (1949) *Acta Chem. Scand.* **1**, 236–267
Chance, B. (1969) *Ann. N.Y. Acad. Sci.* **168**, 354–355
Chance, B. & Oshino, N. (1971) *Biochem. J.* **122**, 225–233

Chance, B., Greenstein, D. S. & Roughton, F. J. W. (1952) *Arch. Biochem. Biophys.* **37**, 301–339
Clayton, R. K. (1959) *Biochem. Biophys. Res. Commun.* **1**, 191–193
Oshino, N., Chance, B., Sies, H. & Bücher, Th. (1973*a*) *Arch. Biochem. Biophys.* **154**, 117–131
Oshino, N., Oshino, R. & Chance, B. (1973*b*) *Biochem. J.* **131**, 555–563
Roach, M. K., Reed, W. N. & Creaven, P. J. (1969) *Biochem. Biophys. Res. Commun.* **36**, 569–602
Sies, H., Bücher, Th., Oshino, N. & Chance, B. (1973) *Arch. Biochem. Biophys.* **154**, 106–116