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Kinetics and Mechanisms of Catalase in Peroxisomes of the Mitochondrial Fraction

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1. The primary intermediate of catalase and hydrogen peroxide was identified and investigated in peroxisome-rich mitochondrial fractions of rat liver. On the basis of kinetic constants determined *in vitro*, it is possible to calculate with reasonable precision the molecular statistics of catalase action in the peroxisomes. 2. The endogenous hydrogen peroxide generation is adequate to sustain a concentration of the catalase intermediate (p_m/e) of 60–70% of the hydrogen peroxide saturation value. Total amount of catalase corresponds to 0.12–0.15nmol of haem iron/mg of protein. In State 1 the rate of hydrogen peroxide generation corresponds to 0.9nmol/min per mg of protein or 5% of the mitochondrial respiratory rate in State 4. 3. Partial saturation of the catalase intermediate with hydrogen peroxide (p_m/e) in the mitochondrial fraction suggests its significant peroxidatic activity towards its endogenous hydrogen donor. A variation of this value (p_m/e) from 0.3 in State 4 to 0 under anaerobic conditions is observed. 4. For a particular preparation the hydrogen peroxide generation rate in the substrate-supplemented State 4 corresponds to $0.17\,s^{-1}$ (eqn. 6), the hydrogen peroxide concentration to 2.5nM and the hydrogen-donor concentration (in terms of ethanol) to 0.12mM. The reaction is 70% peroxidatic and 30% catalatic. 5. A co-ordinated production of both oxidizing and reducing substrates for catalase in the mitochondrial fraction is suggested by a 2.2-fold increase of hydrogen peroxide generation and a threefold increase in hydrogen-donor generation in the State 1 to State 4 transition. 6. Additional hydrogen peroxide generation provided by the urate oxidase system of peroxisomes (8–12nmol of uric acid oxidized/min per mg of protein) permits saturation of the catalase with hydrogen peroxide to haem occupancy of 40% compared with values of 36% for a purified rat liver catalase of

$$k_1 = 1.7 \times 10^7\,M^{-1}\cdot s^{-1} \quad \text{and} \quad k_4' = 2.6 \times 10^7\,M^{-1}\cdot s^{-1}$$

(Chance, Greenstein & Roughton, 1952). 7. The turnover of the catalase ethyl hydrogen peroxide intermediate (k_3') in the peroxisomes is initially very rapid since endogenous hydrogen peroxide acts as a hydrogen donor. k_3' decreases fivefold in the uncoupled state of the mitochondria.

The iron porphyrin proteins or 'haemoproteins', the haemoglobins, myoglobins, cytochromes and peroxidases, represent the best-known class of proteins. Atomic and electronic structures are probed with the most sophisticated magnetic techniques (Wuthrich, Shulman & Yamane, 1968) and, in several cases, with high-resolution X-ray crystallography (Dickerson *et al.* 1971). Reactions with their physiological substrates, for example oxygen or hydrogen peroxide, give compounds of definite structures and valency states of the haem iron atom that are characterized by distinctive absorption bands, thus permitting intensive kinetic studies of their reaction mechanisms. In fact, haemoglobin and catalases or peroxidases serve respectively as prototypes for enzyme–substrate reaction mechanisms of co-operative and non-co-operative natures (Chance, Greenstein & Roughton, 1952; Monod, Wyman & Changeux, 1965; Chance 1943). Whereas the physiological function of the haemoglobin in oxygen transport is relatively well understood *in vitro* (Dickens & Neil, 1963), our knowledge of the biological function of catalases and peroxidases *in vivo* is most rudimentary (Chance, 1952*a*, 1954; Yonetani, 1970).

The localization of catalase in one of the intracellular organelles, peroxisomes (de Duve & Baudhuin, 1966), leads to the possibility of applying the mass of kinetic constants *in vitro* for the catalytic function of the enzyme–substrate compound of

catalase to its function in its native biological structure in isolated cell fractions and *in vivo* (Sies & Chance, 1970). Quantitative evidence for the existence of significant amounts of its oxidizing (H_2O_2) or reducing substrates (generally $\geq$C–OH functional groups; Chance, 1951) in tissue is lacking, since catalase, by virtue of its peculiar capacity for catalatic (eqns. 1 and 2) as well as peroxidatic (eqns. 1 and 3) reactions literally 'destroys the evidence' for free hydrogen peroxide in the cell (Chance, 1951). However, we may identify the presence of hydrogen peroxide from the distinctive spectroscopic properties of the intermediate compounds and peroxides (eqn. 1) generated in the isolated fractions from liver and in the perfused organs (Sies & Chance, 1970). Under conditions *in vitro* comparable with those used in the present investigation, the chemical mechanism for catalase action can be adequately described by three irreversible reactions, the first two being consecutive reactions with hydrogen peroxide (Chance, 1949*a*) and the third reaction responsible for the coupled oxidations described by Keilin & Hartree (1945):

$$\text{Cat-Fe}^{3+} + H_2O_2 \xrightarrow{k_1} \text{Cat-Fe}^{3+}\text{–}H_2O_2 \quad (1)$$

$$\text{Cat-Fe}^{3+}\text{–}H_2O_2 + H_2O_2 \xrightarrow{k_2} \text{Cat-Fe}^{3+} + 2H_2O + O_2 \quad (2)$$

$$\text{Cat-Fe}^{3+}\text{–}H_2O_2 + \text{>C(H)OH} \xrightarrow{k_4} \text{Cat-Fe}^{3+} + \text{>C=O} + 2H_2O \quad (3)$$

Alkyl hydrogen peroxides substitute for hydrogen peroxide in eqns. (1) and (3), but not in eqn. (2) (Chance, 1950). Further details of the reactions are described by Kremer (1970) and by Jones & Suggett (1968).

For the study of catalase action, we have chosen the mitochondrial fraction, because work by Chance & Williams (1955) has shown the intermediate compound to be present at 12% of the cytochrome *a* concentration and this fraction could simulate the function of catalase *in vivo*. At that time, however, our attention was primarily focused on the relative amounts of the components in the mitochondrial fraction. The present work attempts to furnish us with the information on the possible function of catalase in the mitochondria. It reports some of the previous unpublished experiments on the nature of catalase intermediates and on the nature, source and rate of hydrogen peroxide generation, thereby providing an evidence of interaction between mitochondrial function and the hydrogen donor pool available for catalase intermediates. The kinetics and mechanism of catalase function in its physiological state can be quantitatively evaluated.

MATERIALS AND METHODS

The mitochondrial fraction was prepared from rat liver by differential centrifugation (Schneider & Hogeboom, 1950). To eliminate any haemoglobin contamination of the mitochondrial fraction, livers were perfused with cold 0.225M-mannitol–75mM-sucrose–0.1mM-EDTA medium before homogenization. A 10% (w/v) homogenate in the same medium was prepared and centrifuged at 900*g* for 10min. The mitochondrial fraction (including the 'fluffy layer') was obtained by centrifuging the 900*g* supernatant for 10min at 12000*g* and then washed twice by centrifugation at the same speed to remove the soluble proteins, especially catalase from ruptured peroxisomes.

Uricase activity (which served as an estimate of the peroxisome content) was measured by the spectrophotometric method of Schneider & Hogeboom (1952) with a small modification for turbid suspensions. The disappearance of uric acid was followed by a decrease in $E_{293}-E_{320}$ (0.5cm-light-path cuvette) in a Perkin–Elmer dual-wavelength model 365 spectrophotometer. Activity as measured in the mitochondrial fraction was 8–12nmol of uric acid oxidized/min per mg of protein under the conditions employed.

The catalase content was measured spectrophotometrically from $E_{405}-E_{450}$ or $E_{660}-E_{630}$ of both the hydrogen peroxide and ethyl hydrogen peroxide derivative and estimated to be 0.12–0.15nmol of haem/mg of protein. The steady-state concentrations of the catalase intermediate were measured in the mitochondrial fraction with the dual-wavelength spectrophotometric technique as afforded either by the Aminco instrument or by that designed and constructed in the Johnson Foundation. The rapid reactions of the intermediate with its substrate were measured by the regenerative flow method, which has been described elsewhere (Chance, DeVault, Legallais, Mela & Yonetani, 1967). In this case the centre volume of the instrument (15ml) was filled with the mitochondrial fraction of appropriate protein concentrations (see figure legends) and the substrate was delivered from the small side syringe (one-seventieth of the volume of the large syringe). In the cases where hydrogen peroxide generation was measured, oxygen was delivered to the anaerobic suspension from the side syringe as oxygen-saturated water. In the case where kinetics of formation of the ethyl hydrogen peroxide compound were measured, ethyl hydrogen peroxide was placed in the side syringe. The optical path of the regenerative flow apparatus is 0.6cm and the absorption calibrations that appear in the figures below are computed per centimetre. The time-scales are indicated on the abscissa of each of the figures and the top trace indicates the profile of flow-velocity changes, particularly the time at which the flow stopped. The apparatus has a mixing time of a few tenths of a millisecond, a time measured during the flow at the position at which observations are made in this case (1.2ml downstream from the mixing point) of 20ms and a stopping time of approx. 15ms as employed under these conditions.

Ethyl hydrogen peroxide used was obtained from Ferrosan (Malmo, Sweden) or synthesized according to

the Williams & Moscher (1954) procedures (courtesy of Dr G. R. Schonbaum and Dr K. Olafsson).

RESULTS

Choice of wavelengths for measurement of the catalase intermediate. A variety of wavelength pairs suitable for the dual-wavelength technique were tested for their response to the formation of the catalase intermediate. The total absorption changes induced by the addition of uric acid and 6mM-ethanol (Table 1, columns 1 and 2) are compared with those involving the cytochromes and occurring on the transition from State 1 to State 2 and from State 2 to State 5 (Chance & Williams, 1956). These states are defined in Table 2. It is apparent that the intermediate compound is easiest to measure in the Soret region ($E_{405}-E_{450}$ is equal to $45 cm^{-1} \cdot mM$; Chance, 1949*b*, 1952*b*) in spite of the appreciable cytochrome interference at this wavelength pair. The ratio of the catalase to the cytochrome signal is more favourable in the α-bands of the intermediate compound ($E_{660}-E_{630}$) which lies beyond the α-bands of the cytochromes. Appropriate comparisons of the signals at $E_{660}-E_{630}$ and at $E_{405}-E_{450}$ indicate the incremental extinction coefficient at the former wavelength pair to be $6.4 cm^{-1} \cdot mM^{-1}$.

General properties of the catalase intermediate in the mitochondrial fraction. Fig. 1 illustrates four examples of the kinetic and steady-state transitions of the catalase intermediate in the mitochondrial fraction. The preparation is initially present in State 1, which favours the endogenous production of hydrogen peroxide and the generation of the hydrogen donors. Both factors, hydrogen peroxide concentration and that of hydrogen donors, influence the steady state of the catalase intermediate. The four charts indicate the interplay of these factors with additional hydrogen peroxide produced by uric acid, additional hydrogen donor activity induced by ethanol addition and a supplemental substrate from an alkyl hydrogen peroxide. In Fig. 1(*a*) the addition of 30μM-uric acid causes an increase in $E_{660}-E_{630}$ for an interval of about 1 min during which the uric acid is oxidized. The additional hydrogen peroxide generation from the uric acid is observed as a cycle of formation of more of the intermediate for this interval. A second addition of uric acid gives a similar response.

The addition of 6mM-ethanol decreases (in 30s) the concentration of the intermediate compounds practically to zero, as indicated by the fact that the second addition of ethanol causes no further change. The half-maximal effect is usually obtained with 0.05–0.1mM-ethanol, and thus the 6mM ethanol used in the experiment causes a complete decomposition of the intermediate. Similar spectroscopic changes are obtained with methanol and formate, a much smaller effect is observed with propanol (one-tenth of that of ethanol) and no effect was induced by butanol, heptanol and hexanol at 1mM concentration.

The total excursion obtained under such conditions (2.6 scale divisions) can be considered a maximum value and to correspond to a saturation of catalase with hydrogen peroxide. Therefore the absorption change due to the formation of the intermediate from the endogenous hydrogen peroxide (in the absence of uric acid) and corresponding to 1.6 scale divisions indicates 61% of the saturation concentration (the intermediate concentration in the presence of uric acid is taken as 100%).

In Fig. 1(*b*) an initial addition of ethanol demonstrates the existence of the catalase intermediate in the mitochondrial mixture at substantial concentrations. At this point an additional formation of the intermediate can still be induced by the addition of uric acid, although the steady-state concentration reached is only 39% (1.0/2.6) and the

Table 1. *Comparison of the absorption changes due to the catalase intermediate and to the mitochondrial cytochromes*

The reaction mixture contained in 2.5ml: 0.225M-mannitol, 75mM-sucrose, 20mM-tris–HCl buffer, pH 7.4, 2mM-potassium phosphate, 1mM-$MgCl_2$ and 0.1mM-EDTA. The protein concentration used is given in the Table. The saturating amount of the catalase–H_2O_2 intermediate was determined by the additions of uric acid (40 or 20μM) and ethanol (6mM) as illustrated in Fig. 1(*b*). The absorption changes due to mitochondrial cytochromes were induced by the additions of pentachlorophenol (2μM) and succinate (4mM) in the presence of ethanol (12mM). The mitochondrial fraction used contained 0.13nmol of cytochrome $(a+a_3)$/mg of protein and a negligible amount of haemoglobin.

Wavelength (nm)	Concn. of protein (mg/ml)	Absorption change (ΔE)		
		Catalase	State 1 → 2	State 2 → 4
660 – 710	7.1	0.0016	0.00055	−0.0027
660 – 640	7.1	0.0021	0.0001	0.00015
660 – 630	7.1	0.0027	0.0001	0.0002
405 – 450	1.9	0.0051	0.0026	−0.0268

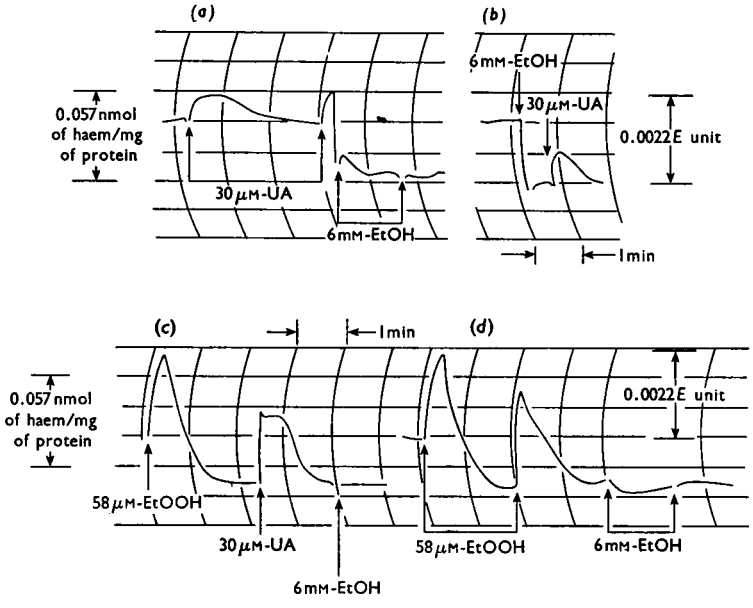

Fig. 1. Steady-state transitions of the catalase intermediate in the mitochondrial fraction. The reaction mixture in 2.5 ml contained: 0.225 M-mannitol, 75 mM-sucrose, 20 mM-tris–HCl buffer, pH 7.4, 2 mM-potassium phosphate, 1 mM-$MgCl_2$ and 0.1 mM-EDTA. Final concentrations of uric acid (UA), ethyl hydrogen peroxide (EtOOH) and ethanol (EtOH) were 30 μM, 58 μM and 6 mM respectively. An upward deflexion of the trace indicates an increase of $E_{660}-E_{630}$ (E units). The relationship between change in $E_{660}-E_{630}$ and concentration of catalase intermediate is indicated at the left-hand side of the figure.

cycle is shortened owing to the increased turnover of the catalase intermediate. Ethyl hydrogen peroxide gives a higher steady-state concentration of the catalase intermediate than does uric acid. As shown in Fig. 1(*c*), when ethyl hydrogen peroxide is exhausted the trace not only returns to its original value, but falls below it. A tentative explanation might be based on eqn. (2): the ethyl hydrogen peroxide intermediate is reduced by endogenously generated hydrogen peroxide and the base-line thus obtained is that of free catalase. This conclusion is further substantiated by the lack of the ethanol effect and the requirement for uric acid as the source of hydrogen peroxide to establish a significant concentration of the intermediate.

The exhaustion of endogenous hydrogen peroxide according to eqn. (2) results in the base-line shift after the first addition of ethyl hydrogen peroxide. Thus the absorption changes are due to the sum of both the ethyl hydrogen peroxide and the hydrogen peroxide compounds. The second addition of ethyl hydrogen peroxide gives absorption changes due to the alkyl hydrogen peroxide compound only.

When the saturation concentrations of the catalase intermediate were measured either by several additions of uric acid or ethyl hydrogen peroxide in experiments (not shown) essentially similar to those of Fig. 1(*c*) the ratio of maximum absorption changes for the first and second additions were respectively 0.43 and 0.37. Since the absorption changes induced by the first and the second additions of uric acid are identical (and equal to 0.4 with respect to ethyl hydrogen peroxide) hydrogen peroxide and not alkyl hydrogen peroxide is the substrate for the endogenous catalase intermediate.

Spectroscopic data. The difference spectrum obtained by the addition of ethanol to respiring mitochondrial preparation is shown in Fig. 2. As discussed above, ethanol stimulates decomposition of the catalase intermediate to free catalase, which exhibits a higher absorption in the Soret region. The difference spectrum of Fig. 2 is identical with that of the catalase compound obtained by the rapid-flow technique (Chance, 1949*a*; Chance & Herbert, 1950).

Fig. 2(*b*) presents difference spectra of the catalase intermediate recorded in the i.r. region under slightly different conditions from those in Fig. 2(*a*). The protein concentration is fivefold higher and the intermediate is generated either by the addition of uric acid to the hydrogen peroxide-depleted mitochondria or by the addition of ethyl hydrogen peroxide, The 64 μM-ethyl hydrogen peroxide used in this

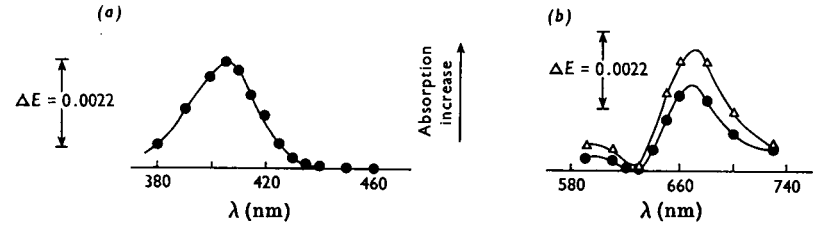

Fig. 2. Difference spectra of mitochondrial fraction with ethanol (*a*) and with uric acid and ethyl hydrogen peroxide (*b*). The reaction mixture is described in Fig. 1. In (*a*) freshly prepared mitochondrial fraction (1.5 mg of protein/ml) was used. In (*b*) mitochondrial fraction kept at 0°C for 2 days was employed at a protein concentration of 5.6 mg/ml. The difference spectra were obtained by plotting the absorption changes caused by the addition of (*a*) ethanol (6 mM) (●), (*b*) uric acid (40 μM) (●) and ethyl hydrogen peroxide (64 μM) (△) at each wavelength with reference to 450 nm (*a*) and 630 nm (*b*).

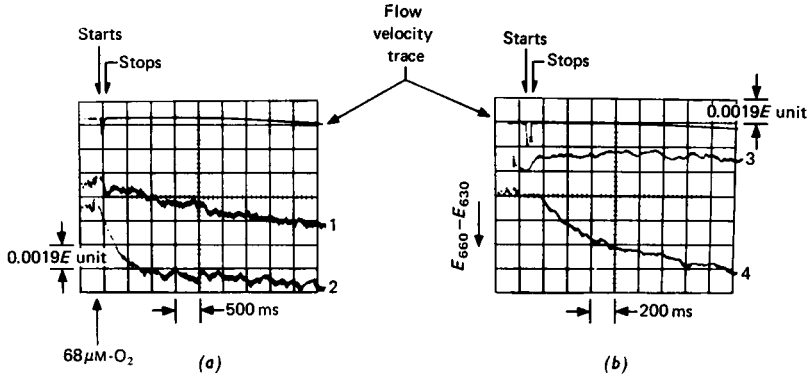

Fig. 3. (*a*) Kinetics of the catalase–H_2O_2 intermediate formation in the absence and in the presence of uric acid. Mitochondrial fraction was used at a protein concentration of 11 mg/ml. Other conditions are described in Fig. 1 and in the Materials and Methods section. The mitochondrial preparation showed 10.4 nmol/min per mg of protein of uric acid oxidase activity at 25°C in parallel experiments. Trace 1, uric acid absent; trace 2, 120 μM-uric acid. (*b*) Kinetics of formation of the catalase–ethyl hydrogen peroxide intermediate. The reaction conditions was as described under (*a*) above. Final concentration of ethyl hydrogen peroxide was 120 μM. Trace 3, ethyl hydrogen peroxide absent; trace 4, 120 μM-ethyl hydrogen peroxide. Formation of the intermediate was followed under anaerobic conditions.

experiment gives 60% saturation of the catalase intermediate, identifying the peroxisomal compound to be similar to that obtained with the purified enzyme.

Rate of hydrogen peroxide generation in the mitochondrial fraction. A direct method for determining the effective rate of hydrogen peroxide arrival at the peroxisomal site of catalase is to measure the initial rate of formation of the intermediate compound at $E_{660}-E_{630}$ in response to oxygen pulses (68 μM) delivered to the previously anaerobic mitochondrial fraction.

The mitochondrial fraction is studied under two conditions: (*a*) with endogenous substrate only; (*b*) with a supplement of 120 μM-uric acid. The downward deflexion of the two traces of Fig. 3(*a*) illustrates the rate of the catalase intermediate formation. The rate is three times as fast in the presence of uric acid and the steady-state concentration of the intermediate is doubled (not shown). The initial rates of the intermediate formation can be calculated from the slopes ($\epsilon_{660}-\epsilon_{630}$ = 6.4 $cm^{-1} \cdot mM^{-1}$, light-path 0.6 cm) and are 0.9 and 11.6 nmol of hydrogen peroxide/min per mg of protein for conditions (*a*) and (*b*) respectively. The increment of hydrogen peroxide generation caused by uric acid oxidation (10.7 nmol/min per mg of protein) agrees very closely with the independently measured rate (10.4 nmol/min per mg of protein). Thus most of the hydrogen peroxide generated from uric acid oxidation reacts with catalase to form the intermediate compound.

Effect of ethanol on the steady-state concentrations of the catalase intermediate. To compute the steady-state hydrogen peroxide concentration and the amount of hydrogen donor present in the mitochondrial fraction we have titrated the concentration of the catalase intermediate with concentrations of ethanol. Hydrogen peroxide generation is afforded in this case by endogenous substrate, and the initial concentration of the intermediate is approx. 70%. Additions of ethanol to the mitochondrial fraction are made either in State 1 or in State 4. Fig. 4 shows a progressive decrease of the amount of intermediate with increasing ethanol concentration that is different for States 1 and 4. Half-maximal effects are obtained with 50 μM-ethanol in State 1 and with 150 μM-ethanol in State 4. This suggests a less active hydrogen peroxide generation in State 1 than in State 4, the higher metabolic activity of the latter state enhancing hydrogen peroxide generation.

If one extrapolates these curves to $p_m/e = 0.4$ (the maximum value found with uric acid supplements) one obtains an estimate of the endogenous hydrogen donor concentration in terms of its ethanol equivalent. The values are 50 μM for State 1 and 120 μM for State 4. There may be a co-ordination of hydrogen peroxide and hydrogen donor generation in the mitochondrial fraction; State 4 favours larger quantities of both substrates for catalase, State 1 smaller quantities. The details of these curves are calculated in the Discussion section below.

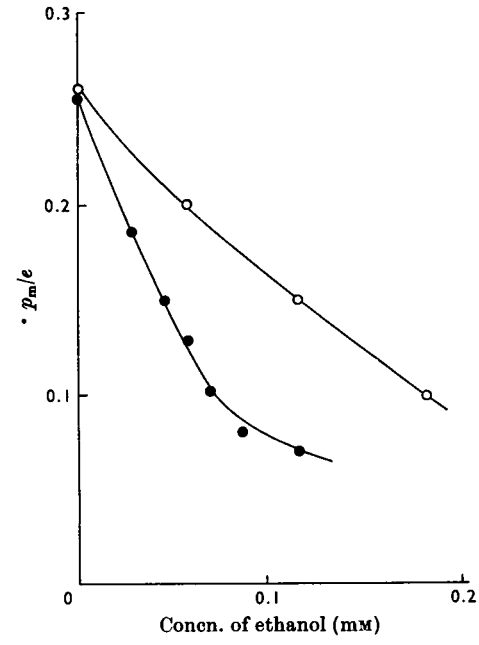

Fig. 4. Effect of the exogenous hydrogen donor (ethanol) on the steady state of the catalase intermediate at States 1 (●) and 4 (○). The reaction mixture described in Fig. 1 contained mitochondrial fraction at 4.3 mg of protein/ml. State 4 was obtained by the addition of succinate and glutamate (each 2 mM). The steady state was determined as illustrated in Fig. 1.

Effect of metabolic state on the concentration of catalase intermediate. In Table 2 the effect of metabolic state on the steady-state concentration of the catalase intermediate (p_m) is evaluated. The latter value is determined by uric acid and ethanol additions according to Fig. 1(*b*), and the value of k'_3 is computed from the second addition of 32 or 64 μM-ethyl hydrogen peroxide according to eqn. (4).

The resting States 1 and 4 (endogenous and added substrate) favour a high concentration of the intermediate compound, whereas States 2 and 3, obtained with ADP and phosphate in excess, a lower concentration of the intermediate. Uncoupling with pentachlorophenol results in an intermediate concentration of the catalase compound.

Rate of formation of the intermediate with ethyl hydrogen peroxide. Fig. 3(*b*) illustrates the formation of the intermediate on the addition of a pulse of 120 μM-ethyl hydrogen peroxide to the anaerobic mitochondrial suspension. The half-time of the reaction is approx. 330 ms and corresponds to the first-order constant of $2 s^{-1}$ and a second-order constant of $1.8 \times 10^4 M^{-1} \cdot s^{-1}$, the latter value being very close to that observed for free catalase ($2.0 \times 10^4 M^{-1} \cdot s^{-1}$) under similar conditions. It appears that there is no detectable effect of the peroxisomal diffusion barrier on the reaction of ethyl hydrogen peroxide with the catalase. However, diffusion effects are more pronounced at the higher reaction rates characteristic of the hydrogen peroxide reaction.

Turnover of the catalase intermediate with ethyl hydrogen peroxide as a substrate. The kinetics of catalase–ethyl hydrogen peroxide intermediate permit a calculation of the hydrogen donor activity from the equation (Chance, 1943):

$$k'_3 = k_4 a = \frac{x_0}{p_m t_{\frac{1}{2}}\text{off}} \qquad (4)$$

k_4 is defined in eqn. (3), x_0 is the initial peroxide concentration, a is the hydrogen donor concentration, p_m is the maximum concentration of the intermediate compound, and $t_{\frac{1}{2}}$off is the time between half-maximal values of p in the cycle of the intermediate (Chance, 1943).

Under aerobic conditions, in addition to the usual hydrogen donor pool in the mitochondrial fraction, hydrogen peroxide may act as a reductant for the catalase intermediate, according to eqn. (2). For this reason the initial experiments with the catalase

Table 2. *Effect of the mitochondrial metabolic states on the steady state of the catalase intermediate and the decomposition rate of the catalase–ethyl hydrogen peroxide intermediate (k_3')*

The reaction mixture as described in the legend of Fig. 1 contained 5.6 and 6.9 mg of protein/ml of mitochondrial fraction for preparations A and B respectively. Final concentrations of uric acid, ethanol and ethyl hydrogen peroxide were: 40 μM, 6 mM and 32 (for A) and 64 (for B) μM, respectively. Percentage saturation of the catalase intermediate with endogenous substrate was estimated as illustrated in Fig. 1(*b*). 100% means the saturation value of the intermediate formation by uric acid. The k_3' value was calculated from the second cycle of the response to ethyl hydrogen peroxide (see Fig. 1*d*) according to eqn. (4).

Preparation	State	Addition	Characteristic	% saturation of the intermediate	k_3' (s^{-1}) Aerobic	k_3' (s^{-1}) Anaerobic
A	1		O_2 present, endogenous substrate	59	3.9	
	2	ADP (0.3 mM)	O_2 present, substrate depleted	36	2.0	
	2	Pentachlorophenol (7 μM)		51	0.8	
	3	β-Hydroxybutyrate (4 mM) ADP (0.3 mM)	Substrate ADP + P_i present	37	5.4	
	3	β-Hydroxybutyrate (4 mM) Pentachlorophenol (7 μM)		50	1.1	
	4	β-Hydroxybutyrate (4 mM)	Substrate present ADP or P_i absent	63	5.0	
	4	Succinate (4 mM)		69	5.9	
B	1		As above	68	3.7	
				0		1.3
	4	Succinate (2 mM) + glutamate (2 mM)	As above	—	9.7	
				0		5.8

intermediate were made under anaerobic conditions to suppress the generation of endogenous hydrogen peroxide (see Table 2). The first addition of ethyl hydrogen peroxide gives a k_3' value of 5.2 s^{-1}; with further additions of ethyl hydrogen peroxide the endogenous substrate is exhausted and k_3' falls to 1.3 and 1.1 s^{-1}. The hydrogen donor pool is reactivated by the addition of succinate and glutamate to the anaerobic mitochondrial suspension and the k_3' rises to 5.2 and 5.8 s^{-1} respectively for the first and second additions of ethyl hydrogen peroxide.

Under aerobic conditions, the first addition of ethyl hydrogen peroxide gives values for k_3' of 10.7 s^{-1}, and for the second addition we have in State 1 3.7 s^{-1}, as indicated in Table 2, a considerably higher value than that for the anaerobic mitochondria. The value for State 4 after addition of succinate plus glutamate is higher in the aerobic state than in the anaerobic state (9.7 versus 5.8 s^{-1}). These State 1 and State 4 values obtained with ethyl hydrogen peroxide are considerably larger than those calculated from ethanol addition to the hydrogen peroxide intermediate, a topic that is discussed below.

Further, in Table 2 are values for k_3' obtained in State 2, either with ADP or pentachlorophenol. Both these treatments cause depletion of the endogenous substrate pool. The addition of an uncoupler to give State 3u (uncoupled) does not increase the endogenous hydrogen donor pool. Maximum values are obtained in State 3 or State 4.

DISCUSSION

The mitochondrial fraction of rat liver provides a unique opportunity to study the action of catalase in coupled oxidations and to shed light on many aspects of catalase action in the intact or perfused liver (de Duve & Baudhuin, 1966; Sies & Chance, 1970; Thurman & Chance, 1969; Sies, Brauser & Bucher, 1969). One of the remarkable results of this investigation is the possibility that there is a co-ordinated production of the oxidizing and reducing substrates necessary for catalase function in its peroxidatic mode. It is further of note that the state favouring the high production of both substrates is near State 4, the state that characterizes the perfused liver (Scholz, Thurman, Williamson, Chance & Bucher, 1969).

Simple physical argument identifies the role of catalase in the mitochondrial fraction to be significantly peroxidatic, as evidenced by the fact that the saturation of the intermediate compound with hydrogen peroxide is in the range 60–70%. The

following mathematical formulation verifies and extends this reasoning.

Utilization of hydrogen peroxide. The equations for identifying the peroxidatic and catalatic contribution of catalase activity in the steady state were discussed by Chance (1969).

$$+\frac{dx_n}{dt} = -\frac{dx}{dt} - \frac{da}{dt}\Big]_{\frac{dp}{dt}=0} = +2p_m(k'_4 x_m + k_4 a) \quad (5)$$

The total utilization, dx_n/dt, consists of two parts, the catalatic reaction, dx/dt, and the peroxidatic reaction, da/dt. Their respective contributions in terms of kinetic constants and concentrations is afforded by the two terms on the right-hand side of the equation. The rate of hydrogen peroxide production, $+dx_n/dt$, is calculated from the initial rate of formation of the intermediate compound from endogenous substrate to be 0.9nmol/min per mg of protein, assuming that $dx_n/dt = dp/dt$ near $t = 0$ and $p = 0$ (Chance *et al.* 1952). The catalase content is 0.15nmol/mg of protein, and when the steady state is reached approx. 25% is converted into the intermediate compound. We may then substitute these particular values in eqn. (5) at 1mg of protein/ml. In this way the two right-hand terms of the equation are evaluated:

$$\frac{1}{2p_m} \cdot \frac{dx_n}{dt} = k'_4 x_m + k_4 a = 0.20\,s^{-1} \quad (6)$$

Thus the total of both peroxidatic and catalatic reactions causes a turnover of catalase of $0.20\,s^{-1}$.

If the hydrogen donor concentrations are equal to zero then the catalatic reaction, $k'_4 x$, has this value and x is equal to 8nM for $k'_4 = 2.6\times10^7\,M^{-1}\cdot s^{-1}$. This result can also be obtained from eqn. (16) of Chance & Higgins (1952).

The hydrogen peroxide concentration estimated above is a maximum value and will be correspondingly less whenever there is a peroxidatic reaction. For example, a value of 1nM was calculated for bacterial catalase previously (Chance, 1950).

Nature of peroxisomal catalase. The steady-state concentration of the catalase intermediate in mitochondrial fraction ($p_m/e \leqq 0.4$) permits a calculation of the k'_4/k_1 value:

$$\frac{p_m}{e} = \frac{1}{1 + \frac{k'_4}{k_1} + \frac{k_2 + k_4 a}{k_1 x_m}} \quad (7)$$

If the rate of hydrogen peroxide generation is increased as the result of a uric acid supplement $k_1 x_m$ becomes large compared with $k_4 a$, and p_m/e approaches 0.4. Thus:

$$p_m/e = 0.4 = \frac{1}{1 + k'_4/k_1} \quad (8)$$

$$k'_4/k_1 = 1.5 \quad (9)$$

The value of k'_4/k_1 of 1.5 is at the low end of the values observed for liver catalases (Table IV in Chance *et al.* 1952). It is unlikely that hydrogen peroxide diffusion would selectively alter the k'_4/k_1 ratio, since the same hydrogen peroxide concentration is available for these two reactions. In addition, hydrogen peroxide is generated within the peroxisome by urate oxidase. The k'_4/k_1 ratio is, however, sufficiently similar to that of liver catalase B of Table IV in Chance *et al.* (1952) ($k'_4/k_1 = 1.7$) that we shall use for k_1 the value $1.7\times10^7\,M^{-1}\cdot s^{-1}$ and 1.5 times this value = $2.6\times10^7\,M^{-1}\cdot s^{-1}$ for k'_4 in the calculations below.

Hydrogen peroxide and hydrogen donor concentrations and the peroxidatic pathway. The peroxidatic mode of catalase action is readily detected by the p_m/e value, which reaches 0.4 if k_2 and a of eqn. (7) are both negligible. We have found $k_2 = 10^{-3}\,s^{-1}$ (Chance & Schonbaum, 1962), and Schonbaum (1970) has proposed k_2 to be zero. Thus p_m/e values less than 0.4 (the 100% is defined above) identify a significant peroxidatic mode of catalase activity.

Eqn. (6) affords an estimate of the relative amounts of peroxidatic and catalatic activity and the concentrations of hydrogen donor. As shown in Fig. 4, the addition of an exogenous hydrogen donor (ethanol) causes a decrease of p_m/e. Thus we can calculate x_m by substituting the following values into eqn. (7): $k'_4/k_1 = 1.5$, $k_2 = 10^{-3}\,M^{-1}\cdot s^{-1}$, $k_1 = 1.7\times10^7\,M^{-1}\cdot s^{-1}$, $k_4 = 10^3\,M^{-1}\cdot s^{-1}$, $k'_4 = 2.6\times10^7\,M^{-1}\cdot s^{-1}$.

The hydrogen peroxide concentration, x_m, can be calculated for the values of ethanol in the range 20–80μM and is 0.6 and 2.0nM in States 1 and 4 respectively.

By extrapolation, the hydrogen donor equivalent to ethanol is 50 and 120μM for States 1 and 4 respectively. Substituting these x_m and a values in eqn. 6 we find 0.07 and $0.17\,s^{-1}$ for both States 1 and 4 as compared with $0.25\,s^{-1}$ from kinetic measurements. Further, the reaction is 76% and 71% peroxidatic in States 1 and 4 respectively. It is probably more than mere coincidence that the catalase system is poised at least in the mitochondrial fraction to be mainly peroxidatic yet partly catalatic, suggesting some co-ordination of hydrogen peroxide and hydrogen donor production.

Values of k'_3 with ethyl hydrogen peroxide as substrate. The value of k'_3 measured with ethyl hydrogen peroxide in the presence of uncoupler is also $0.7\,s^{-1}$, but in other metabolic states k'_3 rises to $5.4\,s^{-1}$.

The high k_5 values can be explained if an extraordinarily high hydrogen peroxide production rate is caused by the presence of ethyl hydrogen peroxide. For example, when the k'_3 value for ethyl hydrogen

peroxide was measured in the presence of uric acid extremely high values were obtained ($k_3 = >25 s^{-1}$); thus we rely on the values obtained with endogenous hydrogen peroxide generation for a calculation of the k'_3 values and the percentage of peroxidatic activity generated.

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