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The Characteristics of the 'Peroxidatic' Reaction of Catalase in Ethanol Oxidation

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Ethanol oxidation by rat liver catalase (the 'peroxidatic' reaction) was studied quantitatively with respect to the rate of H_2O_2 generation, catalase haem concentration, ethanol concentration and the steady-state concentration of the catalase–H_2O_2 intermediate (Compound I). At a low ratio of H_2O_2-generation rate to catalase haem concentration, the rate of ethanol oxidation was independent of the catalase haem concentration. The magnitude of the inhibition of ethanol oxidation by cyanide was not paralleled by the formation of the catalase–cyanide complex and was altered greatly by varying either the ethanol concentration or the ratio of the rate of H_2O_2 generation to catalase haem concentration. The ethanol concentration producing a half-maximal activity was also dependent on the ratio of the H_2O_2-generation rate to catalase haem concentration. These phenomena are explained by changes in the proportion of the 'catalatic' and 'peroxidatic' reactions in the overall H_2O_2-decomposition reaction. There was a correlation between the proportion of the 'peroxidatic' reaction in the overall catalase reaction and the steady-state concentration of the catalase–H_2O_2 intermediate. Regardless of the concentration of ethanol and the rate of H_2O_2 generation, a half-saturation of the steady state of the catalase–H_2O_2 intermediate indicated that about 45% of the H_2O_2 was being utilized by the ethanol-oxidation reaction. The results reported show that the experimental results in the study on the 'microsomal ethanol-oxidation system' may be reinterpreted and the catalase 'peroxidatic' reaction provides a quantitative explanation for the activity hitherto attributed to the 'microsomal ethanol-oxidation system'.

Alcohol dehydrogenase (EC 1.1.1.1) present in the liver is responsible for a major part of the ethanol metabolism in man and animals (Jacobsen, 1952; Lundquist *et al.*, 1963; Tephly *et al.*, 1964; Blomstrand & Theorell, 1970; Krebs & Perkins, 1970). Some part of the metabolism is, however, not sensitive to the inhibitors of alcohol dehydrogenase and can be accounted for by a contribution from liver catalase (EC 1.11.1.6) (Jacobsen, 1952; Van Harken *et al.*, 1965). Recently another new enzyme system, the 'microsomal ethanol-oxidation system', was reported (Orme-Johnson & Ziegler, 1965; Lieber & DeCarli, 1968; Rubin *et al.*, 1968; Lieber & DeCarli, 1970; Lieber *et al.*, 1970; Rubin *et al.*, 1971) and arguments have been presented that 'microsomal ethanol-oxidation activity' is mainly due to the 'peroxidatic' reaction of catalase which contaminates the microsomal fraction (Roach *et al.*, 1969; Tephly *et al.*, 1969; Isselbacher & Caster, 1970; Thurman *et al.*, 1972), but the interpretation of some experimental results is still controversial. From an enzymological point of view, this conflict seems to be due mainly to a lack of basic information about the properties of the 'peroxidatic' reaction of catalase, especially under the conditions of slow H_2O_2 generation.

Catalase catalyses the decomposition reaction of H_2O_2 in its 'catalatic' mode as shown in the following equations with the original notation:

$$\text{Cat} + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cat–}H_2O_2 \text{ (Compound I)} \quad (1)$$

$$\text{Cat–}H_2O_2 + H_2O_2 \xrightarrow{k_4'} \text{Cat} + 2H_2O + O_2 \quad (2)$$

k_1, k_2 and k_4' represent rate constants of the indicated reaction (Chance *et al.*, 1952). The second molecule of H_2O_2 functions as a hydrogen donor in the 'catalatic' reaction. Since the rate constant, k_1, of the formation of Compound I is only slightly smaller than that of the decomposition reaction, k_4' ($k_4'/k_1 = 1.5$), formation of the catalase–H_2O_2 intermediate approaches a certain maximal value [40% of the haem binds with H_2O_2 in the case of rat liver catalase (Chance & Oshino, 1971)] at a particular rate of H_2O_2 generation, while further increase in H_2O_2 generation causes only an increase in the turnover number of the overall reaction (Chance, 1950; Chance *et al.*, 1952). When another type of hydrogen

donor, e.g. ethanol, is present, the catalase–H_2O_2 intermediate (Compound I) is also decomposed to free catalase and H_2O by coupling with the oxidation of ethanol to acetaldehyde (Keilin & Hartree, 1945; Chance, 1949*a*).

$$\text{Cat–}H_2O_2 + CH_3CH_2OH \xrightarrow{k_4} \text{Cat} + 2H_2O + CH_3CHO \quad (3)$$

The reactions (1) and (3) are called the 'peroxidatic' reaction (Keilin & Hartree, 1945). Although the 'peroxidatic' reaction of catalase was studied kinetically by Chance (1950) and Kremer (1970), direct measurement of the ethanol oxidation by catalase has not been performed in detail. The reaction sequence mentioned above introduces considerable complexity into the assay of 'peroxidatic' activity of catalase, and measurement of only the rate of ethanol oxidation, without regard to the proportion of 'peroxidatic' and 'catalatic' reactions in the overall reaction, may lead to a misinterpretation of the experimental results.

The present paper describes the special properties of the ethanol-oxidation reaction of catalase in a phenomenological way, which should be useful in considering contributions of catalase to ethanol and methanol metabolism in the liver.

Experimental

Methods

Rat liver catalase. Rat liver catalase was purified by the method of Price *et al.* (1962). The E_{407}/E_{276} ratio of the preparation was 1.067, and its purity was about 99%, which was measured by the method of Price *et al.* (1962). The haem content of the catalase, which was determined by using $\epsilon_{557} = 34.4\ cm^{-1} \cdot mM^{-1}$ for reduced protohaem pyridine haemochromogen (Paul *et al.*, 1963) and $\epsilon_{407} = 430 cm^{-1} \cdot mM^{-1}$ for rat liver catalase (Price *et al.*, 1962), was 3.76/molecule. The rate constant of the overall reaction, which was measured by following decomposition of 6mM-H_2O_2 at 230nm in 50mM-potassium phosphate buffer, pH7.4 (Beers & Sizer, 1952; Chance & Maehly, 1955), was $1.16 \times 10^7 M^{-1} \cdot s^{-1}$ in terms of haem concentration at 25°C.

Assay of ethanol-oxidation rate. The rate of ethanol oxidation was measured as the rate of NAD^+ reduction by coupling the reaction with that of yeast aldehyde dehydrogenase (EC 1.2.1.3). The reaction mixture contained, in a final volume of 3.0ml, 50mM-potassium phosphate buffer, pH7.4, 10mM-glucose, 1–1.5 i.u. of yeast aldehyde dehydrogenase, 0.1mM-NAD^+ and various concentrations of ethanol and rat liver catalase. Yeast aldehyde dehydrogenase was dissolved in 0.1M-potassium phosphate buffer–1mM-cysteine, pH7.4, before use (Black, 1955). After a very slow reduction of NAD^+, owing to a contaminating alcohol dehydrogenase activity (below 0.1μmol/l per min under the conditions used) was recorded as a blank for 1min, the reaction was started by adding various amounts of glucose oxidase (EC 1.1.3.4) and the rate of NAD^+ reduction was measured at 340nm in a Coleman–Hitachi double-beam spectrophotometer model 124 or at 340 minus 360nm in an Aminco–Chance dual-wavelength spectrophotometer at 25°C.

Measurement of H_2O_2-generation rate. The rate of H_2O_2 generation from the glucose plus glucose oxidase system was measured at 550nm as the rate of ferrocytochrome *c* oxidation by coupling the reaction with that of yeast cytochrome *c* peroxidase (EC 1.11.1.5). The reaction conditions were the same as described for the assay of ethanol oxidation, except that catalase was omitted and 0.1μM yeast cytochrome *c* peroxidase and 25μM-ferrocytochrome *c* (horse heart) were added. $\Delta E_{550} = 19.6 cm^{-1} \cdot mM^{-1}$ (Yonetani, 1965), which was determined by using the cytochrome *c* peroxidase reaction, was used for calculation of the rate of cytochrome *c* oxidation. Then 2mol of cytochrome *c* is oxidized by decomposition of 1mol of H_2O_2 in this reaction (Yonetani, 1965).

Measurement of the steady-state concentration of the catalase Compound I. The concentration of the catalase Compound I at a given steady state was measured either at 660 minus 630nm or at 407 minus 435nm by using an Aminco–Chance dual-wavelength spectrophotometer. Total catalase haem concentration was measured at 660 minus 630nm as the catalase–cyanide complex by adding 0.3mM-KCN. Values of $\Delta E_{660-630} = 3.9$ and $5.4 cm^{-1} \cdot mM^{-1}$ were used for the catalase–H_2O_2 Compound I and catalase–cyanide complex respectively. The former value was obtained by using ΔE_{407} of free catalase minus Compound I ($49 cm^{-1} \cdot mM^{-1}$; Brill & Williams, 1961) and the latter was based on the protohaem content of rat liver catalase. A steady state of Compound I was expressed by a ratio of the catalase haem binding with H_2O_2 at a steady state to the total haem. The saturation value of the Compound I formation was 0.4 in the case of rat liver catalase (Chance & Oshino, 1971). The formation of Compound II also produces absorbance changes both at 660 minus 630nm and 407 minus 535nm; however, its contribution is quite small because of slow conversion of Compound I into II at neutral pH (Chance, 1950; Nicholls, 1961). In our measurements, the amount of Compound II formed was always checked by adding excess amounts of ethanol: a steady state of Compound I was immediately decreased nearly to the original concentration, whereas decomposition of Compound II took quite a long time (Chance, 1950).

Materials

Purified preparations of yeast cytochrome *c* peroxidase were kindly supplied by Professor T.

Yonetani and Mr. K. Takio, Johnson Research Foundation, University of Pennsylvania. Horse heart cytochrome *c*, potassium-activated yeast aldehyde dehydrogenase and glucose oxidase from *Aspergillus niger* were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and used without further purification. Other reagents were analytical grade.

Results

Ethanol oxidation by catalase

The results of a typical experiment for the measurement of ethanol oxidation are shown in Fig. 1. On addition of glucose oxidase, an H_2O_2-generating system, to the reaction mixture containing ethanol, catalase, yeast aldehyde dehydrogenase, NAD^+ and glucose, there is an NAD^+ reduction as indicated by an increase of E_{340}. The linearity of NAD^+ reduction is established within 20s after the addition of glucose oxidase, and the slope of the linear portion of the trace corresponds to the rate of acetaldehyde formation from ethanol in the steady state. At a high concentration of ethanol and a slow rate of H_2O_2 generation, the NAD^+-reduction rate closely follows the rate of H_2O_2 generation, indicating the validity of the assay method (see Table 1). The time-course of NAD^+ reduction was linear until either O_2 concentration in the mixture decreased to a range where glucose oxidase activity was affected or about 60% of NAD^+ was reduced. Alcohol dehydrogenase activity contaminating the enzyme preparation was below 0.1 μmol of acetaldehyde formed/l per min under the assay conditions and it was possible to measure the ethanol-oxidation rate of 0.2 μmol/l per min in the catalase 'peroxidatic' reaction.

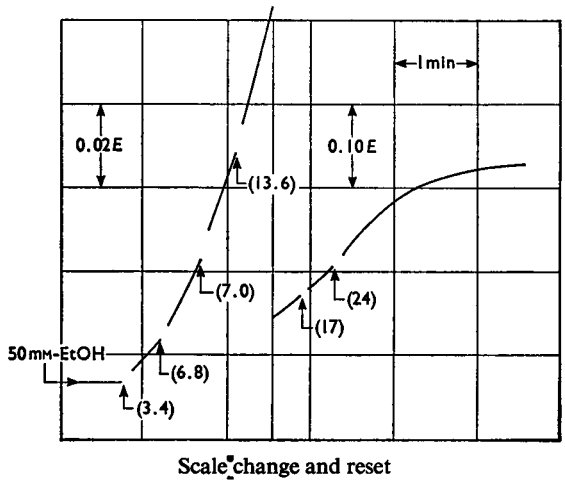

Fig. 1. *Measurement of ethanol-oxidation rate by 'peroxidatic' reaction of liver catalase*

The conversion rate of ethanol into acetaldehyde was measured as an NAD^+ reduction by coupling the reaction with the yeast aldehyde dehydrogenase reaction. The reaction mixture contained, in a final volume of 3.0ml, 50mM-potassium phosphate buffer, pH7.4, 10mM-glucose, 1.5i.u. of yeast aldehyde dehydrogenase, 0.086 μmol of catalase haem/l and 50mM-ethanol. NAD^+ reduction caused by addition of glucose oxidase was followed at 340nm. The numbers in parentheses represent the rates of H_2O_2 generation (μmol of H_2O_2/l per min) from the glucose oxidase system at each steady state, which was determined under similar conditions as described in the Experimental section.

Effect of the catalase concentration on the rate of ethanol oxidation

It was believed, *a priori*, that, at a low concentration of H_2O_2, the 'peroxidatic' reaction of catalase is predominant whereas the 'catalatic' reaction is predominant at high concentration of H_2O_2 (de Duve & Baudhuin, 1966; Deisseroth & Dounce, 1970). To determine the quantitative relationship between the factors governing the 'catalatic' and 'peroxidatic' (ethanol-oxidation) reactions, the rates of ethanol oxidation are, at first, compared by changing both catalase haem concentration from 0.17 to 1.7 μmol/l and H_2O_2-generation rate from 6.9 to 34.3 μmol/l per min in the presence of 50mM-ethanol (Table 1). As long as the ratio of H_2O_2-generation rate to catalase haem concentration is maintained below about 60/min, a change in the catalase haem concentration does not cause any change in the rate of ethanol oxidation. Under such conditions, the H_2O_2 decomposition by catalase is almost 100% 'peroxidatic' and, thus, the rate of ethanol oxidation depends only on the rate of H_2O_2 generation but not on the catalase concentration. When the ratio of the H_2O_2-generation rate to catalase haem concentration exceeds 200/min, a significant decrease in the rate of ethanol oxidation as compared with the total H_2O_2-generation rate is observable and this is due to an increase in the proportion of 'catalatic' decomposition of H_2O_2 described in a following section.

Effect of ethanol concentration on the rate of ethanol oxidation

In the experiment shown in Fig. 2, the rates of ethanol oxidation in the presence of 0.17 μmol of catalase haem/l are compared by varying the rates of H_2O_2 generation from 1.3 to 200 μmol/l per min in various ethanol concentrations. Theoretical curves of 100% 'peroxidatic' and 100% 'catalatic' reactions are

Table 1. *Effect of catalase concentration on ethanol oxidation*

The reaction mixture, in a final volume of 3.0ml, contained 50mM-ethanol, 50mM-potassium phosphate buffer, pH7.4, 10mM-glucose, 1.5i.u. of yeast aldehyde dehydrogenase, 0.1mM-NAD^+ and different concentrations of rat liver catalase. The reaction was started by addition of glucose oxidase. The rates of H_2O_2 generation were varied by changing final concentration of glucose oxidase. $\frac{H_2O_2}{[haem]}$ represents the H_2O_2-generation rate/catalase haem concentration ratio.

H_2O_2-generation rate (μmol/l per min) ...	6.9		10.3		34.3	
Catalase haem (μmol/l)	EtOH oxidation (μmol/l per min)	$\frac{H_2O_2}{[haem]}$ (min^{-1})	EtOH oxidation (μmol/l per min)	$\frac{H_2O_2}{[haem]}$ (min^{-1})	EtOH oxidation (μmol/l per min)	$\frac{H_2O_2}{[haem]}$ (min^{-1})
0.17	7.2	41	12.0	60	25.2	200
0.34	6.9	20	10.0	30	27.0	100
0.51	7.1	13.5	10.4	20	26.0	70
0.89	7.1	8	11.3	12	28.8	38
1.70	7.2	4	11.0	6	30.7	20

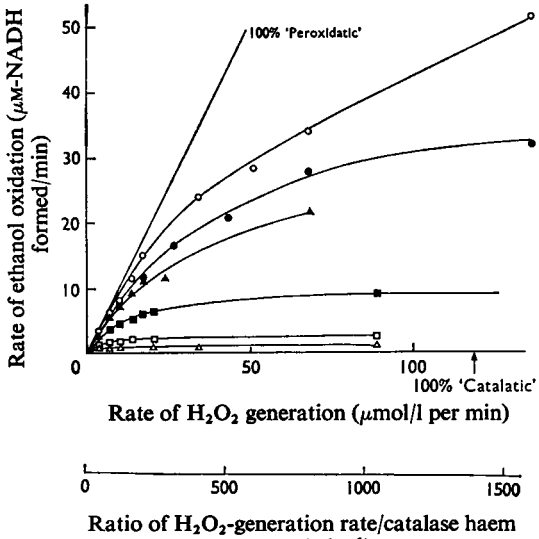

Fig. 2. *Proportion of the 'catalatic' and 'peroxidatic' activities in the overall H_2O_2 decomposition by catalase*

The reaction conditions were as described for Fig. 1, except that ethanol concentration and H_2O_2-generation rate were altered as represented in the figure Ethanol concentrations: △, 0.33mM; □, 1mM; ■, 5mM; ▲, 25mM; ●, 50mM; ○, 100mM. The ordinate is expressed in terms of the rate of ethanol oxidation measured as the rate of appearance of NADH (μM/min). The abscissa is expressed in terms of both the rate of H_2O_2 generation and the ratio of the H_2O_2-generation rate to catalase haem concentration. Theoretical curves for 100% 'peroxidatic' and 100% 'catalatic' reactions are given (see the text). given in Fig. 2. The result indicates that the proportion of the 'peroxidatic' and 'catalatic' reactions in the overall H_2O_2 decomposition is a function of both the ethanol concentration and the ratio of H_2O_2-generation rate to catalase haem concentration. In general, the higher the ethanol concentration, the greater the proportion of the 'peroxidatic' reaction to the overall decomposition of H_2O_2. However, even in the presence of 100mM-ethanol, an increase in the ratio of the H_2O_2-generation rate to catalase haem concentration enhances the 'catalatic' reaction so effectively that H_2O_2 is predominantly decomposed in the 'catalatic' reaction. For example, an increase in the rate of H_2O_2 generation from 10 to 100μmol/l per min (the ratio from 59 to 590/min) alters the ethanol-oxidation rate from 10 to only 44μmol/l per min in the presence of 100mM-ethanol. Therefore a knowledge of the ratio of the H_2O_2-generation rate to catalase haem concentration is necessary for a measurement of the ethanol-oxidation reaction involving catalase.

The ethanol concentration that produces a half-maximal 'peroxidatic' activity at a given rate of H_2O_2 generation can be measured from the results in Fig. 2. The estimated values, which are obtained by using two different concentrations of catalase, are replotted as a function of the ratio of the H_2O_2-generation rate to catalase haem concentration in Fig. 3. The plot is almost linear, although it bends slightly at high values of the ratio of H_2O_2-generation rate to catalase haem concentration. From these results, it is clear that a K_m value for ethanol in the 'peroxidatic' reaction of catalase cannot be measured. An apparent ethanol concentration producing a half-maximal activity can be measured, however; it is dependent on the ratio of H_2O_2-generation rate to catalase haem concentration.

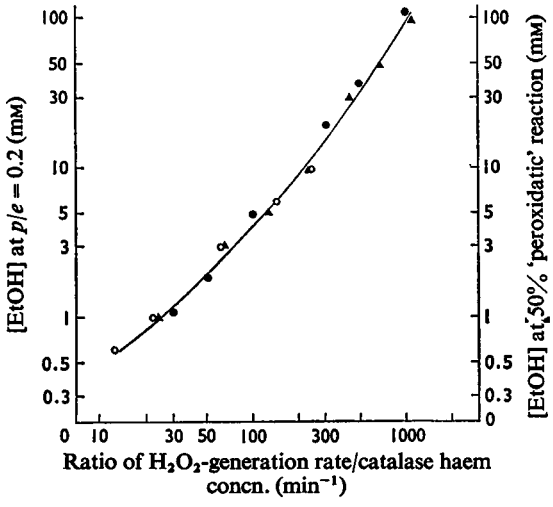

Fig. 3. *Relationship between ethanol concentration and the ratio of the H_2O_2-generation rate to catalase haem concentration in the catalase reaction*

The reaction conditions were as described for Fig. 2 and Fig. 7 except that catalase haem concentrations were: ▲, 0.06; ●, 0.175; o, 0.725 μmol/l. o, Ethanol concentration producing a half-maximal saturation of the catalase-H_2O_2 intermediate ($p/e = 0.2$; where p is the concentration of the catalase-H_2O_2 intermediate and e is catalase haem concentration) at the respective ratios of the H_2O_2-generation rate to catalase haem concentration. ●, ▲, Ethanol concentration producing decomposition of 50% H_2O_2 in the ethanol oxidation.

Cyanide-inhibition of ethanol oxidation

Cyanide, as well as azide, is a strong inhibitor of the 'catalatic' activity (Chance, 1949*a*,*b*). The formation of the catalase–cyanide complex, which has an absorbance maximum at 427nm, is observable at very low concentrations of cyanide even under slow H_2O_2-generation rates (Fig. 4). The results are replotted in Fig. 5, where the ordinate is expressed by percentage of free haem at a given cyanide concentration. The dissociation constant of cyanide is about 8 μmol/l. This value is slightly higher than that reported for horse liver catalase (5 μmol/l) (Chance, 1949*b*). It has been shown that binding of cyanide to catalase haem is accompanied by a parallel inhibition of catalase activity, which is usually measured in the presence of excess of H_2O_2 (Chance & Maehly, 1955; Chance, 1949*b*).

The effect of cyanide concentration on the ethanol-oxidation rate is shown in Fig. 6. In spite of such a strong affinity for the catalase haem, cyanide does not inhibit the ethanol oxidation effectively in the

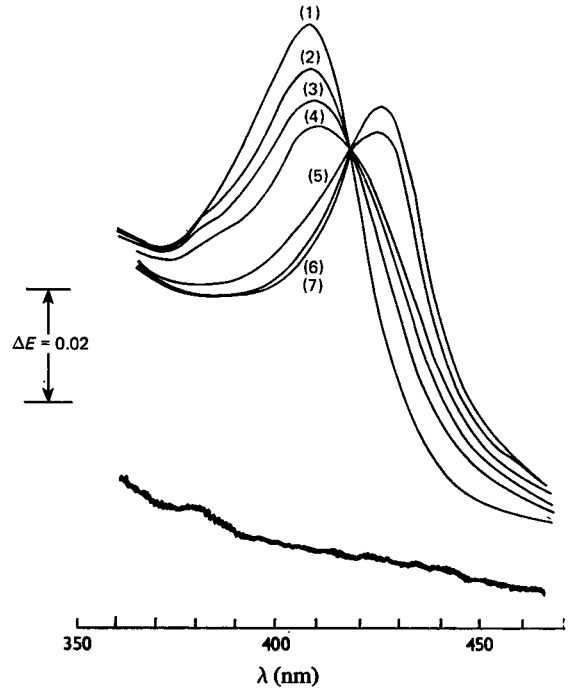

Fig. 4. *Absorption spectrum of catalase–cyanide complex*

The reaction mixture was the same as described for Fig. 2, except that the H_2O_2-generation rate/catalase haem concentration ratio was 0.34 μM-H_2O_2/0.86 μM-catalase haem per min and cyanide concentrations were altered from 0 to 500 μM as follows: (1), 0; (2), 3 μM; (3), 7 μM; (4), 12 μM; (5), 45 μM; (6), 200 μM; (7), 500 μM. The bottom trace represents a base-line in measurement.

presence of 1mM-ethanol. In addition, the effective concentration range of cyanide inhibition is dependent on the ratio of the H_2O_2-generation rate to haem concentration. Fig. 6 includes two different experimental results, one of which is obtained in 0.43 μmol of catalase haem/l, whereas the other is in 0.86 μmol of catalase haem/l. The results indicate that, whatever the catalase concentration, the magnitude of cyanide inhibition and the range of its effective concentration in the ethanol-oxidation reaction are dependent on the ratio of the H_2O_2-generation rate to haem concentration. For example, when this ratio is 0.4/min, 0.35mM-cyanide causes only 50% inhibition of the ethanol oxidation whereas the same concentration of cyanide inhibits the ethanol-oxidation reaction by 95% under the condition where the ratio of H_2O_2-generation rate to catalase haem concentration is 20/min. This phenomenon can be

explained by the changes in the proportion of the 'catalatic' and 'peroxidatic' reactions in the overall H_2O_2 decomposition. Cyanide decreases the effective haem concentration and hence increases the ratio of the H_2O_2-generation rate to catalase haem concentration. As shown in Fig. 2, an increase in this ratio results in a decrease of the contribution of the 'peroxidatic' reaction in the overall H_2O_2 decomposition. For example, in Fig. 2, the ratio of the H_2O_2-generation rate to catalase haem concentration producing 50% 'peroxidatic' decomposition of H_2O_2 in 1 mM-ethanol is about 30/min. When the H_2O_2-generation rate/haem concentration ratio is 0.4/min, 1 mM-ethanol produces 100% 'peroxidatic' reaction. Therefore, to get a 50% inhibition of the ethanol-oxidation rate, the ratio of the H_2O_2-generation rate to haem concentration must be increased from 0.4 to 30/min. Without changing the H_2O_2-generation rate, this can be achieved by 98.7% inactivation of catalase haem by cyanide, which effectively decreases the available haem concentration. When the ratio of the H_2O_2-generation rate to haem concentration is 27/min in Fig. 6, the proportion of the H_2O_2 utilized by the ethanol-oxidation reaction corresponds to 50% of the total H_2O_2 (Fig. 2). To change this proportion from 50 to 25% corresponding to a 50% inhibition of the ethanol-oxidation rate, the ratio must be increased from 27 to 74/min. This can be achieved by only 63% inactivation of catalase haem by cyanide. Therefore, the greater the ratio of the H_2O_2-generation rate to haem concentration, the lower the cyanide concentration necessary to produce a 50% inhibition of the ethanol-oxidation rate. Clearly the discrepancy between the extent of cyanide binding to haem and of cyanide inhibition of ethanol-oxidation rate is greater as the ratio of the H_2O_2-generation rate to catalase haem becomes smaller.

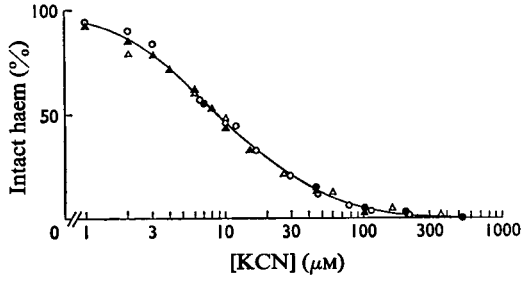

Fig. 5. *Formation of catalase–cyanide complex*

The catalase was titrated with cyanide either at 660 minus 630nm or at 427nm. The catalase haem concentrations used were: ●, 0.86; △, 1.72; ▲, 2.06; ○, 4.8 μmol/l. For ●, the glucose plus glucose oxidase system was added to generate H_2O_2 at the rate of 0.34 μmol/l per min. This condition corresponds to a ratio of the H_2O_2-generation rate to catalase concentration of 0.4 in Fig. 6.

Similarly, therefore, when the ethanol concentration is increased, cyanide becomes less effective in inhibiting ethanol oxidation of catalase. The mechanism of cyanide inhibition of ethanol oxidation is not directly due to the formation of the catalase–cyanide complex, but is due to the change in the proportion of the decomposition of catalase Compound I by the 'catalatic' and 'peroxidatic' reactions of catalase.

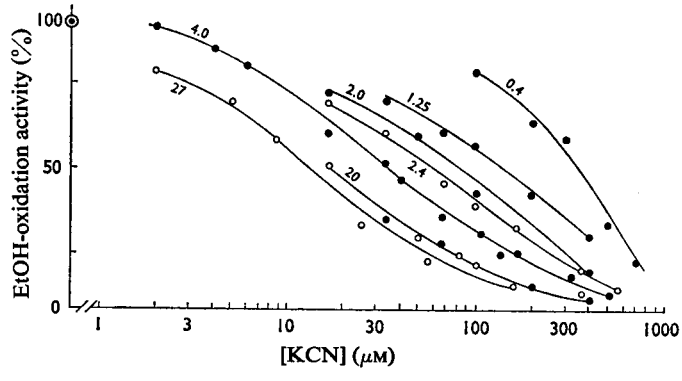

Fig. 6. *Effect of cyanide concentrations on the ethanol oxidation by catalase*

The assay conditions were the same as described for Fig. 1. The catalase haem concentrations used were: ●, 0.86; ○, 0.43 μmol/l; the ethanol concentration was 1.0 mM. Amounts of glucose oxidase were altered to vary the ratio of the H_2O_2-generation rate to catalase haem concentration from 0.4 to 27/min. These rates are represented by numbers in the figure.

Relationship between the 'peroxidatic' activity and the steady state of catalase–H_2O_2 intermediates

As described above, the 'peroxidatic' reaction of catalase with ethanol is restricted by two factors: the ethanol concentration and the ratio of the H_2O_2-generation rate to haem concentration. It is also known that a steady state of the catalase–H_2O_2 intermediate (Compound I) can be changed by varying either ethanol concentration or the ratio of the H_2O_2-generation rate to haem concentration (Chance & Oshino, 1971). A correlation of the 'peroxidatic' activity of catalase with the steady-state concentration of Compound I has been made. The steady-state concentration of Compound I can be expressed by the proportion of the haem bound with H_2O_2 to the total haem. The maximum concentration ratio of Compound I to total haem for rat liver catalase is 0.4 (Chance & Oshino, 1971). As shown in Fig. 7, the steady-state concentration ratio of Compound I to total haem is increased as the ratio of the H_2O_2-generation rate to haem concentration is increased. An increase of the ethanol concentration causes a shift of the plot to the right.

From the curves drawn in Fig. 7, one can measure the ratio of H_2O_2 to catalase, which produces a half-maximal concentration of Compound I (steady-state concentration ratio = 0.2) for a given ethanol concentration. For a comparison with the 'peroxidatic' activity, the results are replotted in Fig. 3. The open circles represent the ratio of the H_2O_2-generation rate to haem concentration that produces a half-maximal concentration of Compound I formation for a given ethanol concentration. The closed symbols represent the ratio of the H_2O_2-generation rate to haem concentration that causes a 50 % decomposition of H_2O_2 through ethanol oxidation for a given ethanol concentration. The two plots are parallel and differ only slightly. The theoretical explanation of these curves is provided by equations (4e) and (4g) in the Appendix (Chance & Oshino, 1973). The results show that when the reaction is nearly 100% 'peroxidatic', the steady-state concentration of Compound I approaches zero [cf. Fig. 1 in the Appendix (Chance & Oshino, 1973)].

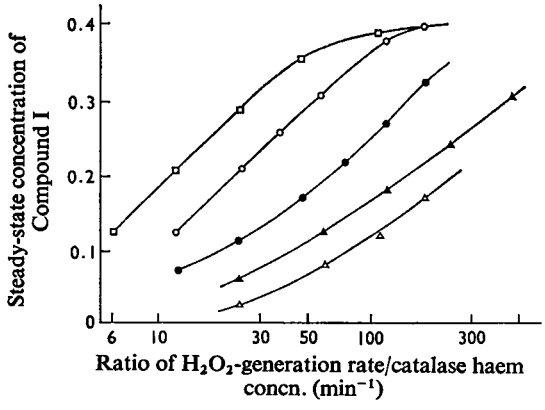

Fig. 7. *Steady-state concentration of the catalase–H_2O_2 Compound I in the presence of ethanol*

The reaction mixture contained, in a final volume of 3.0ml, 50mM-potassium phosphate buffer, pH7.4, 10mM-glucose, 0.725μM-glucose, and different concentrations of ethanol. The steady-state concentration of Compound I on addition of different amounts of glucose oxidase was measured at 407 minus 435nm in the presence of: □, 0.6mM-; ○, 1.0mM-; ●, 3mM-; ▲, 6mM-; △, 10mM-ethanol.

Approximately 45% of the total H_2O_2 is utilized by the ethanol-oxidation reaction when there is a half-maximal concentration of Compound I. Because the catalase–H_2O_2 intermediate can be measured spectrophotometrically, the above finding provides an independent method to study the ethanol-oxidation reaction by catalase and has been applied to the perfused liver system to estimate the H_2O_2-generation rate (Oshino, 1972).

Discussion

The results described in this paper clearly demonstrate the complexity of the ethanol oxidation by catalase, i.e. (*a*) under certain conditions, invariance to the catalase concentration (Table 1 and Fig. 2), (*b*) variability of the inhibitor effect (Fig. 6) and (*c*) variability of the apparent K_m value of ethanol for the ethanol-oxidation reaction (Fig. 2). These phenomena are explained by changes in the contribution of the 'catalatic' and 'peroxidatic' reactions to the overall decomposition of H_2O_2 and can be simplified by introducing, as a parameter, the ratio of the H_2O_2-generation rate to haem concentration. An increase in the ratio of H_2O_2-generation rate to catalase haem concentration, either by increasing H_2O_2 generation or by decreasing effective haem concentration, causes a decrease in the contribution of the 'peroxidatic' reaction and consequently a decrease in an apparent rate of the ethanol oxidation. Because of these properties, the usual enzymological approaches cannot simply be applied to the ethanol-oxidation systems in which catalase may participate. This is especially the case in the studies of the 'microsomal ethanol-oxidation system'. Evidence which has been considered to indicate the existence of the 'microsomal ethanol-oxidation system' is that strong inhibitors of catalase, such as cyanide, azide and 3-amino-1,2,4-triazole, do not inhibit 'microsomal ethanol-oxidation activity' as they do the catalase (Lieber & DeCarli, 1970); 0.1mM-cyanide can inhibit

the 'catalatic' activity almost completely (92%) (Lundquist *et al.*, 1963), whereas only 12% of 'microsomal ethanol-oxidation activity' is sensitive to this concentration of cyanide (Lieber & DeCarli, 1970). Roach *et al.* (1969) seem to have challenged this result, but confirmed the same observation. Later, to eliminate the contribution of catalase to the 'microsomal ethanol-oxidation system', the authors attempted to wash the microsomal fraction repeatedly. The catalase activity ('catalatic') decreased tenfold by this treatment whereas 40% of the 'microsomal ethanol-oxidation activity' remained. Their conclusion was, therefore, that 'about 60% of the activity was due to catalase and 40% to some other enzyme system'. It is worth mentioning that they described the ethanol-oxidation activity as more sensitive to cyanide in washed preparations than in unwashed microsomal preparations. As described in the Results section, this cannot be used as evidence indicating existence of a new 'ethanol-oxidation system'.

An explanation for these observations is clear in the present study: a small variation in the amount of catalase contaminating the microsomal fraction may make a difference in the ratio of the H_2O_2-generation rate to catalase haem concentration, which is one of the key factors in the catalase 'peroxidatic' reaction. Under such conditions, it is reasonable that sensitivity of the ethanol-oxidation activity to cyanide is variable (Fig. 6).

This consideration is supported by the fact that apparent K_m values of the 'microsomal ethanol-oxidation system' for ethanol are 8mM (Lieber & DeCarli, 1970) and 12mM (Thurman *et al.*, 1972), which is variable depending on the microsomal preparations. Since for a given ratio of the H_2O_2-generation rate to catalase haem concentration, the maximal rate of ethanol oxidation expected corresponds to the rate of H_2O_2 generation (Fig. 3), the apparent K_m values quoted above represent the ethanol concentration causing 50% of H_2O_2 to be utilized by the 'peroxidatic' reaction under their conditions. If our results obtained at 25°C (Fig. 3) are converted into their values measured at 37°C (Lieber & DeCarli, 1970; Thurman *et al.*, 1972), the K_m values of 8 and 12mM-ethanol are consistent with the ratios of the H_2O_2-generation rate to catalase haem concentration of 170 and 200/min respectively. The rate of H_2O_2 generation from microsomal fraction is about 1.7nmol/min per mg of protein in 0.1M-potassium phosphate buffer, pH7.4, at 25°C (Boveris *et al.*, 1972), and therefore 0.0085nmol of catalase haem/mg of microsomal protein is sufficient to give the value of 200/min. Higashi & Peters (1963), with catalase antibody, reported that the catalase content of isolated rat liver microsomal fraction is 0.002–0.003nmol of haem/mg of protein. Although precise comparison may not be meaningful because of differences in the experimental conditions, the latter value appears to be in satisfactory agreement with the value estimated above.

The microsomal electron-transport system (Gillette *et al.*, 1957; Thurman *et al.*, 1972; Boveris *et al.*, 1972) as well as the mitochondrial respiratory system (Chance & Oshino, 1971; Boveris *et al.*, 1972) can produce H_2O_2 at rates between 1 (Boveris *et al.*, 1972) and 15 (Thurman *et al.*, 1972) nmol/min per mg of protein. The H_2O_2 production is NADPH-specific (Gillette *et al.*, 1957; Boveris *et al.*, 1972) and partially inhibited by CO (Thurman *et al.*, 1972). Thus the microsomal drug-hydroxylation system is at least partly responsible for the H_2O_2 production. This fact can explain an apparent parallelism between the drug-hydroxylation system and 'microsomal ethanol-oxidation system' (Rubin *et al.*, 1968). It must be emphasized that a comparison of the endogenous activity with the catalase- or H_2O_2-supplemented systems does not provide conclusive evidence to prove or to disprove a possible participation of catalase in the 'microsomal ethanol-oxidation system', unless the ratio of H_2O_2 to catalase is well defined in both systems.

Not only the results observed in the 'microsomal ethanol-oxidation system', but also previous results on methanol metabolism, may have to be re-evaluated. 3-Amino-1,2,4-triazol is a strong inhibitor of catalase and, thus, 10min after injection of the drug, more than 90% of liver catalase is inhibited (Price *et al.*, 1962). Nevertheless, this drug inhibits the rate of methanol oxidation by only 50–70% in intact animal and perfused liver systems (Mannering *et al.*, 1969). Mannering *et al.* (1969) studied this inhibition in detail and reported that 'methanol oxidation was not altered with declining hepatic catalase activities until the catalase activity reached a level of about 400Kat_f unit per g wet wt. of liver'. [The Kat_f unit is defined by Chance & Maehly (1955).] Their interpretation was that 'at levels of catalase activity higher than 400Kat_f unit per g of liver, the steady state among peroxide generating system, H_2O_2, catalase–H_2O_2 complex and methanol functions maximally for whatever amount of H_2O_2 is being produced'. This consideration has been proved in our study quantitatively, because methanol and ethanol are the hydrogen donors for catalase (Chance & Herbert, 1950). It has to be emphasized again that the degree of the inhibition does not tell us anything about the proportion of the contribution of catalase in methanol metabolism. Conclusive evidence has to be drawn by another approach. There is no need for a parallelism between methanol-oxidation activity and liver catalase content or catalase inhibition (Mannering *et al.*, 1969). In the perfused liver and even in the intact rat, the ratio of H_2O_2-generation rate to catalase haem concentration is in the range 4–100/min (Oshino *et al.*, 1973). Under such conditions, the methanol-oxidation

rate is mainly dependent on the rate of H_2O_2 production but not on catalase content.

As described in the Results section and also in the Appendix (Chance & Oshino, 1973), a steady-state concentration of the catalase–H_2O_2 intermediate reflects a ratio of 'catalatic' and 'peroxidatic' activities. A knowledge of the catalase–H_2O_2 intermediate concentration may make possible the evaluation of the catalase reaction in ethanol and methanol metabolism and some work has been successfully accomplished with the perfused liver (Sies *et al.*, 1973; Oshino *et al.*, 1973). The fact that the catalase–H_2O_2 intermediate observable in the perfused liver (Sies & Chance, 1970) and intact animal (B. Chance & N. Oshino, unpublished work) can respond to ethanol and methanol indicates that catalase actually functions in ethanol and methanol metabolism, though the absolute rates are dependent on the rate of H_2O_2 production.

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