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Optical Measurement of the Catalase–Hydrogen Peroxide Intermediate (Compound I) in the Liver of Anaesthetized Rats and its Implication to Hydrogen Peroxide Production *in situ*

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The spectrophotometric determination of the catalase–H_2O_2 intermediate (Compound I) was extended to the liver *in situ* in anaesthetized rats. The rate of H_2O_2 production was determined for the liver *in situ* with endogenous substrates, and in the presence of excess of glycollate. Glycollate infusion doubled H_2O_2 production rate in the liver of air-breathing rats, and caused a fourfold increase when rats breathed O_2 at 1×10^5 Pa. Hyperbaric O_2 up to 6×10^5 Pa did not increase H_2O_2 generation supported by endogenous substrates, nor did it increase H_2O_2 production above that produced by 1×10^5 Pa O_2 in glycollate-supplemented rats. The rates of ethanol oxidation via hepatic catalase and via alcohol dehydrogenase in the whole body were separately measured. The contribution of hepatic catalase to ethanol oxidation was found to be approx. 10% in endogenous conditions and increased to 30% or more of the total ethanol oxidation in rats supplemented with glycollate.

One method of studying biochemical reactions taking place in living isolated organs or in organs of the intact animals *in situ* is to utilize spectrophotometry or fluorimetry without damaging the organs. Skeletal muscle *in vitro* (Millikan, 1937; Ramirez, 1959; Jobsis, 1963*a*,*b*) and isolated organs such as the haemoglobin-free perfused heart (Williamson & Jamieson, 1966; Chance *et al.*, 1972) and liver (Lübbers *et al.*, 1965; Bücher *et al.*, 1972) have been used extensively by several workers for such investigations. However, *in situ*, the presence of haemoglobin in the blood obscures most spectrophotometric measurements and thus has appeared to limit this technique to perfused organs. In certain cases the interference due to haemoglobin may be minimized or circumvented, and the spectrophotometric monitoring method then becomes feasible. In the present study spectrophotometry was extended to the exposed liver of the anaesthetized rat, where the existence of the catalase–H_2O_2 intermediate (Compound I) was established, and used for the study of H_2O_2 generation under a variety of conditions.

As in the perfused liver described previously (Sies & Chance, 1970; Sies *et al.*, 1973), the catalase–H_2O_2 intermediate (Compound I) can be measured at $E_{660-640}$ in liver *in situ*. At this wavelength pair optical interference due to haemoglobin and cytochromes is minimal (Sies & Chance, 1970; Chance & Oshino, 1971). More important, it is possible to use the technique of titrating the steady state of the catalase–H_2O_2 intermediate (Oshino *et al.*, 1973*a*), which is maintained in the presence of a constant supply of H_2O_2, with hydrogen donors such as methanol and ethanol *in vivo*. Thus we are able to calculate the rate of H_2O_2 generation in the liver *in situ* in the rat, as explained below. Although obviously more limited *in vivo* as regards perfusion of substrates and inhibitors, we have been able to study several factors which affect H_2O_2 production in the liver of the whole rat, such as the effect of the peroxisomal substrate, glycollate, and the action of hyperbaric oxygen. Also, we have been able to evaluate the significance of catalase in the oxidation of ethanol in the liver *in vivo*.

Materials and Methods

Optical measurement in hyperbaric chamber

Male Holtzman rats (220–300g wt.) were used. Animals were lightly anaesthetized with urethane (1.0–1.2g/kg), and one or both femoral veins were cannulated as necessary. Cannulas were inserted as deeply as possible and when glycollate was infused in addition to methanol or ethanol, two separate cannulas were used. The liver was exposed and the animal was placed in the pressure chamber (Bethlehem Steel model 1836-HP), which was maintained at a temperature of 30°C. Light-guides were used for absorbance measurements, the light-guides being sealed into two of the penetrating plugs of the pressure chamber. A spade-shaped holder, with

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the light-guide inserted, was placed beneath the largest liver lobe, and the other light-guide was clamped in position approx. 1 cm above the liver; i.e. changes in the transmitted light were measured through the liver lobe as for a perfused organ. In general the method is similar to that previously described for the perfused liver (Theorell *et al.*, 1972). A mechanically driven time-sharing, two-channel, dual-wavelength spectrophotometer was used for these studies. The apparatus, described by Theorell *et al.* (1972), consists of a motor-driven disc containing four interference filters, which are rotated before a tungsten lamp, and provide the four wavelengths for the two dual-wavelength measurements at 660–640 nm for the catalase–H_2O_2 intermediate and 605–620 nm appropriate to the measurement of cytochrome *a*, but used *in situ* largely for measuring haemoglobin absorbance changes resulting from blood-flow changes. Although the method operates similarly to that described previously (Theorell *et al.*, 1972), considerably less light is transmitted through the lobe of the liver *in situ* due to haemoglobin absorbance than through the lobe of the haemoglobin-free perfused liver and consequently higher dynode voltages are required on the photomultiplier. The signal transmitted by the liver lobe may vary with alterations in the haemodynamics in the tissue. This is, however, monitored with an oscilloscope and may be maintained at a constant value manually so that the sensitivity in recording the catalase–H_2O_2 intermediate is constant. Only in extreme cases of blood-flow changes in abnormal animals was this readjustment necessary. However, as the text below explains, the calibration of the catalase–H_2O_2 intermediate is a relative one for a given set of conditions and is determined by a methanol or ethanol titration, and thus the effect of blood flow and its changes are minimized.

Motion of the liver past the light-guide with respiration caused modulation of the signal since the thickness of the optical path varied. This affected the dual-wavelength method only as a second-order effect and was sufficiently small to be neglected. These fluctuations were minimized by a long-time constant (10 s).

Venous cannulas were connected to 26-gauge stainless-steel tubing sealed into two penetration plugs in the pressure chamber. Infusions of drugs were performed from outside the chamber by using infusion pumps (Harvard Apparatus, series 940) with cannulas connected to the 26-gauge stainless-steel tubing inserts in the wall of the chamber. Great care was taken at all times to ensure that all syringes and fittings were leak-free, and the infusion pumps were calibrated up to 7×10^5 Pa pressure in the chamber to confirm that their delivery rates were correct under hyperbaric conditions. The chamber was continuously flushed with O_2 for the 100%-O_2 experiments. In compression experiments, the chamber was flushed with O_2 at a rate of approx. 50 litres for 5–10 min, then the pressure was raised at a rate of approx. 1×10^5 Pa/min.

Blood ethanol determinations

For alcohol determinations, the animals were injected intraperitoneally with 0.2 ml of 10 mM-*n*-propylpyrazole, an alcohol dehydrogenase inhibitor kindly supplied by Dr. H. Drott of Johnson Research Foundation (Drott, 1974). This pyrazole derivative has not previously been used in intact animals, and so was tested as described in the Results section before being used in our experiments. The inhibitor was injected 5 min before placing of the animal in position, with a total time of approx. 20–30 min elapsing between injection and ethanol perfusions. For blood ethanol determinations, a femoral artery was cannulated as well as one or both femoral veins. A control sample of blood was taken before any ethanol infusions were begun. As each steady state was reached, as monitored spectrophotometrically by the fractional saturation of the catalase–H_2O_2 intermediate at a particular rate of infusion of ethanol, a sample of blood (0.1 ml) was withdrawn into a heparinized syringe, and immediately placed into an Eppendorf centrifuge tube containing 0.4 ml of 15% perchloric acid and refrigerated. Usually five or six samples at different rates of ethanol infusion were taken for each animal. Sampling of blood was similarly performed in *n*-propylpyrazole-pretreated rats supplemented with glycollate.

The blood samples were then centrifuged and 0.3 ml of supernatant was removed and neutralized with 0.2 ml of 1.6 M-K_2CO_3. The tubes were centrifuged again and samples (50 μl) of supernatant were taken for analysis, which was performed in triplicate by using the alcohol dehydrogenase reaction (Dickinson & Dalziel, 1967).

Drugs and administrations

Ethanol and methanol were injected intravenously as described above. The strength of solution infused depended on the presence or absence of a substrate which generates H_2O_2, e.g. 0.425 M- or 0.65 M-methanol was used for endogenous conditions, whereas 2–3 M solutions were used when glycollate was also infused as more hydrogen donor must be supplied as the rate of H_2O_2 generation increases. Glycollate, obtained from Calbiochem (San Diego, Calif., U.S.A.), was diluted to 2 M, buffered to pH 7.4, and infused at a constant rate of 60 μmol/min per 100 g body wt. for 5–10 min before methanol or ethanol titrations. The glycollate infusion continued throughout the titration, and the dose was super-maximal for glycollate oxidase activity, by extrapolation from experiments with perfused liver (Oshino *et al.*, 1973*a*).

Results

Detection of the catalase–H_2O_2 intermediate in the liver of anaesthetized rat

When a constant supply of H_2O_2 is maintained in the liver, a steady-state concentration of the catalase–H_2O_2 intermediate (Compound I) is established, and can be measured at $E_{660-640}$ (Sies *et al.*, 1973; Oshino *et al.*, 1973*a*). In the presence of hydrogen donors such as methanol or ethanol, the concentration of the catalase–H_2O_2 decreases and there is a relationship, as determined previously, between the extent of fractional saturation of the catalase–H_2O_2 intermediate, the rate of H_2O_2 generation and the concentration of hydrogen donor (Oshino *et al.*, 1973*a*,*b*; Chance & Oshino, 1973). When, for example, methanol or ethanol was infused at a constant slow rate into the femoral vein of an anaesthetized rat, $E_{660-640}$ decreased, and this change in absorbance could be attributed to a decrease in the steady-state concentration of the catalase–H_2O_2 intermediate, owing to a stimulation of the catalase 'peroxidatic' reaction by methanol or ethanol, as described below. Although other changes in $E_{660-640}$ might be included in the measurement of the liver of anaesthetized rats, such an infusion of methanol or ethanol did not change $E_{605-620}$, which is largely a monitor of haemodynamic changes and which was measured simultaneously until the infusion rate became so rapid (above 0.5ml/min) that a considerable dilution of the blood occurred.

Methanol infusion lowers the concentration of the catalase–H_2O_2 to a new steady state. This effect is seen in Fig. 1, and is shown as a downward deflexion in the trace. Increasing the infusion rate of methanol to a new value then produces another yet lower steady-state concentration of the catalase–H_2O_2 intermediate. Such stepwise titrations *in vivo* were continued until no further change in $E_{660-640}$ was observed (i.e. the catalase–H_2O_2 intermediate concentration approached zero).

With ethanol as hydrogen donor, in contrast with methanol, there was a difference in the response obtained *in vivo*. Infusion of ethanol, as of methanol, produced new lower steady states of the catalase–H_2O_2 intermediate, but when the ethanol infusion was stopped the catalase–H_2O_2 concentration returned towards its baseline value owing to the presumed rapid consumption of ethanol by alcohol dehydrogenase (Fig. 1). The participation of alcohol dehydrogenase was confirmed by measuring surface fluorescence of the liver of anaesthetized rats; nicotinamide nucleotides were reduced and reoxidized on initiation and cessation of ethanol infusion respectively (Chance *et al.*, 1974).

The catalase–H_2O_2 intermediate as Compound I has already been identified in the isolated haemoglobin-free perfused rat liver (Sies & Chance, 1970; Sies *et al.*, 1973), where more detailed identification was possible. Use of the catalase–cyanide compound or complete spectral analysis is obviously not possible *in vivo*. However, additional evidence indicating that the methanol- or ethanol-induced change in $E_{660-640}$ is the change in the steady-state concentration of the catalase–H_2O_2 intermediate was afforded by use of 3-amino-1,2,4-triazole, which is a specific inhibitor of catalase (Price *et al.*, 1962). Fig. 2 shows that changes in $E_{660-640}$ responding to ethanol infusion (36μmol/min per 100g body wt.) become smaller as time proceeds after an intraperitoneal injection of 3-amino-1,2,4-triazole (1 g/kg). At 1h after the drug injection, no change in $E_{660-640}$ could be observed by ethanol infusion. In separate experiments, the extent of the catalase–H_2O_2 signal in the perfused livers from 3-amino-1,2,4-triazole-pretreated rats was compared with that from control rats. It was confirmed that the catalase–H_2O_2 signal, as well as the catalase–cyanide signal, was below 5% the control value at 1h after the drug injection.

The steady-state concentration of the catalase–H_2O_2 intermediate in rat liver *in situ* appears to be approx. 100% of its maximal saturation value. This conclusion was drawn from the effect of glycolate, which is a potent H_2O_2 generator owing to glycollate oxidase activity in the rat; glycollate infusion did cause more than twofold stimulation in the rate of

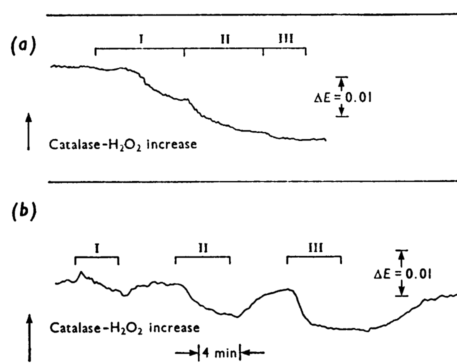
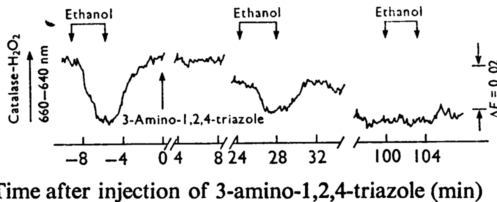


Fig. 1. *Change in the steady state of catalase Compound I in response to methanol and ethanol infusion*

The steady state of catalase Compound I was followed optically at 660–640nm. In (*a*) methanol was infused at the rate of 1.1 (I), 5.2 (II) and 26μmol/min per 100g body wt. (III) for a period as indicated in the Figure. Infusion rate of ethanol in (*b*) was 3.35 (I), 8.35 (II) and 16.7μmol/min per 100g body wt. (III) respectively. Downward deflexion of the trace is a decrease in $E_{660-640}$ and corresponds to a decrease in the steady-state concentration of catalase Compound I. A lag phase in the initial methanol response was due to the time taken to eliminate heparinized saline from the catheter. These traces are representative of eleven (*a*) and eight (*b*) experiments.

H_2O_2 production, yet, in most cases, no change in $E_{660-640}$ was observed. Glycollate infusion sometimes produced up to a 10% increase in the total catalase–



Time after injection of 3-amino-1,2,4-triazole (min)

Fig. 2. *Effect of* 3-*amino*-1,2,4-*triazole on the catalase–H_2O_2 signal in the liver of anaesthetized rat*

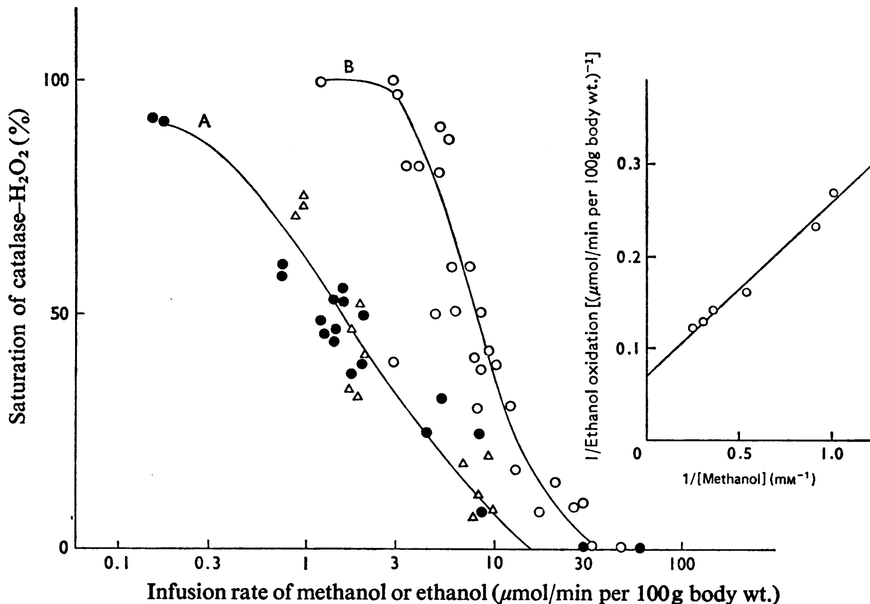
After recording the response of the catalase–H_2O_2 to ethanol (36 μmol/min per 100 g body wt.), 3-amino-1,2,4-triazole (0.1 g/100 g body wt.) was injected intraperitoneally, and the change in the steady-state concentration of the catalase–H_2O_2 was monitored at 660–640 nm. At 25 and 100 min after the drug injection, ethanol infusion (36 μmol/min per 100 g body wt.) was repeated for a period as indicated in the Figure. This trace was representative of three separate experiments.

H_2O_2 signal, but this change, when it occurred, was always accompanied by a change in $E_{605-620}$, and thus appeared to be related to a change in haemodynamics. Thus the initial catalase–H_2O_2 concentration was taken as 100% saturation value, and the steady state concentration when no further change was produced by increased methanol was taken as zero for the catalase–H_2O_2 intermediate. Then each steady state produced by a certain infusion rate of hydrogen donor could be expressed as a fractional saturation of the maximal catalase–H_2O_2 signal. The titration curves thus obtained with methanol and with ethanol are shown in Fig. 3. The infusion rates of methanol and of ethanol required to produce the half-maximal saturation of the catalase–H_2O_2 intermediate were 1.7 and 7.5 μmol/min per 100 g body wt. respectively.

Theoretical basis for determination of H_2O_2 generation rate

The equation previously determined for the catalase reaction of rat liver is:

$$1/e \cdot dx_n/dt = Ka_{1/2} \quad (1)$$



Infusion rate of methanol or ethanol (μmol/min per 100 g body wt.)

Fig. 3. *Relationship between the fractional saturation of the catalase–H_2O_2 and the infusion rate of methanol and ethanol*

The extents of the fractional saturation of the catalase–H_2O_2 observed with continual infusion of methanol and of ethanol at various rates were plotted in a semi-logarithmic scale as a function of infusion rate. ●, Methanol; ○, ethanol; △, ethanol in the presence of *n*-propylpyrazole. Points are those obtained in eleven (●), eight (○) and six (△) separate experiments. Inserted Figure is a double-reciprocal plot of the rate of alcohol dehydrogenase-dependent ethanol oxidation against ethanol concentration (equivalent to methanol concentration producing the same fractional saturation of the catalase–H_2O_2). The rate of ethanol oxidation through alcohol dehydrogenase was calculated from the difference in the infusion rates of methanol and of ethanol which were required to produce a given fractional saturation of the catalase–H_2O_2. Ethanol concentration in liver was determined from a corresponding infusion rate of methanol, by using the result shown in Fig. 6.

where e is the catalase concentration and equals 19.2nmol/g wet wt. of liver (Oshino *et al.*, 1975), dx_n/dt is the rate of H_2O_2 generation (min^{-1}), $a_{1/2}$ is the concentration of methanol or ethanol required to produce the half-maximal saturation of the catalase–H_2O_2 intermediate, and K is $31.5 min^{-1} \cdot mM^{-1}$ (Oshino *et al.*, 1973*a*). This equation allows the measurement of the H_2O_2-generation rate in the liver. However, to calculate the rate of H_2O_2 production from these experimental results in the same way as was established for the perfused liver, the methanol concentration in the liver is required for calculation of the $a_{1/2}$ value, rather than the rate of methanol infusion into the animal. Methanol is not easily determined by currently available methods, although the measurement of ethanol concentration is a well-established technique. Ethanol and methanol are equiactive as hydrogen donors in the catalase 'peroxidatic' reaction and at equal concentrations should produce equal changes in the catalase–H_2O_2 concentration (Oshino *et al.*, 1973*b*; Chance & Oshino, 1973). Thus when compared at the condition where methanol and ethanol infusion produced an identical steady-state concentration of the catalase–H_2O_2 intermediate, blood ethanol concentration may be taken as equivalent to blood methanol concentration if alcohol dehydrogenase activity has been completely inhibited.

Inhibition of alcohol dehydrogenase by n-propylpyrazole in vivo

n-Propylpyrazole has been found to be more active than 4-methylpyrazole *in vitro* (Drott, 1974; Dahlom *et al.*, 1974). To measure the optimal dose of *n*-propylpyrazole for the inhibition of alcohol dehydrogenase *in vivo*, the steady-state concentration of the catalase–H_2O_2 was titrated with increasing doses of *n*-propylpyrazole in the presence of a continual infusion of ethanol (3.8μmol/min per 100g body wt.). Because of the continual consumption of ethanol by alcohol dehydrogenase, this rate of ethanol infusion produced only a slight decrease in the steady-state concentration of the catalase–H_2O_2 (Fig. 4). However, by comparison with methanol titrations (see Fig. 3) such an infusion rate of ethanol would be expected to produce more than 80% decrease in the steady state of the catalase–H_2O_2 intermediate, if other pathways of ethanol oxidation, principally alcohol dehydrogenase, were inoperative. Therefore after establishment of a steady state of the catalase–H_2O_2 with a low infusion rate of ethanol (Fig. 4), *n*-propylpyrazole was injected intravenously in 'pulses' of 30–60s. The *n*-propylpyrazole injection caused corresponding decreases in the steady-state concentration of the catalase–H_2O_2, owing to increases in ethanol concentration as oxidation by

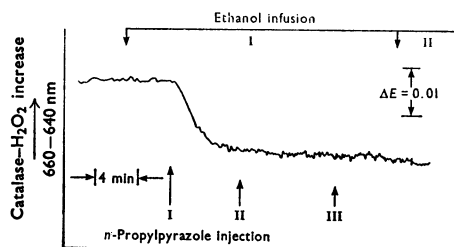


Fig. 4. *Effect of n-propylpyrazole on the activity of alcohol dehydrogenase in situ*

n-Propylpyrazole in 0.9% NaCl solution was infused as 'pulses' of 60s for I and 30s for II and III intravenously under a continual infusion of ethanol at a rate of 3.8μmol/min per 100g body wt. (I). Doses of *n*-propylpyrazole at each injection were 0.8 at I and 0.4μmol/min per 100g body wt. at II and III, respectively. At the end of the titration, ethanol infusion rate was increased to 40μmol/min per 100g body wt. (II). The trace is representative of four separate experiments.

alcohol dehydrogenase was inhibited. These experimental results were obtained with very little variation between animals. A dose of 0.8–1.2μmol/100g body wt. was found sufficient to produce near-maximal inhibition of the alcohol dehydrogenase reaction *in vivo*. Almost complete inhibition of alcohol dehydrogenase by this dose of *n*-propylpyrazole was confirmed by the fact that the titration curves for the catalase–H_2O_2 with ethanol in the presence of *n*-propylpyrazole were almost identical with methanol-titration curves under various conditions as described below (Fig. 3 and Table 1). The intraperitoneal route was found just as satisfactory as intravenous injection. The effect of *n*-propylpyrazole in inhibiting alcohol dehydrogenase lasted at least 2h, as judged by lack of return of the catalase–H_2O_2 signal to the original basal value after an appropriate dose of ethanol in the *n*-propylpyrazole-pretreated rats.

Ethanol concentration in the blood of the n-propylpyrazole-pretreated rats

For blood ethanol determination in the *n*-propylpyrazole-pretreated rats, titrations were carried out as usual, and arterial blood samples were drawn from the animal after allowing appropriate periods for establishment of a steady state, which was monitored at $E_{660-640}$. An example of the results obtained in such titration experiments is shown in Fig. 5. The fractional saturation of the catalase–H_2O_2 is given as a function of both the rate of ethanol infusion and the ethanol concentration in arterial blood as shown in the two abscissa scales

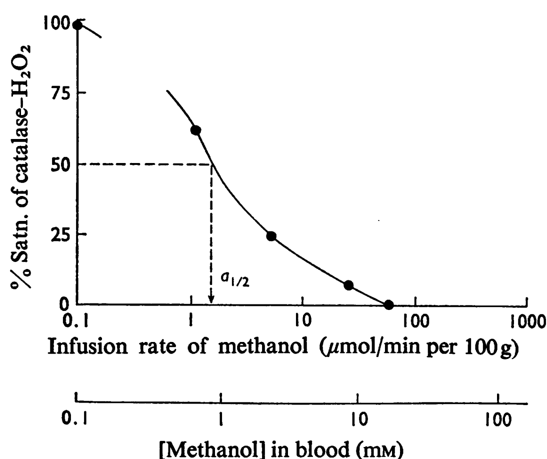


Fig. 5. *Determination of $a_{1/2}$ value for the methanol-titration curve of the catalase–H_2O_2*

The experimental result shown in Fig. 1(*a*) was plotted in this Figure. Two scales are shown for the abscissa, the upper scale being the actual infusion rate of methanol and the lower the blood methanol concentration, which is equivalent to the ethanol concentration in the *n*-propylpyrazole-pretreated rats, and obtained from Fig. 6. $a_{1/2}$ is the concentration of methanol in the liver which was required to produce half-maximal saturation of the catalase–H_2O_2 (% satn. = 50%).

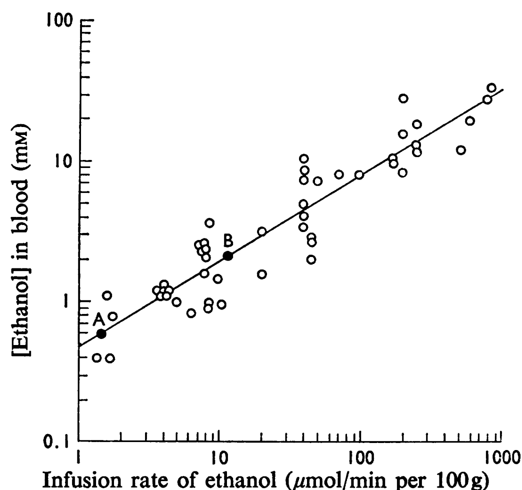


Fig. 6. *Blood concentration of ethanol in the presence of n-propylpyrazole at various infusion rates of ethanol*

The rats used were pretreated with *n*-propylpyrazole (1 μmol/100g body wt.) 5 min before preparation. After establishment of each steady state, judged by attainment of a steady state of the catalase–H_2O_2, arterial blood was withdrawn and the blood ethanol concentration was analysed. To utilize the catalase–H_2O_2 signal as the marker of establishment of the steady state, rats were supplemented with glycollate (60 μmol/min per 100g body wt.) when ethanol infusion rate was above 8 μmol/min per 100g body wt. Two closed circles, marked as A and B, are the mean values of the rate of methanol infusion required to produce half-maximal saturation of the catalase–H_2O_2 in the absence and presence of glycollate respectively (see Table 1 also). Each point represents a mean value of three measurements with one blood sample. The results obtained in nine animals are presented in this Figure.

of the Figure. Compared with the titration curve shown in Fig. 3, it is obvious that the titration curves with ethanol after *n*-propylpyrazole are almost identical with the methanol-titration curves. This result verifies the assumption that if alcohol dehydrogenase activity is completely inhibited a given rate of methanol and ethanol infusion provides the same concentration of hydrogen donor in blood. The relation between the infusion rate of ethanol (equivalent to methanol, as alcohol dehydrogenase is inhibited) and its concentration in the blood thus determined is shown in Fig. 6. From this plot we were able to measure blood methanol concentration at any particular rate of infusion of methanol into the whole animal. The higher values in Fig. 6 belong to the glycollate-supplemented rats, as explained below, and obviously help considerably in measuring an accurate correlation between the infusion rate of hydrogen donor and its blood concentration.

Rate of H_2O_2 production in the liver of the anaesthetized rat

After cessation of methanol infusion (or of ethanol infusion in the presence of *n*-propylpyrazole) the steady-state concentration of the catalase–H_2O_2 intermediate, which had been decreased, was maintained without visible recovery towards its initial higher value within 10 min, indicating that the rate of catalase-dependent methanol oxidation is rather slow compared with the total concentration in blood. Under such conditions methanol (or ethanol in the presence of *n*-propylpyrazole) concentration detected in the arterial blood may be assumed to be equal to that in the liver, and hence it was possible, as described above, to measure the rate of H_2O_2 production in the liver of anaesthetized rats. At half-maximal saturation of the catalase–H_2O_2, the blood ethanol concentration in the *n*-propylpyrazole-pretreated rats was 0.62 mM at an infusion rate of 1.45 μmol/min per 100g body wt. (Table 1). The curves were identical (within the experimental error) with those obtained with methanol; the half-maximal saturation of the catalase–H_2O_2 intermediate was observed at a rate of methanol infusion

Table 1. *Rate of H_2O_2 generation in the liver of anaesthetized rats under various conditions*

H_2O_2-generation rates were calculated from the equation: $1/e \cdot dx_n/dt = Ka_{1/2}$, where e is the catalase content = 19.2nmo of haem/g of liver, $a_{1/2}$ is the concentration of hydrogen donor at half-maximal saturation of catalase Compound I, and $K = 31.5\,min^{-1} \cdot mM^{-1}$ (Oshino *et al.*, 1973*a*). Liver weight of fed rat was assumed to be 4.0g/100g body wt. (Oshino *et al.*, 1975). Values for $a_{1/2}$ were obtained from individual titration curve as shown in Figs. 5 and 7 and given as mean values ±S.E.M. Numbers of animals used are given in parentheses. The other values were calculated from the mean values of $a_{1/2}$

Compounds infused		Respired gas	Infusion rate for 50% saturation of catalase–H_2O_2 (μmol/min per 100g)	Corresponding concentration in blood (mM)	Rate of H_2O_2 generation in liver (μmol/min per g of liver)	(μmol/min per 100g body wt.)
			Methanol			
None	(11)	Air	1.39±0.19	0.60	0.38	1.45
None	(7)	O_2 (6×10^5 Pa)	1.27±0.16	0.56	0.34	1.35
Glycollate	(5)	Air	4.90±0.65	1.20	0.76	2.90
Glycollate	(5)	O_2 (1×10^5 Pa)	12.00±1.81	2.20	1.33	5.32
Glycollate	(5)	O_2 (6×10^5 Pa)	9.90±1.29	1.95	1.18	4.72
			Ethanol			
None	(8)	Air	7.67±0.67	—*		
Glycollate	(6)	O_2 (1×10^5 Pa)	13.71±1.53	—*		
None	(4)	O_2 (6×10^5 Pa)	8.90±2.2	—*		
n-Propylpyrazole	(6)	O_2 (1×10^5 Pa)	1.45±0.17	0.62	0.38	1.50
Glycollate+ *n*-propylpyrazole	(7)	O_2 (1×10^5 Pa)	9.50±1.01	1.95	1.18	4.72

* Actual values could not be determined accurately by our method owing to the presence of alcohol dehydrogenase activity.

of 1.4μmol/min per 100g body wt., which corresponds to the blood methanol concentration of 0.6mM (Table 1). By using eqn. (1) and assuming the liver wt. to be 4g/100g wt. (Oshino *et al.*, 1975), the above values of $a_{1/2}$ give the rate of hepatic H_2O_2 production to be 380nmol/min per g of liver or 1.45μmol/min per 100g body wt.

Stimulation of H_2O_2 production by glycollate

In earlier work with isolated perfused liver (Oshino *et al.*, 1973*a*), it was speculated that the low rate of endogenous H_2O_2 generation in the haemoglobin-free perfused liver was probably due to lack of substrates for H_2O_2 production and this was confirmed by the present measurement *in vivo*, indicating that indeed a higher concentration of substrates for H_2O_2 generation exists in the liver *in situ*. For example, uric acid concentration in rat liver is approx. 0.07mM (Eggleston & Krebs, 1974). Obviously quite high concentrations of fatty acids, lactate and probably xanthine, which enhanced H_2O_2 production in the perfused rat liver (Oshino *et al.*, 1973*a*), will also be present in the intact liver *in situ*. However, the H_2O_2-generation rate with endogenous substrates is still substrate-limited, as could be seen when glycollate was infused into the animal.

An example of the ethanol-titration curve in the presence of glycollate and *n*-propylpyrazole in rats breathing 100% oxygen is shown in Fig. 7. The $E_{660-640}$ under these conditions became quite

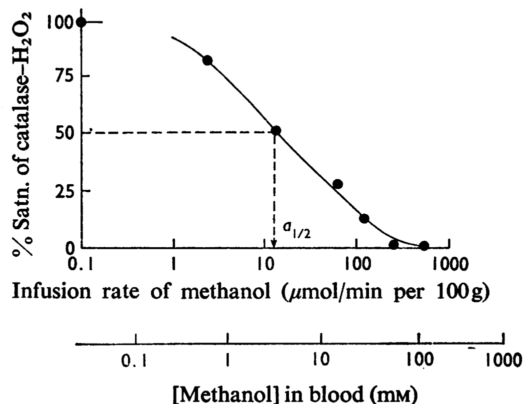


Fig. 7. *Methanol-titration curve obtained in the presence of glycollate*

The result obtained in the experiment shown in Fig. 8 was plotted as a function of both methanol infusion rate and of blood methanol concentration as explained for Fig. 5.

insensitive to ethanol or methanol, and to produce the half-maximal saturation of the catalase–H_2O_2 intermediate approx. 10 times higher infusion rates of ethanol were required compared with those observed in the absence of glycollate (Fig. 5). This is the characteristic property of the catalase reactions predicted theoretically (Chance & Oshino, 1973), and thus the

result observed here confirms our correct measurement of the catalase–H_2O_2 at this wavelength pair. Glycollate was the only substrate as yet found to be suitable for infusion, as uric acid and xanthine are far too insoluble. Table 1 shows that in the air-breathing rat H_2O_2 production in the liver increased twofold when glycollate increased H_2O_2 generation through the glycollate oxidase reaction.

Effect of hyperoxia on hepatic H_2O_2 production

One of the experimental traces obtained under hyperbaric conditions is shown in Fig. 8. Immediately on flushing the chamber with O_2 there was an absorbance change at 660–640 nm and 605–620 nm. As compression began these absorbance changes became more marked, and, as discussed above, were probably due to vasoconstriction induced by hyperbaric O_2. Glycollate infusion was started 1 min after the required pressure (2.4×10^5 Pa in this particular experiment) was reached, then approx. 5 min later methanol infusion was started and the titration was continued stepwise until no further change in the catalase–H_2O_2 signal occurred on addition of methanol. There was a delay in the change in $E_{660-640}$ responding to the initial infusion of methanol (2.8 μmol/min per 100 g body wt.), but this was due to the dead-volume in the cannulation system for infusion.

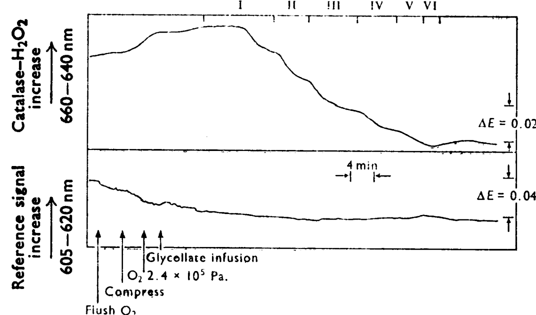


Fig. 8. *Experimental trace of the methanol titration of the catalase–H_2O_2 under hyperbaric conditions*

$E_{660-640}$ represents mainly the change in the steady state of the catalase–H_2O_2 intermediate, and $E_{605-620}$ (reference signal) reflects mostly the change in blood flow under these conditions. The rat was placed in the hyperbaric chamber as described in the Materials and Methods section. Compression with O_2 was started as indicated in the Figure, and during titration the pressure was maintained at 2.4×10^5 Pa. Glycollate infusion was initiated at the indicated point at a rate of 60 μmol/min per 100 g body wt. The rate of methanol infusion for I, II, III, IV, V and VI was 2.8, 13, 70, 130, 260 and 520 μmol/min per 100 g body wt. respectively. The trace is representative of six separate experiments.

The rates of H_2O_2 production calculated from such titration experiments are shown in Table 1. Without exogenous glycollate the rates of H_2O_2 generation were identical in the two groups of air-breathing animals and of animals under O_2 pressure of 6×10^5 Pa, indicating that hyperbaric O_2 did not stimulate H_2O_2 generation under these conditions.

Since glycollate oxidase has a relatively low affinity for O_2 (Oshino *et al.*, 1975) and, in addition, respiration of the urethane-anaesthetized rats may be depressed, there is a definite possibility that the rate of H_2O_2 generation in the glycollate-supplemented rats may become limited by the availability of O_2, and this was the case in our experiments. Table 1 shows that there was a clear and significant increase in H_2O_2 generation of approximately twofold, from 2.9 to 5.3 μmol/min per 100 g body wt., when the glycollate-supplemented animals breathed 100% O_2, compared with the glycollate group breathing air. The increment in the rate of H_2O_2 generation by glycollate was 3.9 μmol/min per 100 g body wt. or 0.97 μmol/min per g of liver, which is in agreement with the maximal rate of glycollate-dependent H_2O_2 production (0.9 μmol/min per g of liver) in the perfused liver, as shown in the preceding paper (Oshino *et al.*, 1975). However, further increases of O_2 pressure up to 6×10^5 Pa did not produce any greater increase in H_2O_2 production than did 100% O_2 (Table 1).

Contribution of catalase to ethanol metabolism in rat liver

As summarized in the titration curves shown in Fig. 3, there is an obvious difference between the curves for methanol (or ethanol in the presence of *n*-propylpyrazole) (curve A) and the curve for ethanol alone (curve B). This difference is attributable to alcohol dehydrogenase as was described for the perfused liver (Lindros *et al.*, 1974). The activity of alcohol dehydrogenase was measured from the difference in the curves at various steady states of the catalase–H_2O_2 intermediate, and then blood ethanol concentration was measured from the infusion rate of methanol which produces corresponding fractional saturation of the catalase–H_2O_2 intermediate, and by using the relation shown in Fig. 6. These values are plotted in a double-reciprocal co-ordinate in an insert to Fig. 3. The $V_{max.}$ obtained for alcohol dehydrogenase of the urethane-anaesthetized rats was approx. 13 μmol/min per 100 g body wt. This value agrees with the value of 34.5 mg of ethanol oxidized/h per kg (13 μmol/min per 100 g body wt.) reported for the rate of ethanol disappearance *in vivo* by Videla *et al.* (1973).

The H_2O_2 generation in the rat liver under endogenous-substrate conditions was 1.45 μmol/min per

100 g body wt. (Table 1), which with the 1:1 stoicheiometry of the peroxidatic reaction corresponds to the maximal catalase-dependent ethanol oxidation in the liver. This value of 1.45 μmol/min per 100 g body wt. will be higher for the total body H_2O_2 generation owing to contribution of H_2O_2 production in extrahepatic tissues. Thus total oxidation of ethanol in the whole rat approximates 13 μmol/min per 100 g for alcohol dehydrogenase activity plus at least 1.45 μmol/min per 100 g body wt. for liver catalase activity, and the contribution of liver catalase in the whole rat is 1.45/14.5 or 10% of the total ethanol oxidation in the rat with endogenous substrates.

We next attempted to increase the rate of ethanol oxidation *in vivo* by increasing the contribution of catalase to ethanol oxidation. To achieve this we infused glycollate to enhance H_2O_2 generation, which of course then results in an increase of hydrogen donor (in this case ethanol) utilization in the peroxidatic reaction. These experiments were carried out in 100% O_2 to overcome the apparent O_2 limitations. As expected, a higher rate of infusion of ethanol was necessary to achieve half-maximal saturation of the catalase–H_2O_2 intermediate under these conditions (Table 1), from 7.7 to 13.7 μmol/min per 100 g body wt. With glycollate and 100% O_2 the rate of generation of H_2O_2 was approx. 5.3 μmol/min per 100 g. If alcohol dehydrogenase activity remained unchanged under these conditions then the contribution of liver catalase should be at least 5.3/(13+5.3) or 30% of total ethanol metabolism.

Discussion

Spectrophotometric measurement of catalase–H_2O_2 intermediate (compound I) in vivo

These results are particularly significant, as they are the first attempt to measure an enzyme–substrate intermediate and its dynamic reactions in a functional preparation *in situ*. Even the presence of haemoglobin, which tends to obscure most spectrophotometric measurements, does not markedly impede measurement of the catalase–H_2O_2 intermediate (Compound I) by dual-wavelength spectrophotometry. Certain changes in the total blood volume in the measuring field, e.g. due to vasoconstriction or vasodilation, do affect the intensity of absorbance at 660–640 nm, but provided that significant flow changes do not occur during a titration, the measurement appears to be reliable and reproducible. The reference signal $E_{605-620}$ used in the present study gave a good indication of haemodynamic changes and thus provided a control against misinterpretation of absorbance measurement due to various amounts of haemoglobin in the field of observation. When large changes in $E_{605-620}$ occurred during a titration, it was even possible to correct such an artifact in $E_{660-640}$ by comparing and calibrating the changes in $E_{660-640}$ and $E_{605-620}$, although such data were not used in this report.

Further justification, besides the data presented in the Results section, of the extension of the measurement of the catalase–H_2O_2 intermediate as an indicator of H_2O_2 generation *in situ* can be obtained from the shape and slope of the methanol titration curve, as shown in Figs. 3, 5 and 7. These are very similar to the methanol-titration curves in the isolated perfused liver, which, in turn, closely approximates the situation found for purified rat liver catalase (Oshino *et al.*, 1973*a*,*b*).

H_2O_2 production under hyperbaric oxygen

Under conditions of endogenous substrates *in vivo*, we failed to detect any increase in the rate of H_2O_2 generation when O_2 pressure was increased up to 6×10^5 Pa. This result agrees with data on the isolated perfused liver (Oshino *et al.*, 1975), but contrasts with studies on isolated mitochondrial fractions, where H_2O_2 production was augmented by increases in the oxygen pressure up to 19.5×10^5 Pa (Boveris & Chance, 1973). It is well known that flavins and flavoproteins can react directly with oxygen, with various flavoproteins differing greatly in their susceptibility to such auto-oxidation (Dixon, 1971). These reactions result in the formation of H_2O_2 or O_2^-, and Boveris & Chance (1973) postulated that flavoproteins were probably the most likely candidates for H_2O_2 generation in mitochondria and that increased O_2 pressure might increase the rate of such types of auto-oxidation reaction in the liver of the intact rat. A primary species of the O_2 by-product from mitochondria was postulated to be O_2^- (Loschen *et al.*, 1974), which, in turn, is converted into H_2O_2. Interaction with the peroxisomal catalase of H_2O_2 produced in the mitochondria has already been demonstrated in the isolated mitochondrial–peroxisomal fractions (Chance & Oshino, 1971) and in the isolated perfused liver (Oshino *et al.*, 1973*a*).

That H_2O_2 generation does not increase in the liver of the anaesthetized rat in hyperbaric O_2 indicates several factors. First, H_2O_2 itself is unlikely to be responsible for the basic mechanism of O_2 toxicity. Secondly, doubt is cast on the role of the superoxide anion (O_2^-) in the mechanism of O_2 toxicity, as proposed by Fridovich (1972), since the presence of abundant superoxide dismutase in the tissues (McCord & Fridovich, 1969) would immediately convert O_2^- into H_2O_2, which should then be detected by the present sensitive method. Similarly H_2O_2 has been implicated in the enhancement of lipid peroxidation in tissues of animals exposed to hyperbaric O_2. For instance, Jerrett *et al.* (1973) found increased amounts of H_2O_2 and a corresponding increase in lipid peroxidation after approx. 1 h of pressurization of rats to 4×10^5 Pa and Johnson *et al.* (1972) demon-

strated haemolysis and increased H_2O_2 production and lipid peroxidation in erythrocytes of rats exposed to hyperbaric O_2. However, such increased amounts of H_2O_2 in these studies were observed in tocopherol-deficient animals only. Also it is not clear whether this accumulation of H_2O_2 is a primary event or a consequence of O_2 toxicity. Thus unless only minute quantities of such oxidizing equivalents are required to exert toxic effects, as could perhaps be inferred from the work of Fong *et al.* (1973), it appears unlikely that superoxide and/or H_2O_2 production itself is the basic mechanism by which hyperoxia exerts its deleterious effect. In intact systems, such as in isolated liver or liver *in situ*, there may be sufficient antioxidant 'buffering' capacity to offset detectable H_2O_2 or O_2^- increases by hyperbaric O_2.

Glycollate infusion in air-breathing rats was found to double the rate of H_2O_2 generation compared with control rats, in agreement with the result of Mannering *et al.* (1969), who found that the rate of methanol oxidation was doubled by glycollate. The rate of methanol oxidation they observed is also similar to the rate of H_2O_2 generation that we detected in the present study. As predicted from the apparent low affinity for O_2 of glycollate oxidase (approx. 0.4mM) (Oshino *et al.*, 1975), the rate of H_2O_2 generation supplemented with glycollate was further doubled in the animal breathing 100% O_2 compared with that in the air-breathing rats (Table 1). Thus the intracellular O_2 concentration did in fact increase under 100% O_2 or hyperbaric O_2. However, the O_2 tension in the liver exposed as in these experiments may not be assumed to be the same as that in the liver of an intact normal animal. Also the possible existence of a steep O_2 concentration gradient between subcellular compartments of mammalian tissues has to be taken into account in interpreting results *in vivo*. As demonstrated in the perfused rat liver, the steady-state concentration of O_2 in the mitochondrial compartment may be lower than those in the peroxisomal and cytosolic spaces (Oshino *et al.*, 1975). Also the degree of this O_2 concentration gradient may be greatly affected by the rate of O_2 consumption in each compartment. The events occurring *in vivo* under hyperbaric O_2 of 6×10^5Pa therefore may be totally different from the phenomena *in vitro*, as was observed with isolated mitochondria under the same O_2 atmosphere.

Catalase and ethanol oxidation

Difficulty in determination of the rate of ethanol oxidation *in vivo* is mainly due to the fact that the rate of ethanol clearance from the blood does not represent the rate of ethanol oxidation itself, but is the sum of the true oxidation rate and the rate of absorption or release of ethanol into or from the whole body spaces of the animal. M. P. Schulman (personal communication) has found that approx. 1h is necessary for full equilibration of ethanol in the whole body of mice. Chance *et al.* (1974) also reported the contribution of ethanol released from the body spaces during the ethanol clearance period. In regard to this point, we allowed 40–50min at each infusion rate of methanol in three experiments, instead of the usual 5–10min, for attaining each steady state of the catalase–H_2O_2 intermediate. Although there was a slight further decline in the steady-state concentration of the catalase–H_2O_2 intermediate between the 10th and 40th min, the actual $a_{1/2}$ values calculated from these experiments were all within the range of values found in the experiments as reported in Table 1. Thus under our experimental conditions, i.e. with a continual infusion of methanol, blood alcohol concentration could be maintained near constant. In addition, the absorption rate of ethanol by whole body spaces and their capacity for absorption may be assumed to be similar to those for methanol. When compared at the same concentration of alcohol in liver, i.e. at the concentration producing the same steady-state concentration of the catalase–H_2O_2 intermediate, the difference in the infusion rates of methanol and of ethanol provides a more accurate rate of ethanol oxidation by extra catalase pathway, with less contribution of ethanol disappearance into body space. Since this difference is totally sensitive to *n*-propylpyrazole (Fig. 3), the values obtained are regarded as the rates of ethanol oxidation via alcohol dehydrogenase in the whole body. As described in the Results section, therefore, the $V_{max.}$ of ethanol oxidation through the alcohol dehydrogenase pathway in the whole body and through the catalase pathway in liver measured separately is approx. 13 and 1.45 μmol/min per 100g body wt. respectively.

Lowering the concentration of ethanol decreases the proportion of the catalase 'peroxidatic' reaction in the overall H_2O_2-decomposition reaction. This change in the proportion of 'peroxidatic' and 'catalatic' modes of the reaction can be measured from the extent of fractional saturation of the catalase–H_2O_2 intermediate (Oshino *et al.*, 1973*b*; Chance & Oshino, 1973) and thus the rate of the catalase-dependent ethanol oxidation at each steady state, as well as the rate of alcohol dehydrogenase-dependent oxidation, could be approximately calculated from results such as shown in Fig. 3. For the sake of better understanding, these values are shown in Fig. 9. These profiles of the activity curves with respect to the rate of ethanol infusion well represent characteristics of both reactions. The rate of ethanol oxidation through alcohol dehydrogenase rises sharply with low concentrations of ethanol and, at higher concentrations, seems to be limited by the redox state of the NADH/NAD$^+$ couple. Inhibition of alcohol dehydrogenase by high concentration of substrate was also

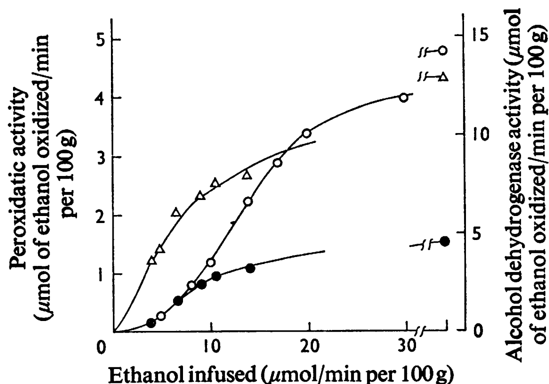


Fig. 9. *Catalase 'peroxidatic' activity and alcohol dehydrogenase activity with respect to the infusion rate of ethanol in situ*

The rate of catalase-dependent ethanol oxidation (●, ○) was computed from the fractional saturation of the catalase–H_2O_2, by using eqn. (4*e*) in a previous paper (Chance & Oshino, 1973) and the rate of H_2O_2 generation of 1.45 for control rats and 5.3 μmol/min per 100g body wt. for the glycollate-supplemented rats in 100% O_2. Alcohol dehydrogenase-dependent ethanol oxidation (△) is as shown in Fig. 3. ●, Catalase-dependent ethanol oxidation; ○, catalase-dependent ethanol oxidation in the presence of glycollate.

reported (Dalziel & Dickinson, 1966). Therefore the $V_{max.}$ value may not be attained *in situ* as was demonstrated in the perfused liver (Lindros *et al.*, 1972). The peroxidatic activity of catalase, on the other hand, varies according to both the rate of H_2O_2 generation and the concentration of ethanol present. Obviously the peroxidatic reaction is operative at higher concentration of ethanol, especially when H_2O_2 generation is increased by some means. Such a situation may occur when acetaldehyde is accumulated, since aldehyde oxidase producing H_2O_2 may start to function under such conditions. Similar conditions may also be produced in chronic alcoholic rats where increases in fat content and uric acid content were reported (see review, Hawkins & Kalant, 1972).

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